

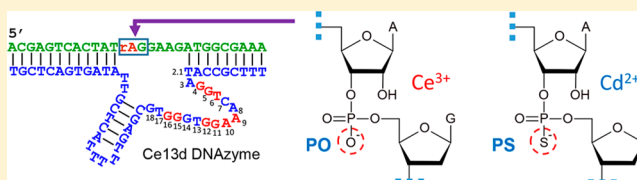
Biochemical Characterization of a Lanthanide-Dependent DNAzyme with Normal and Phosphorothioate-Modified Substrates

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Supporting Information

ABSTRACT: A trivalent lanthanide (Ln^{3+})-dependent RNA-cleaving DNAzyme, Ce13d, was recently isolated via in vitro selection. Ce13d is active in the presence of all Ln^{3+} ions. Via introduction of a single phosphorothioate (PS) modification at the cleavage site, its activity with Ln^{3+} decreases while all thiophilic metals can activate this DNAzyme. This property is unique to Ce13d and is not found in many other tested DNAzymes. This suggests the presence of a well-defined but general metal binding site. Herein, a systematic study of Ce13d with the PO substrate (using Ce^{3+}) and the PS substrate (using Cd^{2+}) is performed. In both the PO and PS systems, the highest activity was with $\sim 10 \mu\text{M}$ metal ions. Higher concentrations of Ce^{3+} completely inhibit the activity, while Cd^{2+} only slows the activity. A comparison of different metal ions suggests that the role of metal is to neutralize the phosphate negative charge. Both systems follow a similar pH–rate profile with a single deprotonation step, indicating similar reaction mechanisms. The activity difference between the R_p and S_p form of the PS substrate is <10 -fold, which is much smaller than most known RNA-cleaving enzymes. Mutation studies identified eight highly conserved purines, among which the two adenines play mainly structural roles, while the guanines are likely to be involved in metal binding. Ce13d can serve as a model system for further understanding of DNAzyme biochemistry and bioinorganic chemistry.



DNAzymes are DNA-based catalysts.^{1–7} Inspired by the chemical functions of ribozymes and the high stability of DNA, DNAzymes were first isolated via in vitro selection in 1994.⁸ Since then, a diverse range of DNAzymes catalyzing various types of chemical reactions have been reported.⁹ Aside from biochemical studies, DNAzymes are quite useful in biosensor development,^{10–12} viral and cancer therapy,^{1,13} and nanotechnology.¹⁴ Among the different types of DNAzymes, those cleaving RNA have attracted the most attention because they are usually small in size and highly active and can be readily used for the applications mentioned above.

To achieve effective DNAzyme catalysis, metal ions are often required.¹⁵ For example, Mg^{2+} is most frequently used because of its physiological abundance and DNAzymes intended for intracellular functions are believed to use Mg^{2+} .¹ Pb^{2+} and lanthanide ions (Ln^{3+}) are used because of their known activity in RNA cleavage.^{8,16–18} Ln^{3+} ions are important probes for nucleic acids.^{19–23} They are quite effective in hydrolyzing RNA,²⁴ and early examples can be traced back to the cleavage of tRNA.²⁵ Ln^{3+} ions inhibit the hammerhead ribozyme and the 8–17 DNAzyme,^{22,26} but accelerate the leadzyme and a Mg^{2+} -dependent ligase.^{33,34} Ln^{3+} ions were also used to select DNA-cleaving DNAzymes together with Zn^{2+} .²⁷ A Pb^{2+} -dependent DNAzyme (GR5) was found to have moderate activity with a few heavy Ln^{3+} ions (the highest rate is $\sim 0.02 \text{ min}^{-1}$).²⁸ We recently isolated a few trivalent lanthanide-dependent RNA-cleaving DNAzymes.^{16–18}

One of those Ln^{3+} -dependent DNAzymes (named Ce13d) is very interesting and deserves more careful studies. First, it works with all Ln^{3+} ions with a similar activity,¹⁶ suggesting that

it has a general Ln^{3+} binding site. Second, with a single phosphorothioate (PS) modification, it becomes active with all thiophilic metal ions (e.g., Cu^{2+} , Hg^{2+} , Cd^{2+} , and Pb^{2+}), while its activity drops significantly with Ln^{3+} .²⁹ Such a broad spectrum of metal activity is not observed with other tested DNAzymes such as the well-known 17E, the uranyl-dependent 39E, and GR5.²⁹ None of these DNAzymes work well with Cd^{2+} even with the incorporation of the PS-modified substrate.²⁹ In this work, we perform a careful biochemical characterization on Ce13d. In particular, the normal phosphate and the PS-modified substrates are compared using the same enzyme but with different metals. New insights are gained by the side-by-side comparison to explore the effect of metal concentration, pH, and mutations.

MATERIALS AND METHODS

Chemicals. All the DNA samples were from Eurofins (Huntsville, AL). The metal salts were from Sigma-Aldrich. The sequences and modifications of the DNA samples are listed in Table S1 of the Supporting Information. The buffers were from Mandel Scientific (Guelph, ON). Milli-Q water was used for making all the solutions and buffers.

pH-Dependent Studies. The DNAzyme complex was formed by annealing $5 \mu\text{M}$ FAM-labeled substrate (PO or PS) and $7.5 \mu\text{M}$ enzyme in water with 125 mM NaCl by warming

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observed in 2 h (black dots). At low Ce^{3+} concentrations, both the cleavage rate and the final cleavage yield increased with an increase in Ce^{3+} concentration. The cleavage activity peaked at $10 \mu\text{M}$ Ce^{3+} , where the final cleavage reached $\sim 80\%$. All the data can fit into a first-order reaction kinetics, and the rate constants are plotted in Figure 2C. At even higher metal concentrations (e.g., $\geq 50 \mu\text{M}$), however, the final cleavage yield dropped. With $50 \mu\text{M}$ Ce^{3+} , only 40% cleavage was achieved; with $100 \mu\text{M}$ Ce^{3+} , the final cleavage was $<20\%$. For these two samples, cleavage occurred in only the first 2 min, after which the enzyme was completely inhibited.

Because of the low final cleavage yield at high Ce^{3+} concentrations, the rate obtained from the fitting still increased (Figure 2C). To avoid this artifact, we did not include the last two data points in our binding curve and the apparent dissociation constant (K_d) was determined to be $27.5 \pm 18 \mu\text{M}$ Ce^{3+} for binding a single metal ion (inset of Figure 2C), which is comparable to the value obtained from the Tb^{3+} luminescence study.³⁴

This result suggests two competing processes at high Ce^{3+} concentrations. The DNAzyme can be either activated or inhibited by lanthanide ions. This is not surprising because Ln^{3+} ions are trivalent cations with strong affinity for the DNA phosphate and base. A high concentration of Ln^{3+} may disrupt nucleic acid secondary structures.³⁵ Our final DNAzyme concentration is $0.7 \mu\text{M}$ for the assays described above, and there are 78 phosphates in each DNAzyme complex, leading to a total phosphate concentration of $55 \mu\text{M}$. Because strong inhibition occurred with $50 \mu\text{M}$ Ce^{3+} , we suspect that when the ratio between phosphate and Ln^{3+} is $\sim 1:1$, Ln^{3+} ions start to disrupt the DNA structure. If this hypothesis is true, we should be able to delay the inhibition effect by using a higher DNA concentration. To test this, we fixed the substrate concentration and increased the enzyme strand concentration by 10-fold. Indeed, only moderate inhibition was observed with $100 \mu\text{M}$ Ce^{3+} (green dots, Figure 3). On the other hand, if the concentrations of both the substrate and enzyme strand were decreased to $0.1 \mu\text{M}$, inhibition occurred at even lower Ce^{3+} concentrations (black dots).

PS-Modified Substrate. Next, we varied the Cd^{2+} concentration for cleaving the PS-modified substrate (Figure 2E). A similar trend was observed, and inhibition occurred with $>50 \mu\text{M}$ Cd^{2+} . The Cd^{2+} -induced inhibition, however, is quite

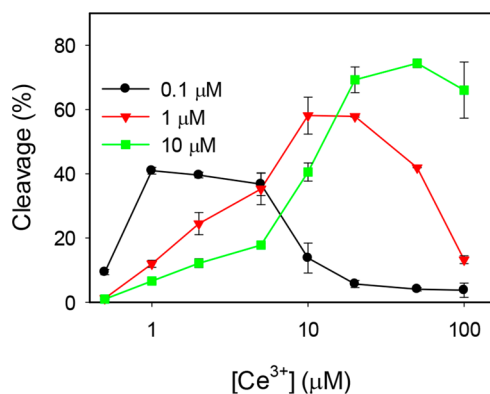


Figure 3. Percentage of the PO substrate cleavage by Ce13d in the presence of various concentrations of Ce^{3+} after 1 h. The DNAzyme concentration was $0.1 \mu\text{M}$ (substrate and enzyme), $1 \mu\text{M}$ (substrate and enzyme), or $1 \mu\text{M}$ substrate with $10 \mu\text{M}$ enzyme strand (green squares). The inhibition effect can be delayed by adding more DNA.

different from the Ce^{3+} inhibition. Even with $100 \mu\text{M}$ Cd^{2+} , time-dependent cleavage was still observed after 3 h, while with $100 \mu\text{M}$ Ce^{3+} , the DNAzyme was completely inactive after 2 min. Therefore, inhibition of the DNAzyme by Cd^{2+} is milder. Unlike a complete disruption of DNAzyme structure by Ce^{3+} , Cd^{2+} might cause only reversible changes in the DNAzyme. The K_d was calculated to be $6.0 \pm 2.8 \mu\text{M}$ for Cd^{2+} using the PS substrate (Figure 2F).

R_p and S_p Diastereomers. A PS modification results in two diastereomers: R_p and S_p .³⁶ The assays described above used a mixture of the two, and the rates obtained are averaged values. In fact, the single-exponential fittings in Figure 2D do not follow all the data points, and the system fits better using a biexponential equation. To gain further insights, we isolated each diastereomer by high-performance liquid chromatography and then tested with the two metals. The separation and chiral assignment were described in another paper.³¹ With Ce^{3+} (Figure 4A), the PO substrate is cleaved ($0.18 \pm 0.02 \text{ min}^{-1}$) 15 times faster than the S_p diastereomer ($0.012 \pm 0.001 \text{ min}^{-1}$), while the S_p substrate is cleaved 8 times faster than the R_p substrate ($0.0016 \pm 0.0002 \text{ min}^{-1}$). Because Ce^{3+} is a hard metal that likes oxygen-based ligands, it is likely that the pro- R_p oxygen is responsible for binding to Ce^{3+} . The assay with Cd^{2+} was further performed (Figure 4B), yielding an R_p rate of $0.12 \pm 0.02 \text{ min}^{-1}$, which is quite similar to that of the PO with Ce^{3+} . Therefore, the activity can be nearly fully rescued by Cd^{2+} . On the other hand, the Cd^{2+}/S_p rate is slightly slower (0.028 min^{-1}), while the PO substrate is essentially inactive with Cd^{2+} .

Most known RNA-cleaving enzymes (e.g., the hammerhead ribozyme,³² HDV ribozyme,³³ 10–23 DNAzyme,³⁷ RNase P,³⁸ and Group II intron³⁹) use pro- R_p for metal binding (mostly Mg^{2+}). This study also indicates that the pro- R_p oxygen is more important for metal binding in the Ce13d DNAzyme. The difference in the rate for the R_p and S_p substrates is quite small (8-fold with Ce^{3+} and 4-fold with Cd^{2+}), suggesting that even the pro- S_p oxygen atoms might contribute to metal binding. For comparison, the hammerhead ribozyme S_p form is >100 times faster than the R_p form in the presence of Mg^{2+} .^{32,40}

In broad terms, the role of metal in RNA cleavage is related to activation of the 2'-OH nucleophile by assisting its deprotonation, or neutralizing the negative charge on the phosphate at the transition state of the reaction.⁴¹ For the Ce13d DNAzyme, all the trivalent lanthanide ions (with PO substrate) and Cd^{2+} , Cu^{2+} , and Pb^{2+} (with the PS substrate) have similar activity (e.g., ranging from 0.02 to 0.18 min^{-1} with $10 \mu\text{M}$ metal ions). These data allow us to conduct an initial analysis. To activate 2'-OH, the metal ion acts as a general base, and the pK_a values of metal bound water need to be compared. For example, the pK_a for Pb^{2+} is 7.2 and for Cd^{2+} is 10.1.^{42,43} This approximately three-decade pK_a difference results in only an ~ 1 -fold difference in rate of the PS substrate. For the PO substrate, the pK_a of Ce^{3+} is 9.3, which is between those of Pb^{2+} and Cd^{2+} , yet Ce^{3+} has the fastest rate. Therefore, the pK_a of metal-bound water does not correlate with the observed activity, and the role of metal is thus unlikely for direct activation of the 2'-OH. This is quite different from the case of the 17E DNAzyme, for which a nice correlation can be observed between the metal pK_a and activity.^{42,44}

The switching of metal preference by the PS modification directly points at the importance of metal binding to phosphate. Compared to most divalent metal ions, which bind to nucleotide acids with millimolar affinity (e.g., using pUpU as

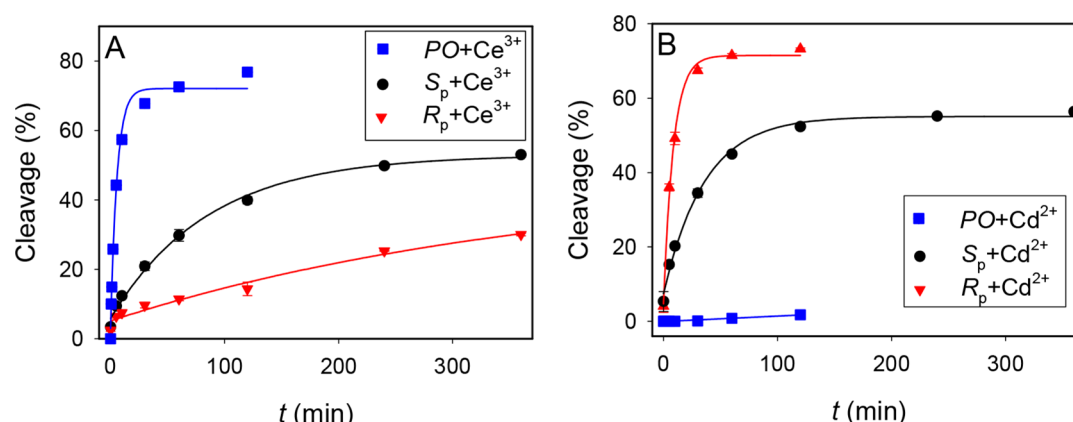


Figure 4. Cleavage kinetics of the three types of substrates (PO , R_p , and S_p) by the Ce13d DNAzyme in the presence of $10 \mu M Ce^{3+}$ (A) or Cd^{2+} (B).

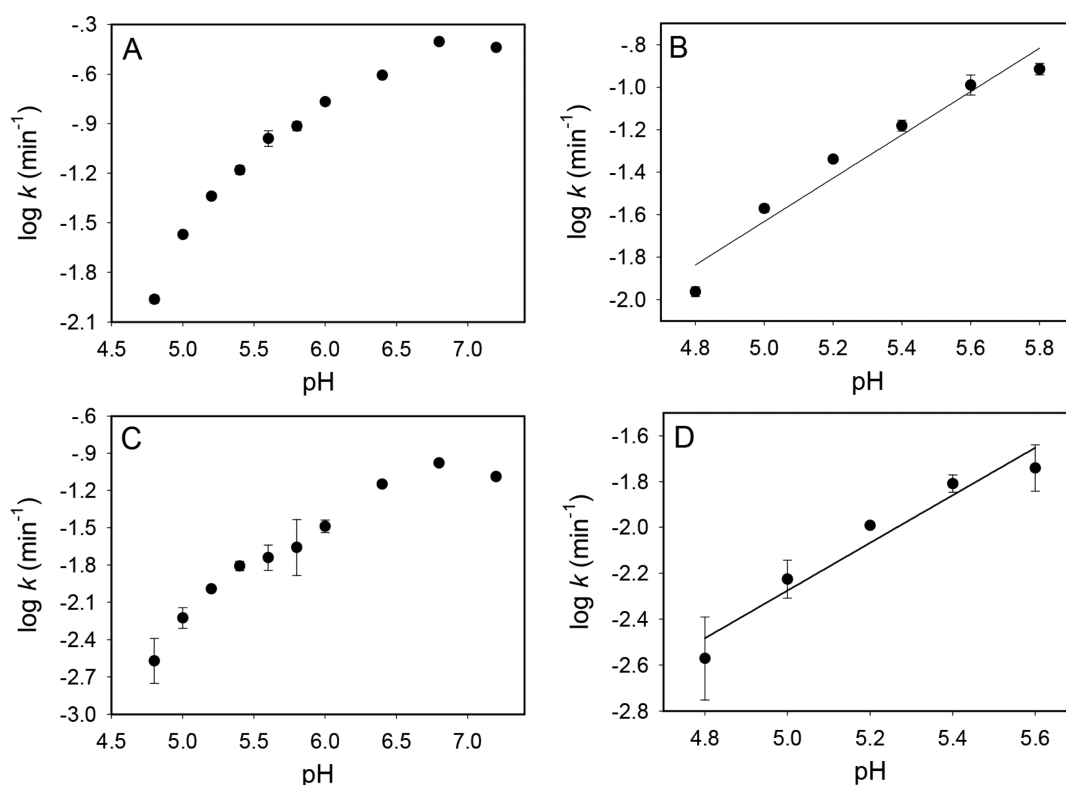


Figure 5. pH-dependent activity of the Ce13d DNAzyme with (A) Ce^{3+} for the PO substrate or (C) Cd^{2+} for the PS substrate. Fitting of the data in the pH region from 4.8 to 5.8 for (B) Ce^{3+} for the PO substrate or (D) Cd^{2+} for the PS substrate.

a model),³⁰ lanthanide binding yields micromolar affinity.⁴⁵ This is probably a primary reason that lanthanides are generally more active for Ce13. It needs to be noted that the +3 charge alone is insufficient, because Ce13d cannot be activated by Sc^{3+} .¹⁶ In addition to the phosphate coordination, the metal must also bind to other sites in the DNAzyme, and the binding pocket is sensitive to the metal size, as well. When the PS modification is introduced, binding to thiophilic metal ions is enhanced. For example, the log of the binding constant of $pUp(s)U$ to Cd^{2+} increases ~ 0.7 unit compared to that of $pUpU$.⁴⁶ Taken together, the metals are likely to play a role in neutralizing the phosphate charge.

Effect of pH. To gain further insights into the mechanism of Ce13d catalysis, we studied the enzyme activity as a function of pH first using the PO substrate and Ce^{3+} . In Figure 5A, the log

of the cleavage rate as a function of pH is plotted. In general, the rate is faster at higher pH values from pH 4.8 to 7. At even higher pH values, the kinetics are complicated and cannot fit to a single rate. Therefore, we focus our discussion in the slightly acidic region. After pH 6, the rate of increase slowed. In the pH 4.8–5.8 region, a linear relationship is obtained with a slope of 1.02 ± 0.12 [close to 1.0 (Figure 5B)], suggesting a single deprotonation step in the reaction process. This is a typical pH response for RNA-cleaving DNAzymes.⁴⁴ As explained in the previous section, this deprotonation is unlikely to be directly related to the metal ion.

A similar observation was made with the PS substrate in the presence of Cd^{2+} (Figure 5C). The slope in the pH region from 4.8 to 5.8 is 1.04 ± 0.15 (Figure 5D), also indicating a single deprotonation step. At each pH, the rate of this system is

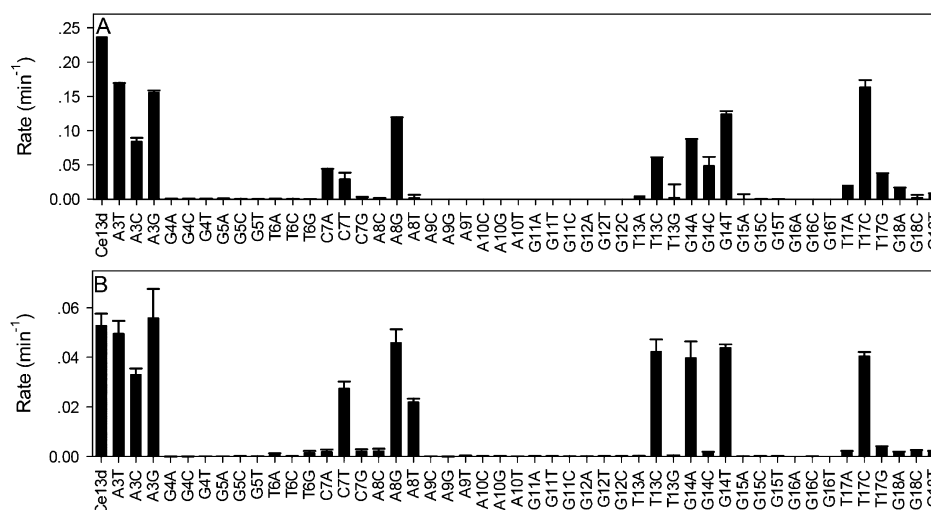


Figure 6. Activity of Ce13d mutants with (A) the PO substrate in the presence of 10 μM Ce^{3+} and (B) the PS substrate in the presence of 10 μM Cd^{2+} .

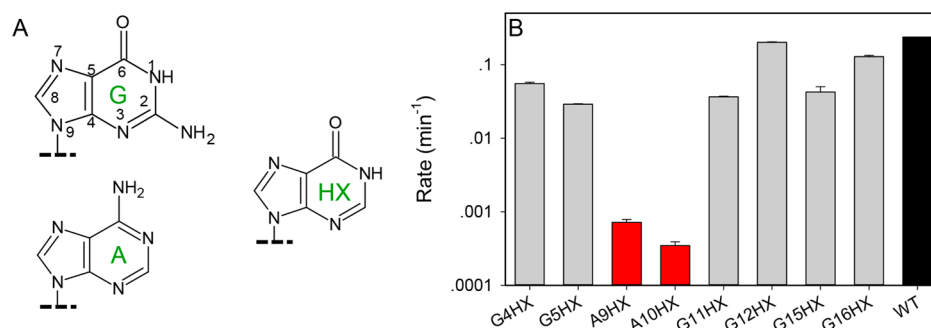


Figure 7. (A) Structures of adenine, guanine, and hypoxanthine (HX). Note that the amino group in G is required for quadruplex formation. (B) Activity of HX-substituted Ce13d mutants plotted on the log scale. All these nucleotides are highly conserved on the basis of the previous round of mutational studies. The rate for cleavage of the PO substrate in the presence of 10 μM Ce^{3+} is shown. WT denotes the wild-type DNAzyme. Bars for the two adenine mutations are colored in red, and their activity dropped by >100-fold compared to that of the wild type.

slightly slower than that of the normal PO/ Ce^{3+} system, but the trend of the rate change is comparable. From the pH probing standpoint, our results suggest that the mechanism of these two systems might be the same. The coordination environment of the metal is likely to be largely maintained in spite of the PO/PS switching. The only difference is that the metal ion is changed from Ce^{3+} to Cd^{2+} because of the PS modification, and the pH titration does not probe this metal binding to the phosphate.

Enzyme Mutation Studies. The Ce13d DNAzyme has a bulged hairpin structure, and our preliminary studies indicated that the hairpin plays only a structural role and catalytically important nucleotides reside in the bulged loop.¹⁶ To identify the important nucleotides and their roles in catalysis, we next performed a systematic mutation study. Mutation of conserved nucleotides often abolishes catalysis, thus giving us clues about the mechanism of DNAzyme action. Such mutation studies may also help us to identify novel DNAzyme variants with better activity.

First, the DNAzyme loop region was studied. The numbering of the nucleotides in the loop is shown in Figure 1A. We systematically mutated a total of 16 nucleotides, from A₃ to G₁₈. Each nucleotide was changed to the other three (e.g., A to C, G, and T), giving a total of 48 mutants. We first observed that the activity patterns for these mutants are very similar between the PO substrate with Ce^{3+} (Figure 6A) and the PS with Cd^{2+}

(Figure 6B), suggesting that the nucleotides in the loop are playing a similar role in both cases. A₃ is relatively well tolerated with respect to all the applied mutations, suggesting that this position does not serve an important functional purpose. On the other hand, eight purines (G₄, G₅, A₉, A₁₀, G₁₁, G₁₂, G₁₅, and G₁₆) are highly conserved (colored red in Figure 1A). Mutating any of them to any other nucleotide abolishes the enzyme activity. On the other hand, mutations in other nucleotides such as C₇, T₁₃, and G₁₄ do not completely disturb the enzyme activity; they are probably more important for the stabilization of the enzyme structure. Another observation is that some nucleotides can tolerate purine-to-purine or pyrimidine-to-pyrimidine mutations but not otherwise, for example, C₇T, T₁₃C, and T₁₇C. Overall, the current enzyme appears to be an optimal sequence, and most of the mutations decreased the enzyme activity.

Conserved Purine-to-Hypoxanthine (HX) Mutations.

Of these eight conserved purines, there are three pairs of guanine dimers (G₄G₅, G₁₁G₁₂, and G₁₅G₁₆). In addition, two unpaired guanines are present near the cleavage junction in the substrate. These four pairs may form a G-quadruplex structure, which is observed in a number of aptamers but not yet reported in RNA-cleaving DNAzymes.⁴⁷ To test this, we further mutated each guanine to HX. The structures of A, G, and HX are shown in Figure 7A. HX can be considered to be an intermediate molecule between A and G. Compared to G, HX misses the 2-

amine group, while compared to A, the 6-amino group in A is mutated to an oxo group.

Mutations to HX from G₄, G₅, G₁₁, and G₁₅ only slightly decreased the activity (<5-fold), while G₁₆ or G₁₂ to HX had almost no effect (Figure 7B). These results indicate that the 2-amino groups in these guanines are not involved in important structural or functional roles. The fact that all the important guanines can be mutated to HX rules out the possibility of a G-quadruplex, because in a G-quadruplex, the 2-amino group is critical for maintaining the hydrogen bonding network and HX cannot support it. Most of the discussions mentioned above are focused on metal binding, and we cannot rule out base mutation-induced changes in hydrogen bonding patterns in the DNAzyme and the related tertiary structure changes.⁴⁸ The resolution of biochemical assays cannot probe such effects, and more accurate spectroscopic methods are needed to fully understand it.

To gain further insights, we also mutated the two conserved adenines to HX. Interestingly, both mutations abolished the activity. This is in sharp contrast to the guanine mutations mentioned above. The 6-amino group in adenine is not a good metal ligand because its free electron pair is shared with the conjugated ring. On the other hand, the 6-oxo group in HX is a better metal ligand. Therefore, the abolished activity for these two substitutions is unlikely to be related to the loss of metal coordination. The 6-amino group in adenine is a hydrogen bond donor, while the 6-oxo in HX is a hydrogen bond acceptor. Therefore, these two adenines might be critical in hydrogen bonding, and they play a structural role.

CONCLUSIONS

In summary, we performed a careful biochemical study on a new lanthanide-dependent DNAzyme, Ce13d. Ce13d is the only known DNAzyme that works with all the lanthanides with the normal PO substrate, but with all soft metals with the PS substrate. The PS-modified substrate indicates a relatively weak thio effect for both the R_p and S_p forms, while metal binding is likely via the pro-R_p oxygen in the original substrate. We concluded that the metal is mainly responsible for neutralizing the phosphate charge instead of activating the 2'-OH. Both the PS and PO substrate cleave by the same mechanism as indicated by their similar pH profiles, where a single deprotonation step is involved. For metal concentration-dependent studies, both Ce³⁺ and Cd²⁺ inhibit their corresponding DNAzyme complexes at high concentrations. However, Ce³⁺ inhibition is stronger and takes place more quickly. The mutation studies have identified eight highly conserved purines, where the two adenines are believed to play mainly structural roles, while the six guanines might be involved in metal binding. The presence of a guanine rich metal binding pocket is a special feature of this DNAzyme.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00691.

A table with DNA sequences used in this work (PDF)

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Notes

The authors declare no competing financial interest.

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