Effect of Structural Modifications on the Activity of the Leadzyme†

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ABSTRACT: The structure/function properties of functional groups in the leadzyme have been studied by assaying the activity of analog ribozymes generated by the systematic substitution of modified nucleotides in the internal loop region of the ribozyme. Guanosine analogs introduced at positions 4 and 7 occupied by guanosine in the wild-type molecule severely diminished cleavage. The substitution of deoxycytidine for cytidine at the cleavage site completely eliminated the activity of the leadzyme, as expected if the adjacent 2′-OH were the nucleophile in the cleavage reaction. On the other hand, substitution of an abasic nucleotide for adenosine at position 8 did not affect the activity of the ribozyme. An analysis of the activity of these analogs gives rise to the proposal of a triple-base pair motif implicating C_1, G_4, and G_7.

The leadzyme ribozyme is one of the smallest RNA catalytic domains and thus one of the smallest biological macromolecular catalysts. The domain is formed by two helical regions separated by an internal, asymmetric loop of six nucleotides (Pan & Uhlenbeck, 1992). Unlike many other ribozymes such as the hammerhead, hairpin, and VS[1] ribozyme, which are dependent on magnesium, leadzyme activity is dependent on the presence of a lead ion for promotion of the cleavage of a specific phosphodiester bond. Cleavage takes place between positions C_1 and G_2 (Figure 1) and yields 2′,3′-cyclic phosphate and 5′-OH end products in the first step of the reaction. Another distinguishing feature of this ribozyme is that it promotes the opening of the 2′,3′-cyclic phosphate generating the 3′-phosphate end product in the second step of the reaction (Pan & Uhlenbeck, 1992).

Because of its small size and unique lead-based chemistry, this ribozyme is an excellent model for understanding the structural basis of RNA catalysis. Major steps were taken toward that understanding when Pan and Uhlenbeck (1994), using *in vitro* selection of active ribozymes, demonstrated that C_1, G_4, and G_7 were present in all active forms. Moreover, only purines were found at position 3; the identity of positions 2 and 8 did not affect activity (Figure 1). These data, however, offer no clues as to which functional groups of those nucleotides are important for catalysis.

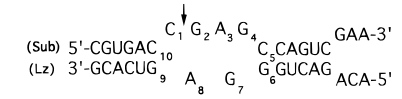

FIGURE 1: RNA structure of the ribozyme (Lz) and the substrate (Sub). The cleavage site is indicated by an arrow.

Chemical synthesis of RNA using modified nucleotides illustrated in Figure 2 has permitted the evaluation of functional groups in RNA domains such as the hammerhead, hairpin, and group I introns (Bratty et al., 1993; Chowrira & Burke, 1991; Usman & Cedergren, 1992; Strobel & Cech, 1995). We report here the application of this technique to the identification of functional groups important for the catalytic activity of the leadzyme.

MATERIALS AND METHODS

Oligonucleotide Synthesis and Purification. All oligomers and modified nucleotides were synthesized using chemical procedures described previously (Scaringe et al., 1990; Beigelman et al., 1994, 1995a,b; Wincott et al., 1995). Oligonucleotides were purified on 15% polyacrylamide/7 M urea gels; the product bands were excised and eluted with water. After isolation, the polymers were desalted on Sephadex G-50 (Pharmacia) and lyophilized to dryness.

Radioisotopic Labeling. The substrate was 5′-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (NEN Dupont). A 5 μL reaction mixture containing 40 pmol of substrate, 50 pmol of ATP, and 1 μg of BSA in 10 mM $MgCl_2$ and 20 mM Tris-HCl at pH 8 was incubated for 30 min at 37 °C. The reaction mixture was analyzed on a 15% polyacrylamide/7 M urea gel and isolated as described above.

Cleavage Conditions. The standard buffer for the cleavage reactions was 0.1 M NaCl and 200 μM $Pb(OAc)_2$ in 15 mM HEPES at pH.7; incubation was at 25 ± 1 °C. Oligonucleotides were first heated to 70 °C in 8 μL of the HEPES/NaCl buffer for 90 s before being rapidly cooled on ice. Reactions were initiated by adding 2 μL of 1 mM $Pb(OAc)_2$ and quenched with 10 μL of a formamide dye mix loading buffer (1 M formamide, 7 M urea, 0.05% XC, 0.05% BPB, and 0.5× TBE buffer) and loaded onto a 15% polyacrylamide/7

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Lz, leadzyme; Sub, substrate; I and dI, inosine and deoxyinosine; r2AP, 2-aminopurine ribonucleotide; rP, ribopurine; VS, Varkud Satellite; TBE, Tris/borate/EDTA; XC, xylene cyanol; BPB, bromophenol blue. Positions of modifications in nucleotide analogs are indicated by numbers, whereas positions in the leadzyme domain are designated by subscripts.

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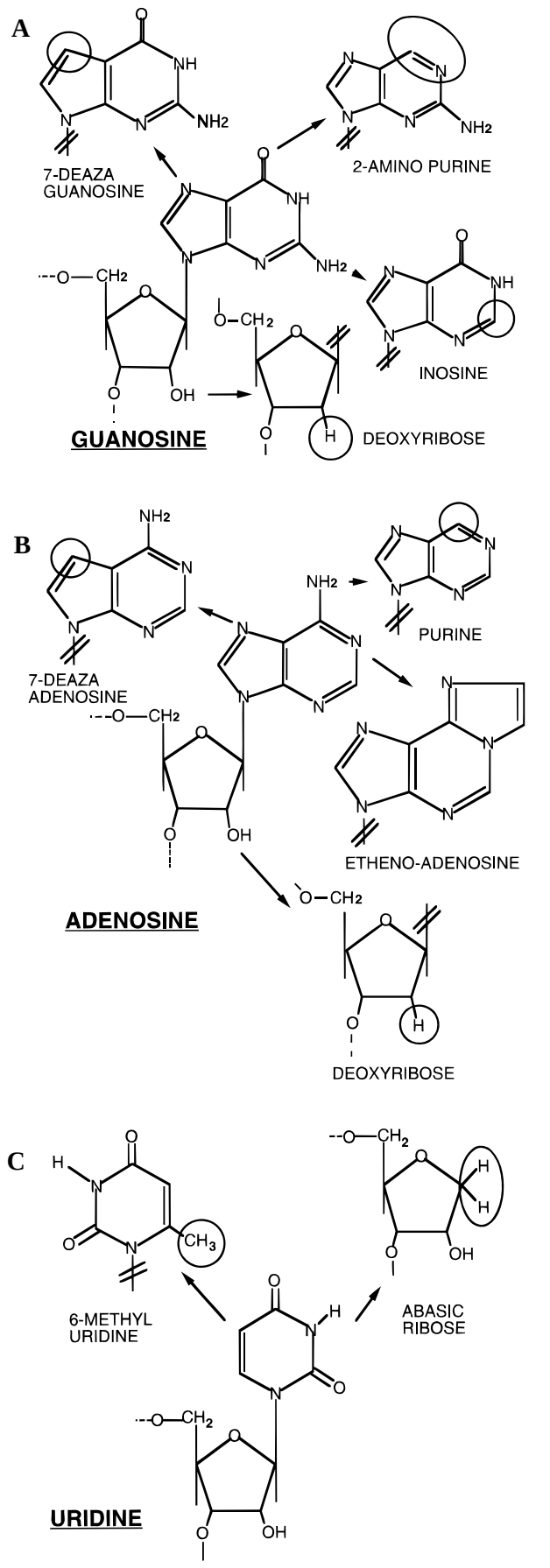

FIGURE 2: (A) Nucleotide analogs of guanosine. (B) Nucleotide analogs of adenosine. (C) Nucleotide analogs of uridine. Circles indicate modifications.

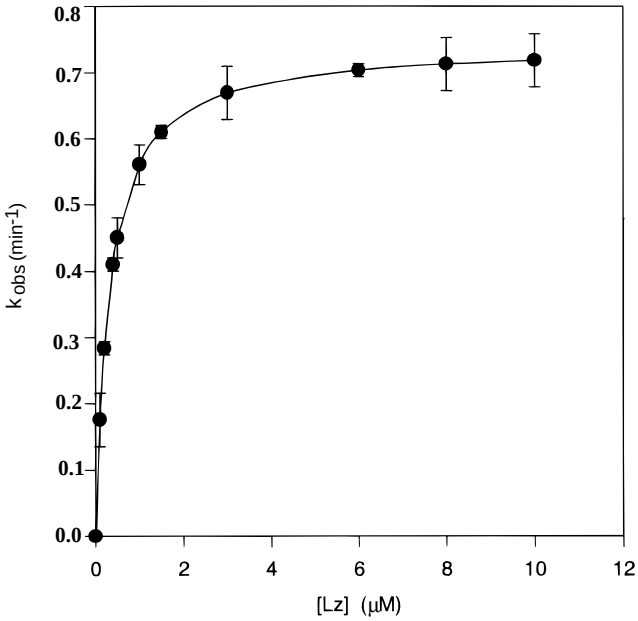

FIGURE 3: Dependence of k_{obs} on the ribozyme concentration. Ribozyme concentrations up to 10 μM were used with 5 nM ^{32}P-labeled substrate. The cleavage rate increased to a maximum of 0.74 min^{-1} at saturation of the substrate by the ribozyme.

M urea gel. The gel was subjected to autoradiography, and the radioactive bands were cut out and quantified using an LKB β counter.

Kinetic Analysis. Single-turnover experiments with an excess of ribozyme over the 5′-^{32}P-labeled substrate were used to measure the first-order rate constant of the reactions catalyzed by the various leadzyme analogs. These reactions were performed under the standard conditions above using 5 nM labeled substrate and 10 μM ribozyme. Aliquots (1 μL) were removed at appropriate intervals, quenched in the formamide dye buffer mix, and analyzed by gel electrophoresis. Rate constants (k_{obs}) were obtained from semi-logarithmic plots according to the equation $\log[A] = (-k/2.303)t$. Each rate constant is the average of at least three measurements.

Nondenaturing Gel Analysis. The oligonucleotides and solutions were mixed as above with 200 μM lead acetate in 10 μL and incubated at 25 ± 1 °C for 5 min. The samples, to which was added 10 μL of a solution containing 10% glycerol, 0.05% XC, 0.05% BPB, 0.1 M NaCl, and 200 μM $Pb(OAc)_2$ in 15 mM HEPES at pH 7, were loaded onto a 10% polyacrylamide gel containing 50 mM HEPES at pH 7.0. The gel was developed at 25 °C in a 50 mM HEPES buffer at pH 7.0 and 100 V.

RESULTS

Activity of the Leadzyme Analogs. For the purposes of this study, the original leadzyme domain used by Pan and Uhlenbeck (1992) was modified so that the substrate (Sub) was *trans* to the catalytic fragment (Lz, Figure 1). This manipulation reduced the length of the oligonucleotides to be synthesized and facilitated the measurement of the kinetic parameters. Reactions were performed under single-turnover conditions, that is with an excess of ribozyme over the substrate. The observed rate constant k_{obs} was increased until the ribozyme concentration reached around 4 μM (Figure 3) at a substrate concentration of 5 nM. All reactions were

Table 1: Activity Data of Modified Nucleotides Incorporated into the Leadzyme Domain

position	substitution	k_{obs} (min^{-1})	k_{rel}[a] (ribo)	k_{rel}[b] (deoxy)	$\Delta\Delta G^{\ddagger}$[c] (kcal/mol)
−	none	0.74 (±0.04)	1.0	−	−
Sub C_1	dC	inactive	−	−	−
Sub C_1	rU[d]	0.0017 (±0.0002)	0.004	−	−3.3
Sub G_2	dG	0.37 (±0.02)	0.5	−	−0.4
Sub G_2	dI	0.4 (±0.04)	0.54	1.1	−0.06
Sub G_2	7−deaza-dG	0.028 (±0.002)	0.04	0.08	−1.5
Sub A_3	dA	0.24 (±0.03)	0.32	−	−0.6
Sub A_3	rP	0.02 (±0.002)	0.03	−	−2.1
Sub A_3	7−deaza-dA	0.034 (±0.005)	0.046	0.14	−1.2
Sub G_4	dG	0.56 (±0.08)	0.76	−	−0.1
Sub G_4	dI	0.017 (±0.002)	0.023	0.03	−2.1
Sub G_4	r2AP	0.0002 (±0.00004)	0.0003	−	−4.8
Sub G_4	7−deaza-dG	0.0045 (±0.0005)	0.006	0.008	−2.8
Lz G_7	dG	0.149 (±0.003)	0.2	−	−0.9
Lz G_7	rI	0.0032	0.0043	−	−3.2
Lz G_7	r2AP	0.000 52 (±0.00007)	0.0007	−	−4.3
Lz G_7	7−deaza-dG	0.000 39 (±0.00001)	0.0053	0.0026	−3.5
Lz A_8	rP	>0.8	−	−	−
Lz A_8	7−deaza-dA	0.77 (±0.04)	1	−	0
Lz A_8	dI	0.57 (±0.07)	0.77	−	−0.1
Lz A_8	6-methyl-U	>0.8	−	−	−
Lz A_8	etheno dA	0.43 (±0.04)	0.58	−	−0.3
Lz A_8	ribo abasic	>0.8	−	−	−
Lz G_9	dG	0.056 (±0.001)	0.076	−	−1.5
Lz G_9	7−deaza-dG	0.0095 (±0.0003)	0.013	0.17	−1
Lz G_9	rC[e]	0.039 (±0.002)	0.052	−	−1.8

[a] k_{rel} (ribo) = k_{obs} (mutant)/k_{obs} (wild type). [b] k_{rel} (deoxy) = k_{obs} (mutant)/k_{obs} (deoxy analogs at the same position). [c] $\Delta\Delta G^{\ddagger}$ was calculated with k_{rel} (deoxy) in the case of deoxyribonucleotides and with the k_{rel} (ribo) with the ribonucleotide analogs. Values of T = 298 K (25 °C) and R = 1.987 cal K^{-1} mol^{-1} were used. [d] Measured with the etheno-dA_8 Lz derivative in order to avoid U_1−A_8 base pairing. [e] Measured with a substrate containing an rG instead of an rC at position 10.

therefore carried out at 10 μM to ensure that the ribozyme was in excess. Under these conditions, the k_{obs} reflects the rate constant for the chemical step of substrate cleavage (k_2) and is independent of the kinetics of product release. The value obtained here (k_{obs} = 0.74 min^{-1}) is similar to that obtained by Pan and Uhlenbeck (1992) with a *cis* ribozyme−substrate system (0.7 min^{-1}).

Oligonucleotide analogs of the leadzyme were made by incorporating modified nucleotides at every position of the asymmetric internal loop of the leadzyme. The modified nucleotides used in this study are shown in Figure 2A−C, and the effect of each structural modification on leadzyme activity is shown in Table 1. The relative activity of the analogs is shown with reference to the wild-type leadzyme [k_{rel} (ribo)]; those in the deoxyribonucleotide series were compared as well to that of the parent deoxyribonucleotide at the same position [k_{rel} (deoxy)] to distinguish between effects of 2′-hydroxyl and base modification. In this regard, substitution by a deoxyribonucleotide generally diminished the activity by a factor of less than 3, except for the guanosine at position 9 (factor of 10) and the cytidine at the cleavage site, where substitution completely inactivated the ribozyme (Table 1). This latter result supports the view that the adjacent 2′-hydroxyl is the nucleophile in the attack on the scissile phosphodiester group. As seen in Table 1, all base modifications of nucleotides C_1, G_4, and G_7 led to a reduction of leadzyme activity by at least 1 order of magnitude. These results are consistent with the selection experiments done by Pan and Uhlenbeck (1994).

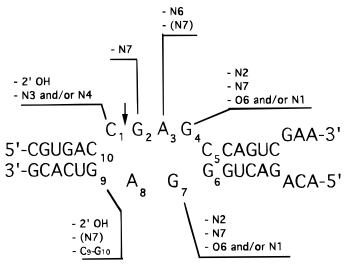

FIGURE 4: Summary of the analog data. Modification of the functional groups indicated for each nucleotides led to a decrease of the activity of the leadzyme by more than 1 order of magnitude. The modification of the functional groups in parentheses decreased the activity between 10 and 5 times.

To avoid base pairing with A8 when uridine replaced cytidine at position 1, the catalytic fragment was also modified with an etheno-dA at position 8 (see Figure 2B). Since the etheno substitution possesses approximately 50% of the wild-type activity, the major factor in the reduction of activity for the doubly substituted leadzyme is most probably due to the incorporation of uridine at position 1. This result suggests that the C requirement is due to either one of its distinguishing functional groups, the exocyclic 4-amino group or the N3 imino group.

Substitution of the guanosine at position 2 shows that inosine is well-tolerated but that deletion of the N7 group is very detrimental to activity. It had been shown previously that position 3 of the asymmetric loop of the leadzyme domain had a purine requirement (Pan & Uhlenbeck, 1994). In addition, we find that lack of the exocyclic amino group of adenosine decreases the activity much more than elimination of the N7 group (a factor of 30 versus 7, respectively).

In contrast to other positions in the leadzyme domain, incorporation of a wide variety of modified nucleotides at position 8 such as purine, 6-methyl-U (which locks the base in the *syn* conformation; Beigelman et al., 1995a), and the 7-deaza-dA invariably gave an increase in the catalytic activity of the leadzyme. To confirm this lack of restrictions at position 8, an abasic ribonucleotide (Beigelman et al., 1994, 1995b) was incorporated into this position and was also found to increase the activity compared to that of the wild-type sequence. These data suggest that the base moiety at this position has no role in catalysis, since the only derivative which showed a diminution in activity was etheno-dA, and this result could be explained by the steric hindrance of the large ethenoadenine moiety.

Finally, the inversion of the G_9·C_{10} base pair to C_9·G_{10} leads to a 20-fold decrease in the activity of the leadzyme, whereas the deletion of the 2′-OH and the N7 of G_9 results in a 12- and 6-fold decrease, respectively. A summary of the functional groups essential for the activity of the leadzyme is given Figure 4.

The relative activity of each mutant (k_{rel}, Table 1) has been used to obtain the change in the apparent free energy of transition-state stabilization ($\Delta\Delta G^{\ddagger}$) using the equation

$$\Delta\Delta G^{\ddagger} = RT \ln(k_{rel})$$

This value measures the energetic penalty for the removal of a functional group involved in the stabilization of the transition state (Fersht, 1988). Even though the binding energy due to a single hydrogen bond is 1−2 kcal/mol (Turner & Bevilacqua, 1993), and is subject to a context

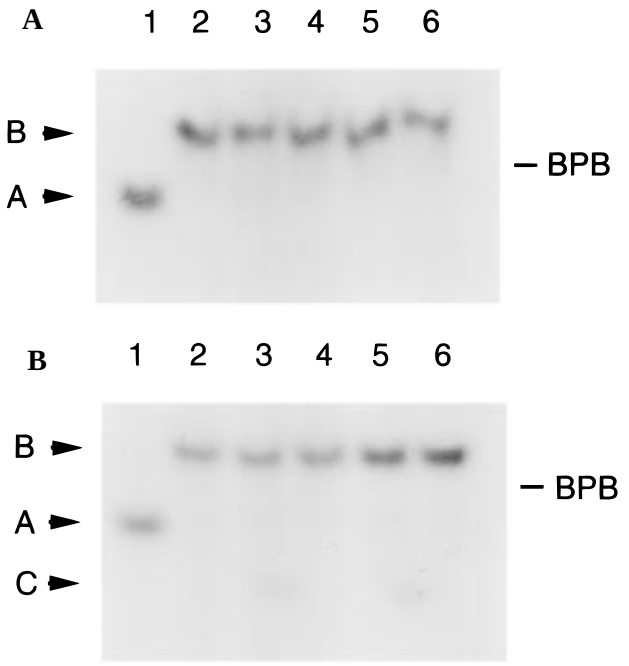

FIGURE 5: Nondenaturing gel of the leadzyme−substrate complex with wild type and mutants leadzyme (Lz) and ^{32}P-labeled substrate (Sub). (A) Lane **1**, dC_1−Sub; lane **2**, dC_1−Sub + Lz; lane **3**, dC_1−Sub + I_7−Lz; lane **4**, dC_1−Sub + $r2AP_7$−Lz; lane **5**, dC_1−Sub + 7-deaza-dG_7−Lz; and lane **6**, dC_1−Sub + 7-deaza-dG_9−Lz. (B) Lane **1**, dC_1−Sub; lane **2**, dC_1−Sub + Lz; lane **3**, dI_4−Sub + Lz; lane **4**, $r2AP_4$−Sub + Lz; lane **5**, 7-deaza-dG_4−Sub + Lz; and lane **6**, U_1−Sub + etheno-dA_8−Lz. **BPB**, bromophenol blue. A, free substrate. B, ribozyme−substrate complex. **C**, cleavage product.

effect (SantaLucia et al., 1992), the deletion of the N6 of A_3, the N2 of G_4, the 2′-OH of G_7 and G_9, as well as the N7 of G_2, A_3, and G_9 would likely disrupt only one hydrogen bond in the transition-state structure of the leadzyme on the basis of $\Delta\Delta G^{\ddagger}$ values between −0.9 and −2.1 kcal/mol (Table 1). On the other hand, the $\Delta\Delta G^{\ddagger}$ value of −3.1 kcal/mol for the deletion of the N2 of G_7 suggests a more extensive disruption involving hydrogen bonds and/or other conformational effects. The incorporation of 7-deaza-dG and r2AP at positions G_4 and G_7 gives $\Delta\Delta G^{\ddagger}$ values between −2.8 and −4.8 kcal/mol which are also too high to be interpreted in terms of the disruption of a single hydrogen bond. Nondenaturing gel electrophoresis was used to determine whether decreases observed in the activity of some analogs could be due to a decrease in the stability of the ribozyme−substrate complex or to the formation of another complex (Fedor & Uhlenbeck, 1990). The gels shown in Figure 5 indicate that the analogs that have the biggest impact on catalytic activity form a complex electrophoretically indistinguishable from the wild-type ribozyme, suggesting that modifications impose only subtle changes on the structure of the complex. Nondenaturing gels also show that these oligonucleotides do not form intramolecular complexes (data not shown).

Rate constant measurements were performed at Pb^{2+} concentrations ranging from 1 to 2000 μM to identify possible Pb^{2+} binding site(s) in the catalytic core. The cleavage rates at various Pb^{2+} concentrations for the wild type (Lz) and mutants 7-deaza-dG_4−Sub and rI_7−Lz are shown in Figure 6A,B. Because the leadzyme is inhibited by NaCl at low Pb^{2+} concentrations (data not shown), the kinetics were performed at 60 μM ribozyme without NaCl.

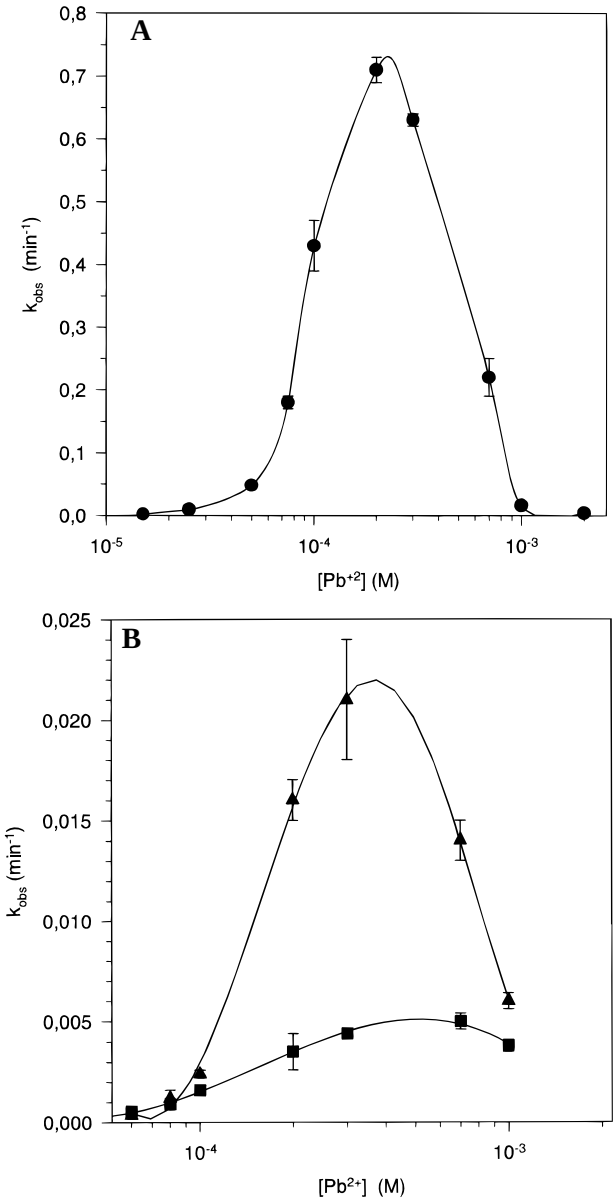

FIGURE 6: (A) Effect of Pb^{2+} concentration on the activity of the leadzyme: Lz (●). B) Effect of Pb^{2+} on the activity of mutants 7-deaza-dG_4−Sub (▲) and rI_7−Lz (■).

For both wild-type and mutant leadzymes, the activity increases with the Pb^{2+} concentration until a maximum is reached between 100 and 500 μM and then decreases at higher concentrations of Pb^{2+}. It is known that Pb^{2+} concentrations adopts a polymeric form at alkaline pH or at near neutral pH at high Pb^{2+} concentrations (Baes, 1976). The highest activity of the leadzyme is observed at 200 μM, which is close to the highest concentration of the mononuclear $(PbOH)^+$ species at pH 7 (Hugel, 1965). In a similar experiment, Pan and Uhlenbeck (1994) reported an optimal activity of around 30 μM of Pb^{2+}, i.e. 10-fold less than that for the ribozyme used in this paper. A possible explanation is that the ionic requirement for the formation of the ribozyme−substrate complex in *trans* could be higher than that for a *cis* complex.

These data were transformed (see Table 2) to calculate the Hill number (n) for the wild-type leadzyme and five analogs (Segel, 1993). This approach has been used by Smith and Pace (1993) for the detection of 2′-OH groups involved in magnesium binding in the ribonuclease P

Table 2: Hill Number for the Leadzyme Ribozyme and Analogs

position	substitution	Hill number (n)[a]
–	none	3.6
Sub C_1	rU[b]	2.6
Sub G_4	7-deaza-dG	3.1
Lz G_7	rI	2.0
Lz G_9	dG	2.2

[a] The Hill number (n) was obtained from the slope of the curve generated by plotting the $\log(v/V_{max}-v)$ versus $\log[Pb^{2+}]$ (Figure 6; Segel, 1993). V_{max} was taken as 0.74 min^{-1}. This value is almost identical to that of Pan and Uhlenbeck (1994), who determined the V_{max} at both ~30 μM lead and 200 μM Pb^{2+}/10 mM Mg^{2+}. [b] Measured with the ribo abasic-A_8 Lz derivative in order to avoid U $_1$·A_8 base pairing.

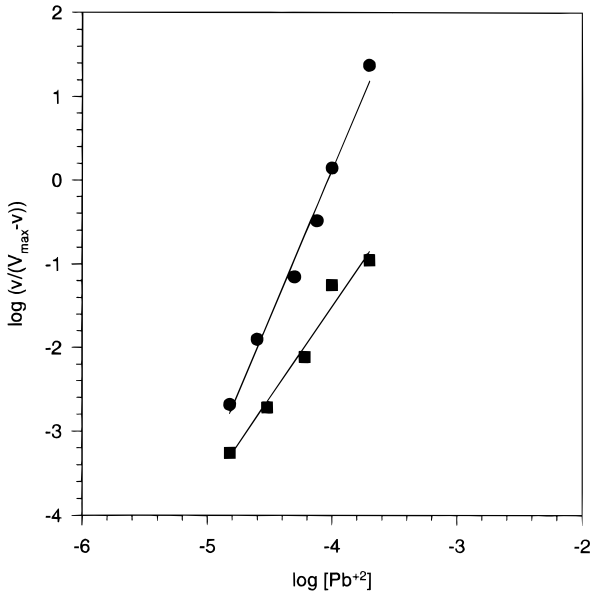

FIGURE 7: Hill plot of the wild-type ribozyme (●) and dG_9−Lz (■).

ribozyme. Our results show a strong cooperativity between catalytic activity and the Pb^{2+} concentrations with a Hill number of 3 for the leadzyme (Figure 7). The value for the different analogs varies between 2 and 3 (Table 2). For example, the I_7 and the deoxy-G_9 analogs show Hill numbers of 2, suggesting the loss of a cooperatively bound Pb^{2+}.

DISCUSSION

This work attempts to define the nitrogen base-derived functional groups in the asymmetric loop of the leadzyme that are important for the maintenance of catalytic activity. The three most important nucleotides, C_1, G_4, and G_7, thought to be essential in a previous study (Pan & Uhlenbeck, 1994), are indeed found to be crucial, since modification of the functional groups in these nucleotides leads to great losses in activity (Table 1).

The requirement for the N3 position or the N4 exocyclic amino group of C_1 has been evaluated using a uridine ribonucleotide. The high loss of activity seen in the U analog is large enough to sustain the idea that both functional groups could be involved in specific interactions in the active site (see below). The reduction in activity due to inverting the G_9·C_{10} base pair to C_9·G_{10} is unlikely to be due to the modification of a single functional group, since the effects of substitution by either dG or 7-deaza-dG at position 9 do not approach the magnitude of that of the inverted base pair.

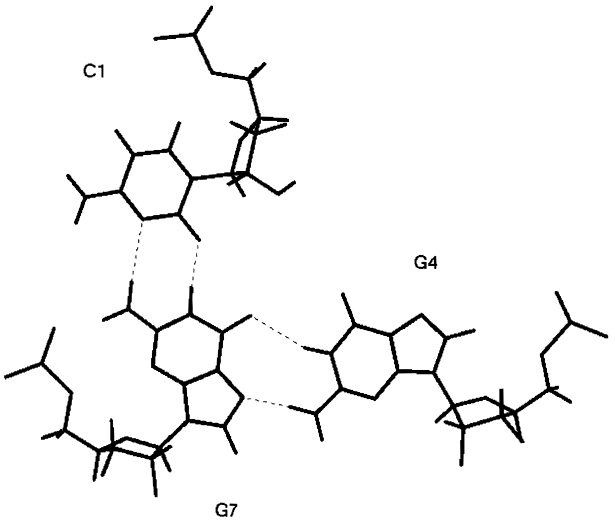

FIGURE 8: Proposed triple-base structure involving nucleotides C_1, G_4, and G_7.

The incorporation of modified nucleotides at position 8 revealed no structural requirement. Only guanosine was not incorporated since it might fold into the stacked loop and base pair with C_1. Even the presence of the large etheno-dA substitution decreased the activity by only 40%. Deletion of the entire base actually increased the activity. These results strongly suggest that this nucleotide is bulged out of the asymmetric loop and argue against the possibility that the protonation of the N1 group of the A_8 at pH 6.5 proposed by Legault and Pardi (1994) could be involved in the cleavage mechanism of the leadzyme. Alternatively, the increase in activity found with many analogs at position 8 compared to that of the wild-type sequence or to that of standard nucleotide substitution experiments (Pan & Uhlenbeck, 1994) could be due to the inability of modified nucleotides to engage in competing, inactive structures.

The high $\Delta\Delta G^{\ddagger}$ values observed with mutants of O6 and/or N1 of G_4 and G_7, and the N7 of G_7 (between −3.5 and −4.8 kcal/mol), support the idea that these functional groups are involved in important structural interactions. We have examined different pairing patterns of these nucleotides in light of their effect on activity. One attractive and sterically feasible possibility is that G_4 and G_7 interact in a non-canonical G·G base pair. The lack of an interaction between A_8 and C_1 would allow C_1 to interact with either G_9, G_7, or G_4. We have tested the feasibility of each of these possibilities by model building and find that, whereas interactions with nucleotides G_9 and G_4 are sterically impossible, interactions with G_7 are feasible. In fact, the finding that the N2 and N7 of G_7 are essential to the activity of the leadzyme is compatible with the idea that G_7 interacts with two different nucleotides. This logic leads to the proposal of a base triple between nucleotides C_1, G_7, and G_4 as shown in Figure 8. This structure requires a type VI G_4·G_7 base pair and a reverse Watson−Crick (type XXII) C_1·G_7 base pair (Saenger, 1984). Even if a base triple incorporating a reverse Watson−Crick C_1·G_7 pair has not yet been found in an RNA structure, it is theoretically possible, and a Watson−Crick base pair is sterically untenable in this context. We are currently exploring the catalytic core of the leadzyme for alternative structures using computer simulations with MC-SYM (Major et al., 1991).

In order to attempt an identification of the groups involved in lead binding, the activity of different analogs at various Pb^{2+} concentrations was measured (Figure 6). Because the saturation of the leadzyme by high concentrations of lead cannot be reached, it is not possible to determine if some analogs have a decreased affinity for Pb^{2+} compared to that of the wild type. Nevertheless, using Hill plots, it has been possible to identify functional groups whose removal results in a reduction of the cooperativity between the Pb^{2+} concentration and leadzyme activity. Thus, one explanation could be that the functional groups such as the N2 of G_7 and the 2′-OH of G_9 could participate in binding site(s) for Pb^{2+}. But since these groups are not good ligands for innersphere binding to Pb^{2+}, they would have to bind Pb^{2+} *via* water molecules.

Finally, our data open the door to a speculative but interesting proposal of a Pb^{2+} binding site in the vicinity of positions 3 and 4 of the asymmetric loop of the leadzyme. This A_3G_4 sequence can be compared to the Pb^{2+} binding site $A_{36}Y_{37}$ in the yeast $tRNA^{Phe}$ (Brown et al., 1985) in that the functional groups that we find important in the A_3G_4 sequence are the same as those involved in the Pb^{2+} binding in the $A_{36}Y_{37}$ sequence. The N6 and N7 functional groups of A_3 and the O6 and N7 of G_4 greatly affect the activity of the leadzyme and are not involved in the formation of the proposed base triple. In the $A_{36}Y_{37}$ sequence, the Pb^{2+} cation is bound to the N7 group of Y_{37} (a modified guanosine) and makes long contacts to the O6 of Y_{37}, to the N7 and N6 of A_{36}, and to the O2 of C_{32} and U_{33} (Brown et al., 1985). This hypothesis would explain the mutagenesis data of Pan and Uhlenbeck (1994) showing that only purines can be tolerated at position 3, since the replacement of A_3 by a G could maintain the same contacts with the Pb^{2+} cation, but substitution by a pyrimidine would not be tolerated. The effect on Pb^{2+} binding of the N7 of G_4 could not be measured because of the polymerization of lead at high concentrations. Even so, Hill plots of the 7-deaza-G_4 analog showed no reduction in the Pb^{2+} binding cooperativity (Table 2), although noncooperative binding could be involved.

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