Properties of an *In Vitro* Selected Pb^{2+} Cleavage Motif†

Tao Pan,‡ Bernhard Dichtl, and Olke C. Uhlenbeck*

Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado 80309-0215

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ABSTRACT: The addition of Pb^{2+} to a small RNA molecule consisting of an asymmetric internal loop of six nucleotides results in site-specific cleavage followed by hydrolysis of the 2′,3′-cyclic phosphate intermediate [Pan, T., & Uhlenbeck, O. C. (1992) *Nature 358,* 560–563]. Here we show that the reaction is highly specific for Pb^{2+} and the cleavage rate increases exponentially with pH from 5.5 to 7.0, both in the presence and in the absence of Mg^{2+}. This suggests that the reaction mechanism involves Pb^{2+} hydroxide acting as a base. Several sequence variants of the RNA are found to be equally active in both steps of the reaction, suggesting that they fold into a similar structure.

The property of yeast $tRNA^{Phe}$ to cleave at a specific site with Pb^{2+} (Dirheimer et al., 1972; Brown et al., 1985; Behlen et al., 1990) was used as a selective pressure in an attempt to identify $tRNA^{Phe}$ variants that had alternative tertiary interactions (Pan & Uhlenbeck, 1992a). Among the many RNA molecules that were isolated in this *in vitro* selection experiment, one was shown to contain an asymmetric internal loop of six nucleotides that was extremely susceptible to cleavage by Pb^{2+} (Pan & Uhlenbeck, 1992b). The reaction was found to occur in two steps, where a unique phosphodiester bond in the RNA was first cleaved to give a 2′,3′-cyclic phosphate intermediate and the cyclic phosphate was then hydrolyzed selectively to give a 3′-phosphate. In this paper we present an analysis of the metal ion requirements of the reaction, the pH dependence of the cleavage rate, and some of the sequence requirements of the motif. While several properties of the reaction resemble the Pb^{2+} cleavage of $tRNA^{Phe}$, others are quite different.

MATERIALS AND METHODS

Preparation of RNAs and Activity Assays. All RNAs were synthesized by *in vitro* transcription of synthetic oligodeoxynucleotide templates using T7 RNA polymerase (Milligan & Uhlenbeck, 1989) and purified on denaturing polyacrylamide gels. To assay RNAs for cleavage, 1 μM RNA in 15 mM MOPS,[1] pH 7.0, was heated at 85 °C for 2 min and cooled to 22 °C and $MgCl_2$ was added to the appropriate concentration (usually 10 mM). To initiate the cleavage reaction, freshly prepared $Pb(OAc)_2$ was added to the desired concentration and the reaction mixture was incubated at 25 °C. Aliquots of reaction mixtures were removed, and the reaction was stopped by addition of an equal volume of 9 M urea/50 mM EDTA. The unreacted RNAs and products were separated on 15% denaturing gels, and the radioactive bands were quantitated using a Phosphorimager (Molecular Dynamics). Cleavage rates were calculated as described in Pan and Uhlenbeck (1992b).

To assay the second step in the reaction, 2′,3′-cyclic phosphate hydrolysis, 1.5 μM purified 3′ cleavage product was combined with 0.3 μM 5′ cleavage product containing 2′,3′-cyclic phosphate ([5′-^{32}P]pGCGACC>p) in 15 mM MOPS, pH 7.0 and heated and cooled as described above; $MgCl_2$ was added to 2.5 mM, and the reaction was initiated with 50 μM $Pb(OAc)_2$ at 25 °C. Reaction aliquots were analyzed on 15% denaturing polyacrylamide gels to permit resolution of the hydrolysis product, [5′-^{32}P]pGCGACCp3′, from the starting material.

Analysis of RNA Mixtures. Two RNA mixtures were prepared from synthetic DNA templates containing equal amounts of the four nucleotides at specific positions. Mixture I was randomized at loop positions 1 and 4, and mixture II was randomized at loop positions 2, 3, 5, and 6 (Figure 3). Both mixtures were cleaved in 15 mM MOPS, pH 7.0, 10 mM $MgCl_2$, and 0.1 mM $Pb(OAc)_2$ at 25 °C for 3 min. The 5′ and 3′ cleavage products were purified from a denaturing gel.

To analyze the cleavage products at loop position 1, mixture I was prepared with [α-^{32}P]GTP, and the isolated 5′ cleavage product was digested in 5 μL of reaction mixture containing 0.2 unit of nuclease P1 and 0.3 unit of calf intestine alkaline phosphatase in 50 mM NH_4OAc, pH 7.0, for 30 min at 37 °C. The reaction mixture consisting of nucleoside 2,3′-cyclic phosphates (N>p) and inorganic phosphate was analyzed on polyethylenimine cellulose TLC plates (J.T. Baker Inc., Phillipsburg, NJ) developed with 1 M NH_4OAc as eluent. The relative amounts of N>p were quantitated on a Phosphorimager.

To analyze the cleavage products at loop position 2, the 3′ cleavage product from mixture II was phosphorylated with [γ-^{32}P]ATP and T4 kinase and then repurified on a denaturing gel. The resulting full-length RNA was digested with 0.2 unit of nuclease P1 in 50 mM NH_4OAc, pH 7.0, and the resulting ^{32}P-labeled nucleoside monophosphates were analyzed by two-dimensional TLC (Nishimura, 1979) and quantitated using a Phosphorimager.

To analyze the cleavage products at loop positions 3–6, the method of Silberklang et al. (1979) was used with modifications. The purified 3′ cleavage products from each mixture were first phosphorylated with nonradioactive ATP. The

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* Corresponding author.

‡ Present address: Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637.

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; GTP, guanosine 5′-triphosphate; LZ2, the *in vitro* selected RNA molecule that cleaves with Pb^{2+}; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TLC, thin-layer chromatography.

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repurified 5′-phosphorylated product was then partially hydrolyzed in 1 mM glycine and 0.4 mM $MgSO_4$, pH 9.5, by boiling for 70 s. The solution was then neutralized in 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol, and the RNA fragments were 5′-phosphorylated by addition of [γ-^{32}P]ATP and 3–5 units of polynucleotide kinase and incubation at 37 °C for 5 min. The mixture was loaded on a denaturing gel, and the RNA fragments of appropriate lengths were cut out, eluted, and ethanol precipitated. Each RNA was then digested with nuclease P1, analyzed by two-dimensional TLC, and quantitated as described above.

Cloning of Variant RNAs. A synthetic DNA oligonucleotide of the sequence 5′-pTGG TGC GAA TTA C($A_{1/3}$/$G_{1/3}$/$T_{1/3}$)($A_{1/2}$/$C_{1/2}$) CCA GAT CTT CAT CTG GC($C_{1/2}$/$T_{1/2}$) ($A_{1/3}$/$C_{1/3}$/$T_{1/3}$)GG TAA TCC GCT ATA GTG AGT CGT ATT ATA-3′ was annealed with the primer 5′-pTAA TAC GAC TCA CTA TAG CGG ATT-3′ by heating at 90 °C for 2 min, followed by incubation at 22 °C for 5 min. The primer was extended with murine Moloney reverse transcriptase at 37 °C for 20 min. The resulting double-stranded DNAs were then cloned into the blunt-end *Sma*I site of the plasmid pUC18. When the plasmid DNAs with inserts were sequenced, all contained two variant sequences of inverted orientation. This was due to our inadvertent choice of the primer, which was two nucleotides shorter at the 5′ end, resulting in extended double-stranded DNAs which had one blunt-end and one 5′-overhanging TA. Thus, two of the synthetic inserts were joined at these 5′-TA ends before ligation into the plasmid vector. Despite this drawback, the RNA transcripts of individual variants could still be isolated by cleaving the DNA plasmids with the restriction enzymes *Bam*HI and *Kpn*I to produce a 133-nucleotide DNA fragment with two divergent T7 promoters. RNA variants produced by transcription terminating at the *Bam*HI site contained seven additional nucleotides, 5′-GGGGAUC-3′, while variants produced by transcription terminating at the *Kpn*I site contained only two additional nucleotides, 5′-GG-3′. This difference of five nucleotides was sufficient to allow separation of the two variants on a denaturing gel and subsequent testing of the cleavage properties of individual variants. In a few cases, more than one clone of the same sequence was obtained which contained either the seven or the two additional nucleotides. In all cases tested, the cleavage efficiency appeared to be identical.

RESULTS

Divalent Metal Ion Requirements of the Cleavage Reaction. Since the original RNA motif was selected to cleave with Pb^{2+} in the presence of Mg^{2+}, it was of interest to determine whether Mg^{2+} was an essential component of the reaction. Using LZ2, an RNA similar to the one originally selected (Figure 1A), cleavage rates were determined in 15 mM MOPS, pH 7.0, with and without 10 mM $MgCl_2$ at varying Pb^{2+} concentrations (Figure 1B). In the absence of Mg^{2+}, specific cleavage is observed and the cleavage rate increases with Pb^{2+} concentration up to 25 μM and then decreases abruptly. Although the initial increase in rate may reflect an increase in binding of Pb^{2+} ion to LZ2, it is unlikely that saturation is reached. Instead, at higher Pb^{2+} concentrations, relative amounts of other forms of aqueous Pb^{2+} ion, such as Pb^{2+} polyhydroxides or polyhydrates, increase substantially at pH 7.0 (Kragten, 1978). These polymeric forms presumably cannot bind to the active site, resulting in reduced cleavage rates. In the presence of 10 mM Mg^{2+}, the cleavage rate is much slower at low Pb^{2+} concentrations but increases to a

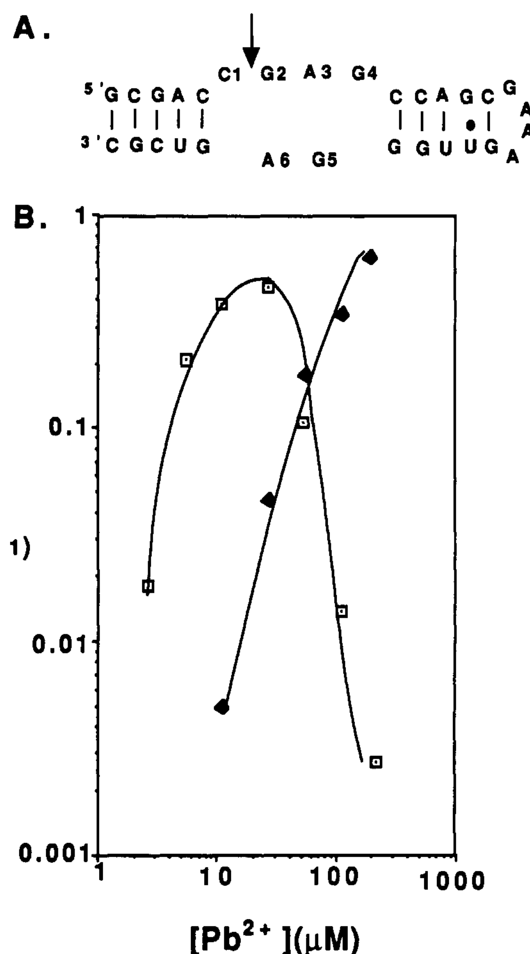

FIGURE 1: (A) Sequence of the selected RNA [LZ2 in Pan and Uhlenbeck (1992b)]. The cleavage site is indicated by an arrow. (B) Rates of cleavage in 15 mM MOPS, pH 7.0, with varying concentrations of Pb^{2+} in the absence (▫) or presence (◆) of 10 mM $MgCl_2$.

similar maximal value at higher Pb^{2+} concentrations. This suggests that Mg^{2+} can compete with Pb^{2+} for binding at a catalytic site. Mg^{2+} also appears to prevent the formation of Pb^{2+} polyhydroxide to some degree; this effect is likely to be due to increased ionic strength. In any case, the data clearly show that Mg^{2+} is not required either for the folding of LZ2 into an active conformation or for the catalytic mechanism. Pb^{2+} can promote cleavage by itself.

Most of the self-cleaving motifs studied thus far are active with a number of different divalent ions (Marciniec et al., 1989; Dahm & Uhlenbeck, 1991; Chowrira et al., 1993). LZ2 RNA was tested for cleavage in reaction mixtures containing 15 mM MOPS, pH 7.0, in which the Pb^{2+} was replaced by 1 mM Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+} or 50 μM Eu^{3+}, both in the absence and in the presence of 10 mM Mg^{2+}. No detectable cleavage was observed after incubation for 60 min at 25 °C (data not shown). Since the detection limit was about 0.05% product in these experiments, the rate of cleavage with the other metal ions is less than 10^{-3} min^{-1}, or about 10^3-fold slower than with Pb^{2+}.

Cleavage Rate Dependence upon pH. Changes in reaction rates as a function of pH can be a useful indicator of participation of basic and acidic groups in the rate-determining step. For LZ2, the cleavage rate increases exponentially with pH between 5.5 and 7.0 with or without 10 mM Mg^{2+} (Figure 2). At higher pH values, the rate decreases, presumably due to the formation of Pb^{2+} polyhydroxides or polyhydrates (Kragten, 1978). Since the slope of the linear part of the pH

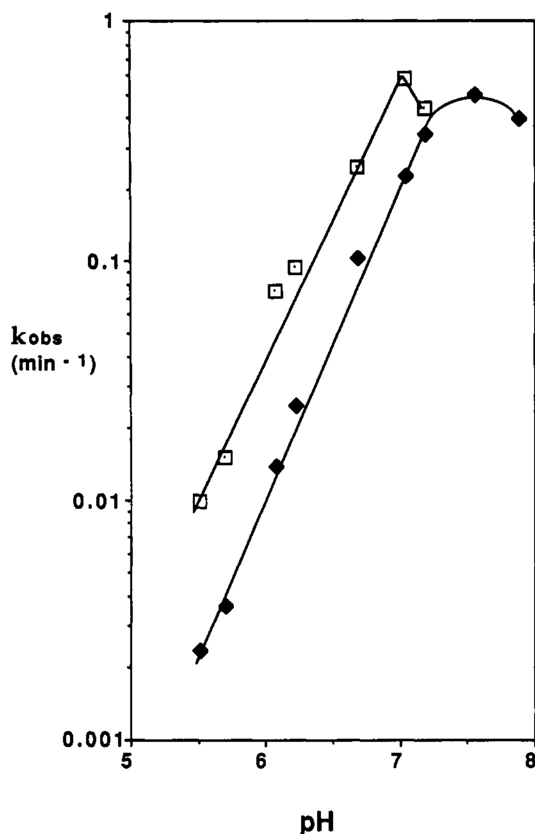

FIGURE 2: pH dependence of cleavage rates of LZ2 in 25 μM Pb^{2+} alone (⊡) or 50 μM Pb^{2+} and 10 mM Mg^{2+} (◆). The buffers used in this experiment are MES, pH 5.5–6.2; PIPES, pH 6.6–7.0; and MOPS, pH 7.0–7.9. The cleavage rates increase linearly with pH up to 7.0 (Pb^{2+} only) or 7.2 (Pb^{2+} + Mg^{2+}).

rate profiles is close to 1, the data is consistent with a basic group being involved in the rate-limiting step of the cleavage reaction. While it is likely that this base is the Pb^{2+} monohydroxide ($(Pb(OH))^+$), similar to the base in the cleavage of yeast $tRNA^{Phe}$ (Brown et al., 1985), the data cannot eliminate another basic group.

Although the cleavage experiments are not optimal to obtain rates of the second step of the reaction, it is clear from examining the relative amount of 5′ product with cyclic phosphate or 3′-phosphate that the second step is also considerably slower at lower pH (data not shown).

Active Sequence Variants of LZ2. We were interested in determining which nucleotides in LZ2 are required for cleavage and whether any RNAs with closely related sequences are active. While these goals could be reached by another *in vitro* selection using a library of sequences with a high level of mutagenesis in the region of interest (Bartel et al., 1991), the selection protocol we developed to find this motif (Pan & Uhlenbeck, 1992a) uses T4 polynucleotide kinase and T4 RNA ligase, whose sequence specificities are likely to introduce biases in the selection. Instead, a strategy was adopted that first analyzed RNA mixtures to identify combinations of sequences that were active and then tested cloned individual variants for activity. Attention was focused on the six nucleotides that formed the internal loop of LZ2.

RNA mixture I contained the 16 combinations of nucleotides at loop positions 1 and 4 (Figure 3A). This mixture cleaved to only 3–4% of the extent of LZ2, suggesting that only one or two of the 16 variants were active. This was confirmed by determining the identity of the nucleotides at positions 1 and 4 present in the products. Only C was present at position 1, and only G was present at position 4 (Table 1). Thus, when

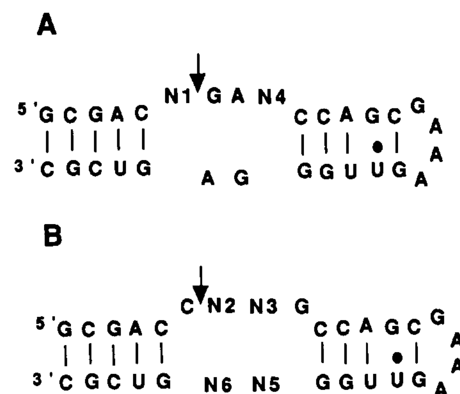

FIGURE 3: (A) Sequence of the mixture I containing randomized loop nucleotides 1 and 4. The arrow indicates the cleavage site. (B) Sequence of the mixture II containing randomized loop nucleotides 2, 3, 5, and 6.

Table 1: Nucleotide Composition (%) in Cleavage Products from RNA Mixtures

nucleotide position	U	C	A	G
mixture I				
1	*a*	>85		
4				>85
mixture II				
2	34		26	35
3			24	66
5	17		7	71
6	24	36	35	

[a] <5%.

loop positions G2A3G5A6 are fixed, the only active sequence is that of the original LZ2.

RNA mixture II contained 256 sequences where nucleotides at loop positions 2, 3, 5, and 6 were randomized (Figure 3B). This mixture also produced 3–4% of the amount of product generated by LZ2, suggesting that most of the sequence combinations were inactive. The identities of the nucleotides at each of these positions present in the products are summarized in Table 1. Each position showed a strongly biased sequence composition, clearly supporting the view that only a limited number of sequences were active. Position 2 contained little C; position 6, little G; position 3, little U or C; and position 5, little A or C. Thus, no more than 36 of the 256 sequences are active for cleavage in this mixture.

A DNA library containing these potential 36 active sequences and an upstream T7 promoter was cloned into a plasmid vector. Individual colonies were sequenced, and RNAs were synthesized by run-off transcription. A total of 20 different sequences were tested for Pb^{2+} cleavage (Table 2). Nine showed specific cleavage at the same site as LZ2, while the remaining 11 showed little or no cleavage. Since not all active variants were identified, it is not possible to define the sequence requirements with certainty. However, some trends are quite clear. All variants containing U5 cleave poorly, suggesting that the G5 is the preferred residue at this position. The small amount of U5 (Table 1) is likely due to contamination in the TLC analysis. However, we cannot rule out the possibility that the active variants containing U5 are not present in the sequences tested. The remaining three positions can accommodate several nucleotides, and there is some evidence that compensatory changes can occur. For example, although the G2U and A3G point mutants are active, they show decreased cleavage with respect to both LZ2 and the G2U,A3G double mutant.

Table 2: Pb^{2+} Cleavage of LZ2 Variants

nucleotide position				rel Pb^{2+} cleavage extent[a]
G2	A3	G5	A6	(1.00)
U	G	G	A	0.92
A	A	G	U	0.85
A	A	G	C	0.50
U	G	G	C	0.42
U	G	G	U	0.31
U	A	G	A	0.23
G	G	G	A	0.16
G	G	G	C	0.08
G	G	G	U	<0.04
A	A	U	A	<0.04
A	A	U	C	<0.04
A	G	U	C	<0.04
G	A	U	U	<0.04
G	G	U	A	<0.04
G	G	U	U	<0.04
U	A	U	C	<0.04
U	A	U	U	<0.04
U	G	U	A	<0.04
U	G	U	C	<0.04

[a] Cleavage was carried out with 1 μM ^{32}P-labeled RNA in 15 mM MOPS, pH 7.0, 10 mM $MgCl_2$, and 0.1 mM $Pb(OAc)_2$ for 3 min at 25 °C. The amount of product for each variant was normalized to the amount of cleavage of the cloned variant containing the wild-type loop sequence.

Table 3: Reaction Rates of LZ2 and Two Mutants

variant	cleavage rate (min^{-1}) Pb^{2+} + Mg^{2+}	Pb^{2+} only	hydrolysis rate (min^{-1})
LZ2	0.28 (1.00)	0.67 (1.00)	0.4[a]
G2A3 → U2G3[b]	0.10 (0.36)	0.21 (0.31)	1.8
G2A6 → A2C6[b]	0.087 (0.31)	0.34 (0.51)	1.6

[a] From Pan and Uhlenbeck (1992b). [b] The remaining sequence is identical to that of LZ2.

Two of the most active variants were chosen for further kinetic analysis (Table 3). The rates of cleavage of the G2U, A3G and G2A,A6C mutants were between 30 and 50% of that of LZ2 under two different conditions. Interestingly, the rate of hydrolysis for the two mutants was significantly greater than that of LZ2. Thus, the selected ribozyme is a member of a family of RNA sequences that can catalyze cleavage and hydrolysis reactions at identical sites.

DISCUSSION

We have described a number of properties of LZ2, an *in vitro* selected RNA motif that cleaves with Pb^{2+} to give a 2′,3′-cyclic phosphate. It is instructive to compare these properties to those of two other self-cleaving RNAs that give 2′,3′-cyclic phosphates and have been similarly characterized. One is the hammerhead ribozyme, a motif found in a variety of plant pathogenic RNAs that evolved by natural selection (Prody et al., 1986; Long & Uhlenbeck, 1993), and the other is the cleavage of yeast $tRNA^{Phe}$ by Pb^{2+}, a cleavage reaction of no known biological function that was found accidentally (Dirheimer et al., 1972; Werner et al., 1976). While all three RNAs require divalent ions for cleavage, their properties are quite different. In the hammerhead ribozyme, some metal ions, such as Mg^{2+} and Mn^{2+}, are capable of promoting both folding and catalysis, while others, such as Cd^{2+} and Co^{2+}, cannot catalyze the cleavage reaction efficiently unless another counterion, Na^{+} or spermine, is also present (Dahm & Uhlenbeck, 1991; Dahm et al., 1993). A similar situation is seen for the cleavage of $tRNA^{Phe}$ by Pb^{2+}. Although Mg^{2+} competes with Pb^{2+} at the cleavage site, a certain amount of Mg^{2+} is needed to fold $tRNA^{Phe}$ into the tertiary structure needed for cleavage (Krzyzosiak et al., 1988; Behlen et al., 1990). Thus, $tRNA^{Phe}$ will not cleave in Pb^{2+} alone. In contrast, while LZ2 was selected with Pb^{2+} and Mg^{2+}, cleavage by Pb^{2+} can occur in the absence of Mg^{2+}. Perhaps the structure of the small internal loop of LZ2 is simple enough that the low concentration of monovalent ions in the buffer is sufficient to permit proper folding. As is the case with $tRNA^{Phe}$ cleavage, Mg^{2+} competes with Pb^{2+} at the cleavage site.

The metal ion specificities of the three RNA cleavage reactions are quite different. When an RNA folding agent such as spermine is present, the hammerhead will cleave with a large number of different divalent ions (Dahm & Uhlenbeck, 1991). Pb^{2+}, however, is inactive in the hammerhead reaction. While Pb^{2+} is the most active ion for the cleavage of yeast $tRNA^{Phe}$, several other ions can substitute, e.g., Eu^{3+} (Marciniec et al., 1989) and even Mg^{2+} at higher pH values (Wintermeyer & Zachau, 1973; Marciniec et al., 1989). In contrast, LZ2 is highly specific for Pb^{2+}. Perhaps due to the selective pressure, the catalytic divalent ion binding site in LZ2 cannot accommodate other divalent ions.

A common feature of all three cleavage reactions is the rate dependence upon pH. The 10-fold increase in cleavage rate per pH unit clearly suggests that at least one basic group is involved in the catalysis. The function of this basic group is presumably to deprotonate the 2′-OH at the cleavage site to facilitate the nucleophilic attack of the 2′- oxyanion at the adjacent phosphodiester bond. It is likely that a specifically bound $M(OH)^{+}$ ion is the basic group in all three cleavage reactions. In the case of the hammerhead, this is supported by the fact that the reaction rate correlates reasonably well with the pK_a of the metal ion in the reaction (Dahm et al., 1993). The specific binding of a Pb^{2+} close to the cleavage site of $tRNA^{Phe}$ located by X-ray crystallography strongly supports such a mechanism (Sundaralingam et al., 1984; Brown et al., 1985). Like that of $tRNA^{Phe}$, the cleavage rate of LZ2 increases with pH until a maximum is reached around pH 7. Although these data are consistent with the pK_a of 7.2–7.7 for $Pb(OH)^{+}$, it is not possible to make such a conclusion with any certainty due to the increasing propensity of aqueous Pb^{2+} ions to form polyhydroxides and polyhydrates as the pH is increased above 7. It is likely that this side reaction is responsible for the decrease in both LZ2 and $tRNA^{Phe}$ cleavage rates above pH 7.0 since the concentration of free $Pb(OH)^{+}$ is reduced. Although the presence of Mg^{2+} alleviates the aggregate formation somewhat, the same effect is seen. Thus, the solution properties of Pb^{2+} ions must be considered when one uses Pb^{2+} cleavage as a tool for monitoring RNA structural changes (Clouet d'Orval et al., 1991; Streicher et al., 1993).

In the context of a defined RNA secondary structure, the sequence requirements for LZ2 activity are considerably simpler than those of the hammerhead and $tRNA^{Phe}$. Only two of the 11 single-stranded nucleotides in the hammerhead core can be mutated without reducing the cleavage rate (Ruffner et al., 1990). In contrast, while only 272 of the 4096 combinations of the six-residue internal loop of LZ2 were tested, a substantial number of these variants were active. In the background of LZ2, three nucleotides (C1, G4, and G5) could not be mutated, while the other three could vary. In one case, cleavage was more efficient with a double mutant (G2U,A3G) than with either of the single mutants. While the sequence requirements of LZ2 and the hammerhead will not be understood until a structure is available, the structure of $tRNA^{Phe}$ permits an understanding of the quite complex

sequence requirement of its cleavage by Pb^{2+} (Behlen et al., 1990). While most of the sequence is required to maintain the folded structure of the tRNA, a few nucleotides are also needed to bind the Pb^{2+} specifically.

In summary, while the *in vitro* selected Pb^{2+} cleavage motif is similar to other cleavage motifs in the catalytic requirements of metal ions and the rate dependence on pH, it is different in the requirements of divalent ions in folding and metal ion specificity in catalysis. Even with only six loop nucleotides, other active variants can be found with similar activities. Taken together with the results of the *in vitro* selection experiment that produced LZ2 (Pan & Uhlenbeck, 1992a), it is clear that many other RNA structures can utilize Pb^{2+} or other divalent metal ions to catalyze cleavage of specific phosphodiester bonds in RNA.

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