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Roles of 2′-Hydroxyls of Leadzyme on an RNA Cleavage

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The roles of two different 2′-hydroxyls near to a catalytic site of a leadzyme (Pb^{2+} ribozyme), CUGGGAGUCC, have been investigated kinetically. These roles of two different 2′-hydroxyls were clearly distinguished upon a cleavage reaction of an RNA substrate. One 2′-hydroxyl of G of the leadzyme in an asymmetry internal loop contributed to only the binding constant for the leadzyme with the substrates. The other 2′-hydroxyl of A in the asymmetry loop contributed to only the cleavage rate constant. On the basis of the results, we propose the possibility of the formation of a hydrogen bond between the 2′-hydroxyl of G of the leadzyme and the substrate and the binding between Pb^{2+} ion and the 2′-hydroxyl of A.

Ribozyme catalysis is very sensitive to mutagenesis of the base or a functional-group modification near to the active site, since non-Watson–Crick base pairs near to the active site play important roles for the structural stabilization of the folded ribozyme.[1—6] The 2′-hydroxyls of the ribozyme or the substrate near to the active site are also required for the ribozyme reaction. The 2′-hydroxyl adjacent to the cleavage site attacks phosphorus by an in-line S_N2.[7,8] The 2′-hydroxyls in the consensus region of the ribozyme or the substrate RNA contribute to the stabilization of the ribozyme-substrate complex in the groundstate or the transitionstate.[9—12] Thus, these specific roles of 2′-hydroxyls are key interactions in the ribozyme reaction. Leadzyme is a ribozyme that requires Pb^{2+}. The leadzyme, CUGGGAGUCC, binds to the RNA substrate, GGACCGAGCCAG, and acts as a ribozyme, so that it cleaves the RNA substrate at one site, as shown in Fig. 1.[13,14] This ribozyme activity was enhanced by the combination effect of Pb^{2+} and Nd^{3+}.[15] The addition of Nd^{3+} in the presence of Pb^{2+} influenced the complex

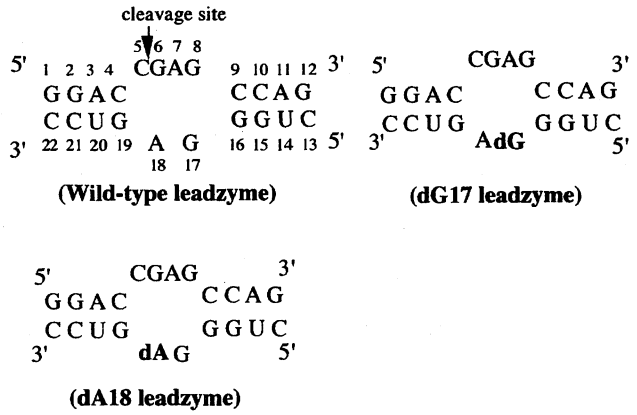

Fig. 1. Secondary structures of the complexes of the wild-type and the chimeric leadzymes with the RNA substrate used in this work. The arrow indicates the cleavage site.

stability, and directly catalyzed the cleavage reaction.[16] Information concerning the functional-groups of the leadzyme is necessary to understand in more detail the combination effect of the metal ions on the ribozyme function. In this work, we investigated the roles of the 2′-hydroxyls in the active center of the ribozyme and clearly distinguished different roles of two 2′-hydroxyls on the substrate cleavage reaction by using chimeric leadzymes, CUGG(dG)AGUCC and CUGGG(dA)GUCC, as shown in Fig. 1.

Experimental

Preparation of Oligonucleotides. RNA substrates, wild-type and chimeric leadzymes, were synthesized on a solid support by a phosphoramidite method on an Applied Biosystems model 391 DNA/RNA synthesizer.[17] The synthesized oligomers were removed from a solid support, and base-blocking groups were removed by a treatment with concentrated ammonia in ethanol (3 : 1, v/v) at 55 °C for 3 h. After drying in a vacuum, the 2′-silyl protection groups were removed by resuspending the pellet in 50 equiv of tetrabutylammonium fluoride (TBAF) per equivalent of silyl; the mixtures were incubated overnight in the dark at room temperature.[18] Then, the samples were passed through a C18 Sep-Pak cartridge (Waters) to be desalted and purified by HPLC on a C18 column (TOSOH) with a gradient of 0—50 % methanol/H_2O containing 0.1 M triethylammonium acetate (TEAA) (1 M = 1 mol dm^{-3}), pH 7.0. After purification by HPLC, the oligomers were desalted again with a C18 Sep-Pak cartridge. The final purities of the oligomers were checked by HPLC and were greater than 98%. The concentrations of the purified oligonucleotides were determined spectrophotometrically with a Hitachi U-3210 spectrophotometer.

Cleavage Reaction. The rate constants for the cleavage reactions by the ribozyme were determined under single-turnover conditions. Single-turnover experiments with the ribozyme in excess over the substrate RNA were carried out in 15 mM NaMOPS (pH 7.5) at 25 °C. The ribozyme (2.5—8.0 μM) and the 5′ end-labeled substrate RNA (250 nM) were heated together to 90 °C for 2 min, cooled slowly, and incubated at 25 °C for 30 min in 7 μL of 15 mM NaMOPS, pH 7.5. Cleavage was initiated by the addition of 7 μL of 15 mM NaMOPS buffer containing Pb^{2+}. Re-

actions were terminated by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of 200 mM Na_2EDTA, 8 M urea, 0.02% Bromophenol Blue, and 0.02% xylene cyanol. The labeled product and substrate were separated by denaturing 20% polyacrylamide gel electrophoresis. The radioactivity of the substrate and the product was analyzed with a Bio-Image Analyzer (BAS 2000; Fuji Film, Tokyo). The observed rate constants were obtained by plotting the natural log of the product vs. time. When the amount of product after 24 h was chosen as the amount at the endpoint of the reaction, all of the plots were linear. Nonlinear least-squares fits of plots of the observed rate constants vs. ribozyme concentration to kinetic equations were performed with a software Igor (Wava Metrics) to obtain separately the binding constant and the cleavage rate constants.

Energy Minimization Calculations. The initial coordinate of the leadzyme-substrate complex for the calculation was generated as follows: (i) Canonical A-RNA duplex, CUGGGUCC/GGACCCAG, was generated by 'Nucleic acid builder' of QUANTA 4.0 (Molecular Simulations Inc.). (ii) The asymmetric loop, CGAG/GA, was inserted in CUGGGUCC/GGACCCAG duplex by using 'Nucleic acid builder' of QUANTA 4.0. We were then able to obtain the coordinate of the leadzyme-substrate complex. Energy-minimization calculations were performed using CHARMm 23.1 (Molecular Simulations Inc.) with parameters taken from the work of Nilsson and Karplus.[19] Also, the energy of the leadzyme-substrate complex was minimized by the adopted basis set Newton–Raphson (ABNR) method to exclude poor bond lengths and angles or bad contacts. In the calculations the stem regions were kept as an A-RNA duplex when the structure was minimized. In the asymmetric internal loop, the sheared G·A base pair was used as the initial coordinate of A7 and G17. However, we did not constrain the two hydrogen bonds in the G·A base pair when the energy-minimization calculations were performed. The resultant structure was displayed in QUANTA 4.0. All of calculations were performed on a Silicon Graphics Indigo2 workstation running IRIX 5.3.

Results

RNA Cleavage by Chimeric Leadzymes. The structures of the complexes of the substrate with the wild-type and chimeric leadzymes used in this study are shown in Fig. 1. The wild-type leadzyme, CUGGGAGUCC, binds to the RNA substrate, GGACCGAGCCAG, and acts as a ribozyme in the presence of 50 μM Pb^{2+} and 15 mM NaMOPS (pH 7.5), so that it cleaves the substrate RNA at one site.[14,15] The cleavage yield for a 20 min incubation in the presence of 50 μM Pb^{2+} at 25 °C was 13.5%. Figure 2 shows an autoradiogram of the cleavage reaction in the presence of the wild-type leadzyme or the chimeric leadzymes, rCUGG-(dG)rAGUCC (dG17 leadzyme) and rCUGGG(dA)rGUCC (dA18 leadzyme) at 50 μM Pb^{2+} for 20 min. The experiment conditions are the same as described above. The product band catalyzed by dG17 or dA18 leadzyme was not observed for a 20 min incubation. If this observation is simply due to the destabilization of the leadzyme-substrate complex by the introduction of the DNA–RNA junction, the product band would be observed by the addition of polyamine spermine, because polyamine spermine is known to stabilize high-order nucleic acid structures at a low concentration.[20] In fact, the addition of the spermine enhanced the activity of the hammerhead ribozyme under the low metal ion concentration in which the ribozyme-substrate complex was not stabilized.[21] However, the product cleaved by dG17 or dA18 leadzyme was not observed even with increasing spermine from 0.1 to 12.5 μM (data not shown).

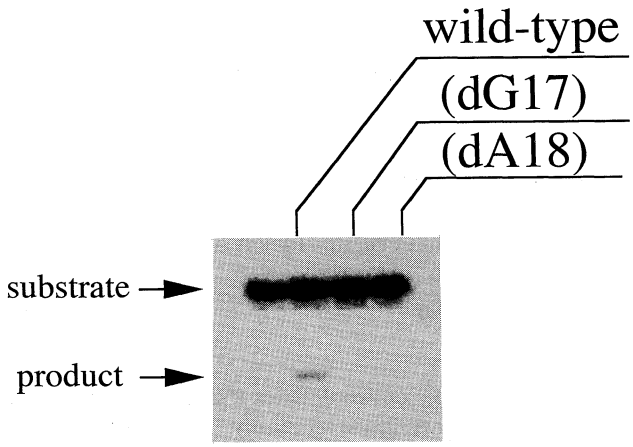

Fig. 2. Autoradiogram of denaturing 20% polyacrylamide gels showing the cleavage of 250 nM substrate by 15 μM wild-type, dG17, or dA18 leadzyme in 15 mM NaMOPS (pH 7.5) solution containing 50 μM Pb^{2+} at 25 °C for 20 min.

To investigate in more detail the property of the chimeric leadzymes, we measured the time course of the cleavage reaction by the chimeric leadzymes. The product band cleaved by dG17 or dA18 leadzyme was observed during long-time incubation in the presence of 50 μM Pb^{2+} and 15 mM NaMOPS (pH 7.5), as shown in Fig. 3. The cleavage site was not changed by the chimeric leadzymes. We determined the rate constants by plotting the natural log of the product concentration vs. time. The values of the observed rate constant (k_{obs}), for the wild-type, dG17, and dA18 leadzymes were 5.4×10^{-3}, 2.5×10^{-3}, and 5.7×10^{-4} min^{-1}, respectively. Thus, two 2′-hydroxyls of G17 and A18 influence the reaction rate for RNA substrate cleavage.

Effect of 2′-Hydroxyl on the Complex-Formation Step and Cleavage Step in the Presence of Pb^{2+}. The cleavage reaction of the RNA substrate by the ribozyme consists of at least two steps, binding and cleavage. We measured the cleavage kinetics in order to investigate the roles of two 2′-hydroxyls on the leadzyme catalysis. Because the kinetic measurements were done under the conditions where the substrate was expected to form a Michaelis–Menten complex with excess and sufficiently high concentrations of the leadzyme, and the reverse ligation reaction was not observed under the condition (data not shown), the cleavage reactions are

$$R + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R\cdot S \xrightarrow{k_2} R\cdot P_1\cdot P_2, \quad (1)$$

where k_1, k_{-1}, k_2 are the rate constants, R is the leadzyme, S is the substrate RNA, R·S is a the leadzyme-substrate complex, and R·P1·P2 is the leadzyme-products complex. When the first step in Eq. 1 is much faster than the second step and $[R]_0 \gg [S]_0$, the equilibrium constant K_1 (= k_1/k_{-1})

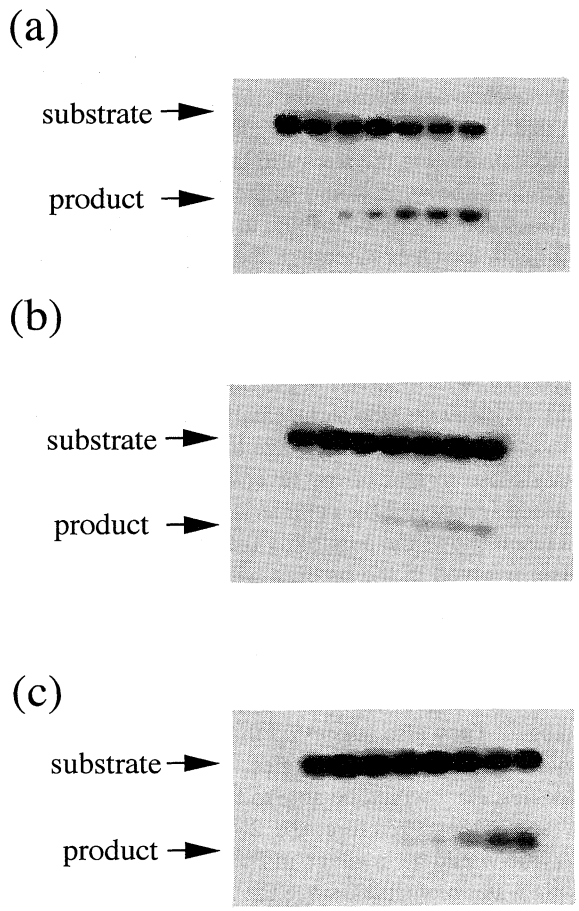

Fig. 3. (a) Autoradiogram of denaturing 20% polyacrylamide gel showing time dependence of the substrate cleavage by wild-type leadzyme. From left to right in the gels, incubation times are 0, 0.5, 1, 1.5, 2, 3, and 4 h, respectively. (b) Autoradiogram of denaturing 20% polyacrylamide gel showing time dependence of the substrate cleavage by dG17 leadzyme. From left to right in the gels, incubation times are 0, 0.5, 1, 1.5, 2, 3, and 4 h, respectively. (c) Autoradiogram of denaturing 20% polyacrylamide gel showing time dependence of the substrate cleavage by dA18 leadzyme. From left to right in the gels, incubation times are 0, 1, 2, 3, 4, 6, 8, and 10 h, respectively. All of the substrate cleavages by wild-type, dG17, or dA18 leadzyme were done in a solution containing 125 nM RNA substrate, 37.5 μM wild-type, dG17, or dA18 leadzyme, 15 mM NaMOPS (pH 7.5), and 50 μM Pb^{2+} at 25 °C.

in the first step and the cleavage rate constant k_2 in the second step can be obtained with non-linear least-squares fits of the data to Eq. 2:

$$k_{obs} = \{k_2[R]_0/([R]_0 + 1/K_1)\}. \quad (2)$$

The fitting curves are shown in Fig. 4. The observed rate constants reach to k_2 at high concentrations of the leadzyme. Higher leadzyme concentrations are required to reach to saturation with dG17 leadzyme than the wild-type and dA18 leadzymes. On the other hand, the k_2 values of the wild-type and dG17 leadzymes were higher than that of the dA18 leadzyme. The values of k_2, K_1, and binding free-energy increment, ΔG_{25}, complex are listed in Table 1. Table 1 shows that the 2′-hydroxyl of dG17 contributes to only the binding constant for the leadzyme with the substrates, while that of dA18 contributes to only the cleavage rate constant. These results indicate that the contributions of 2′-hydroxyl of dG17 and dA18 on the cleavage reaction are different.

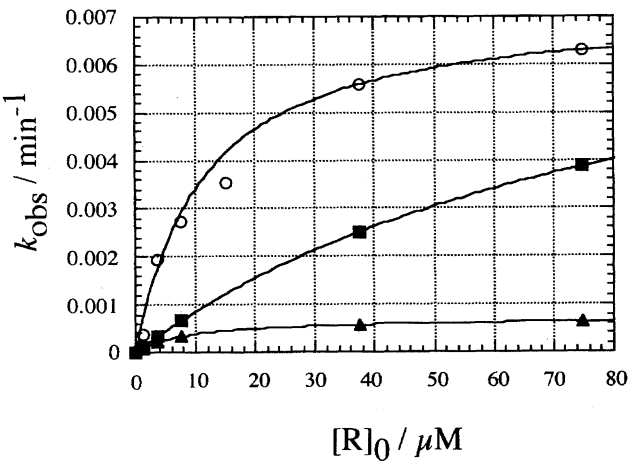

Fig. 4. Plots of k_{obs} at 25 °C vs. the concentration of (○) wild-type, (■) dG17, or (▲) dA18 leadzyme in 15 mM NaMOPS (pH 7.5) solution containing 50 μM Pb^{2+}. Solid curves were obtained with the non-linear least-square fits to Eq. 2.

Table 1. Kinetic Parameters for Cleavage[a)]

Ribozyme	$K_1 \times 10^{-5}$ / M^{-1}	$\Delta G^\circ_{25,complex}$[b)] / kcal mol^{-1}	$k_2 \times 10^2$ / min^{-1}
rCUGGGAGUCC[c)]	0.93	−6.8	0.72
rCUGG**dG**rAGUCC	0.12	−5.5	0.82
rCUGGG**dA**rGUCC	1.23	−6.8	0.07

a) All experiments were done in ribozyme excess condition with its concentration ranging from 1.25 to 40 μM and [5′-^{32}P] substrate concentration of less than 0.125 μM. b) The value calculated from K_1. c) Ref. 16.

Discussion

The RNA cleavages by a ribozyme depends on the specific interaction of a base or 2′-hydroxyls. The leadzyme is a relatively small RNA catalyst consisting of six-membered asymmetry internal loops. The results reported here provide information near the active site in the simple cleavage motif.

One of the present results about the roles of specific 2′-hydroxyls is that the binding constant for the leadzyme with the substrate depends on the 2′-hydroxyl of G17, but not on that of A18. Further, the 2′-hydroxyl of G17 affects only the binding step, but not the cleavage step in the experiments. The 2′-hydroxyl of G17 contributes 1.2 kcal mol^{-1} to binding free energy increment of the wild-type R·S complex at 25 °C. This value is very close to the free-energy increment predicted for a hydrogen bond formation between base pairs in the absence of the competing stacking interaction.[22)] This value is also similar to the free-energy increment of partic-

ular 2′-hydroxyls which make a contribution to the binding of the substrates to a group I ribozyme.[10] In addition, two 2′-hydroxyls of CUCU substrate are known to contribute about 1 kcal mol^{-1} each to the binding free energy.[11,12,23,24] Thus, considering these results, the 2′-hydroxyl of G17 in the asymmetric internal loop would contribute to the binding step.

Recent X-ray and NMR studies show that the 2′-hydroxyls contribute to the stability of the tertiary or secondary structure of the folded ribozyme.[2,25–27] In a hairpin loop ribozyme containing an asymmetric internal loop as well as the leadzyme, a 2′-hydroxyl contributing to a hydrogen bond near to the active site was found.[25] A hydrogen bond formed between the O6 carbonyl of G of the hairpin ribozyme and the 2′-hydroxyl of G of an RNA substrate. In a hammerhead ribozyme-substrate complex, the complex with a 2′-*O*-methyl at the active site to prevent bond cleavage also had hydrogen bonds between the 2′-hydroxyl of U16.1 and O4 of U7, and between the 2′-hydroxyl of G12 and N6 of A9.[2] These interactions are likely to facilitate substrate interaction with the active site. P4–P6 domain of a group I ribozyme had interaction with a tetraloop receptor containing hydrogen bonds between the 2′-hydroxyl and bases or other 2′-hydroxyls.[26,27] Thus, 2′-hydroxyl is a good donor or acceptor of hydrogen bonds to phosphate, bases, and other 2′-hydroxyls.

To estimate the detail interaction of the 2′-hydroxyl of G17, energy-minimization calculations were performed using CHARMm 23.1. As a result, it was shown the possibility that the leadzyme-substrate complex had a hydrogen bond between a 2′-hydroxyl of G17 and O6 of G8, as shown in Fig. 5. Since the mutant substrate that removed G8 from the active site was not cleaved by the leadzyme (unpublished results), the calculations also suggest the important role of the 2′-hydroxyl in the complex formation.

Another result concerning the roles of specific 2′-hydroxyls is that the cleavage rate depends on the 2′-hydroxyl of A18, but not on that of G17. Additionally, the substitution of 2′-hydroxyl for H of A18 hindered the cleavage step without affecting the binding step. In the case of an endonucleolytic cleavage reaction by the group I ribozyme, when the 2′-hydroxyl contributing directly to the chemical step was replaced by H, the cleavage rate was 1700-fold slower.[11,12] The cleavage rate by dA18 leadzyme was about 10-fold slower than the wild-type leadzyme. The decrease in the rate of the substrate cleavage by using the chimeric leadzyme is smaller than that of the endonucleolytic cleavage reaction. This difference concerning the rate ratio between the leadzyme and the group I ribozyme would suggest that the 2′-hydroxyl of A18 does not directly contribute to the cleavage step. On the other hand, the cleavage rate of the reverse-cyclization reaction of the group I ribozyme by a dimer oligonucliotide was 5-fold smaller when the 2′-hydroxyl near to the active site of the dimer was replaced by H.[10] This result suggested that the 2′-hydroxyl was involved in the binding of Mg^{2+} contributing to the cleavage step at the catalytic site. The decrease in the rate of the substrate cleavage by the chimeric leadzyme is very close to that of the reverse-cyclization reaction of the group I ribozyme by a chimeric dimer oligonucliotide.[10] This result shows the possibility that the 2′-hydroxyl of A18 is also involved in the binding with a metal ion contributing to the chemical step at the catalytic site. Leadzyme is a ribozyme that requires Pb^{2+} instead of Mg^{2+}. Cedergren and his coworkers indicated that the 2′-hydroxyl of G19 of the closing base in a similar leadzyme was involved in Pb^{2+} binding.[28] They also suggested that Pb^{2+} might bind to the 2′-hydroxyl via water molecules. If Pb^{2+} binds via water molecules, Pb^{2+} would be able to bind both 2′-hydroxyls of G19 and A18. Therefore, the 2′-hydroxyl of A18 in our leadzyme would be involved in Pb^{2+} binding at the catalytic site via water molecules, since the 2′-hydroxyl is not a good ligand for innersphere binding to Pb^{2+} as well as Mg^{2+}.

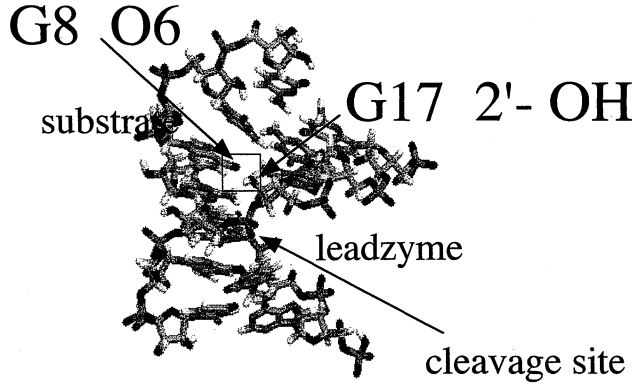

Fig. 5. Structure of the leadzyme-substrate complex.

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