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In Vitro Selection of a DNAzyme Cooperatively Binding Two Lanthanide Ions for RNA Cleavage

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Abstract

Trivalent lanthanide ions (Ln^{3+}) were recently employed to select RNA-cleaving DNAzymes, and three new DNAzymes have been reported so far. In this work, dysprosium (Dy^{3+}) was used with a library containing 50 random nucleotides. After six rounds of in vitro selection, a new DNAzyme named Dy10a was obtained and characterized. Dy10a has a bulged hairpin structure cleaving a RNA/DNA chimeric substrate. Dy10a is highly active in the presence of the five Ln^{3+} ions in the middle of the lanthanide series (Sm^{3+}, Eu^{3+}, Gd^{3+}, Tb^{3+} and Dy^{3+}), while its activity descends on the two sides. The cleavage rate reaches 0.6 min^{-1} at pH 6 with just 200 nM Sm^{3+}, which is the fastest among all known Ln^{3+}-dependent enzymes. Dy10a binds two Ln^{3+} ions cooperatively. When a phosphorothioate (PS) modification is introduced at the cleavage junction, the activity is dropped by more than 2500-fold for both the R_p and S_p diastereomers; and thiophilic Cd^{2+} cannot rescue the activity. The pH-rate profile has a slope of 0.37 between pH 4.2 to 5.2, and the slope was even less at higher pH. Based on these data, a model of metal binding is proposed. Finally, a catalytic beacon sensor is constructed which can detect Ho^{3+} down to 1.7 nM.

Since 1994,[1] *in vitro* selection has been used to isolate RNA-cleaving DNAzymes. DNAzymes are attractive because of their excellent catalytic efficiency, stability, cost-effectiveness, and programmability.[2-5] Divalent metal ions were conceived to be an indispensable part of DNAzyme catalysis.[2,6,7] The first DNAzyme was isolated in the presence of Pb^{2+} for RNA cleavage.[1] Many subsequent selections were carried out with physiological metals (Mg^{2+} and Ca^{2+}) for intracellular RNA cleavage.[8-11] To facilitate spectroscopic studies and further promote cleavage, transition metal ions were also employed, such as Mn^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}.[2,12-16] For environmental monitoring, selections were also carried out with toxic heavy metals such as Ag^{+}, Cd^{2+}, Hg^{2+} and UO_2^{2+}.[17-20]

In contrast, trivalent metals have not been systematically explored until recently. We hypothesize that trivalent lanthanide ions (Ln^{3+}) might be efficient for RNA cleavage, since Ln^{3+} are hard Lewis acids with strong affinity to DNA phosphate. In fact, even free Ln^{3+} can catalyze RNA cleavage, although it requires a high metal concentration with little sequence specificity.[21] The effect of Ln^{3+} was studied in a few known enzymes. For example, Ln^{3+} enhances the activity of the leadzyme by several folds.[22] The GR5 DNAzyme can also be moderately activated by Ln^{3+}.[23] On the other hand, Ln^{3+} inhibits the hammerhead ribozyme,[24] and the 8-17 DNAzyme.[25] In addition to RNA cleavage, Ln^{3+} ions also catalyze other reactions such as DNA cleavage and RNA ligation.[26,27]

Given these examples, we reasoned that *in vitro* selection in the presence of Ln^{3+} might produce new enzymes. Recently, we reported four Ln^{3+}-dependent RNA-cleaving DNAzymes.[28-31] The Ce13d DNAzyme was isolated using a Ce^{4+} salt and an N_{50} DNA library (e.g., a library with 50 random nucleotides).[28] Ce13d has a similar activity across the whole Ln^{3+} series (but is inactive with Ce^{4+}); and it also requires Na^{+}.[32-34] Next, Lu12 was isolated using a N_{35} library in

the presence of Lu^{3+}, a heavy lanthanide. Interestingly, Lu12 is more active with the light Ln^{3+}, showing a descending activity trend with the last few heavy Ln^{3+}.[29] This pattern is similar to another DNAzyme cleaving 2′-5′ linked RNA.[31] Finally, using the N_{35} library, we carried out three selections in the presence of Ho^{3+}, Er^{3+} and Tm^{3+}, respectively. A new DNAzyme named Tm7 was isolated. Tm7 is nearly inactive with the first seven light Ln^{3+} but is highly active with the heavy ones.[30] Tm7 binds three Ln^{3+} ions cooperatively, which has never been observed previously for DNAzymes.

To date, our N_{50} library was used only once with a light lanthanide (Ce). In our continuous effort to search for new Ln^{3+}-dependent DNAzymes, we herein employed the N_{50} library with a heavy lanthanide, dysprosium (Dy^{3+}). Dysprosium strongly absorbs neutrons and it has a high magnetic susceptibility, allowing applications in nuclear reactors and data storage. A new DNAzyme, named Dy10, was isolated and characterized. It has very high affinity for Ln^{3+}, binding two Ln^{3+} cooperatively for catalysis.

Materials and Methods

Chemicals. The *in vitro* selection related DNA and the beacon DNA samples were from Integrated DNA Technologies (Coralville, IA). All the other DNAs were from Eurofins (Huntsville, AL). See Table S1 and S2 for DNA sequence details. The lanthanides and other metal salts were from Sigma-Aldrich at the highest available purity. Tris(hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)ethanesulfonic acid (MES), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 3-(N-morpholino)propansulfonic acid (MOPS), EDTA disodium salt dihydrate, sodium chloride, sodium acetate, and ammonium

acetate were from Mandel Scientific Inc. (Guelph, Ontario, Canada). *In vitro* selection related molecular biology reagents were from New England Biolabs.

In vitro selection. The methods for *in vitro* selection, PCR, cloning, and sequencing were previously reported,[28] and are not repeated here. The only difference was that Dy^{3+} was used to induce cleavage. The selection conditions for each round are presented in Table S3. The round 6 library was cloned and sequenced, yielding a total of 40 sequences.

Gel-based assays. DNAzyme activity assays were performed with the FAM-labeled substrate (Sub-FAM, 0.7 μM) and the Dy10a enzyme (or other enzyme sequences, 1.1 μM). The DNAzyme complex was annealed in buffer A (50 mM MES, pH 6.0, 25 mM NaCl) before adding a metal solution to initiate the cleavage reaction. For the pH-dependent studies, the acetate, MES and MOPS buffers (50 mM with 25 mM NaCl) were used. The reaction products were separated on a denaturing polyacrylamide gel (dPAGE) and analyzed using a Bio-Rad ChemiDoc MP imaging system. Separation of the two diastereomers of the PS-modified substrate was performed following our previously reported procedure.[17]

Sensing. The sensor performance was studied using a microplate reader (SpectraMax M3). The sensor complex was formed by annealing the FAM-labeled substrate and the quencher-labeled enzyme (1:1.5 ratio) in buffer A. In each well, 100 μL of the complex containing 50 nM FAM-labeled substrate was diluted in 10 mM HEPES (pH 7.5). 1 μL of metal ion was added after 5 min of background reading and the signaling kinetics was monitored.

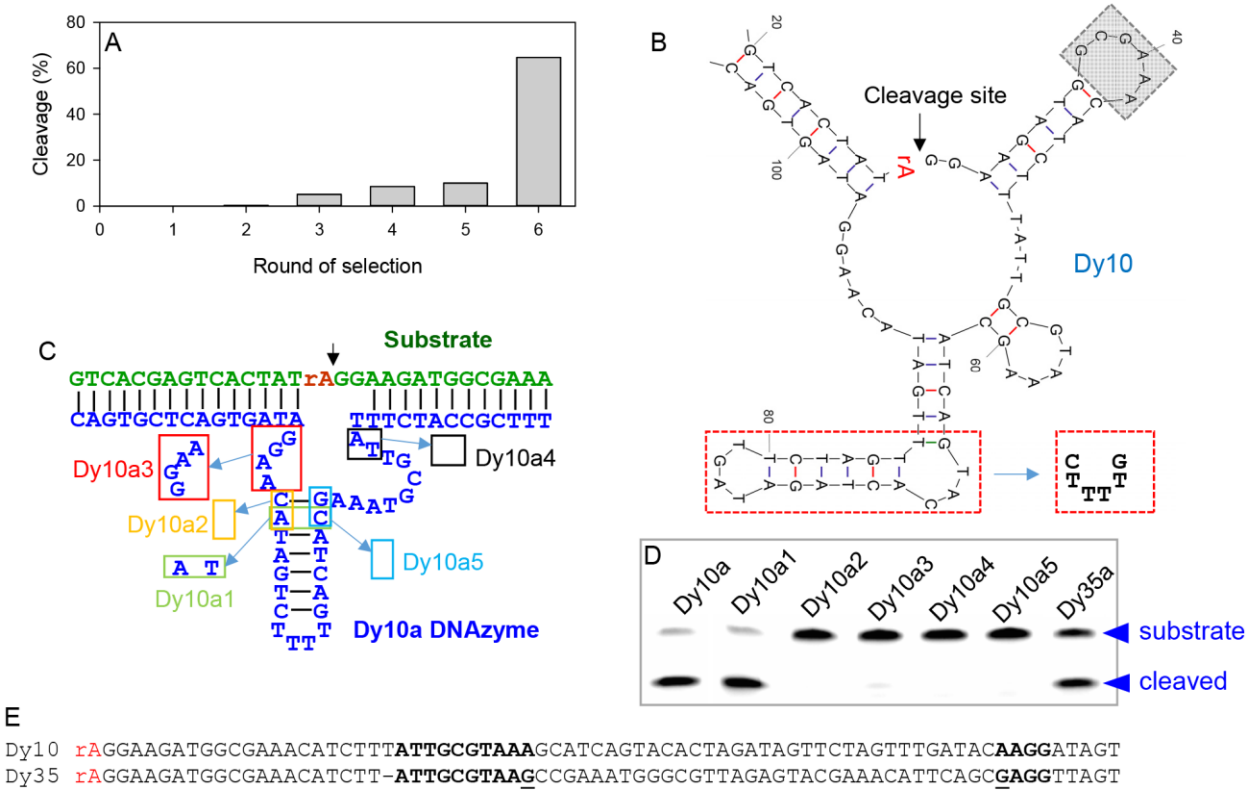
Results and Discussion

In vitro DNAzyme selection. With a DNA library containing 50 random nucleotides (N_{50}), we carried out *in vitro* selection in the presence Dy^{3+} following a previously established method.[28] The whole library had only a single RNA linkage serving as the putative cleavage site, while the rest are DNA. At each round, a fraction of the library may undergo self-cleavage after adding Dy^{3+}. This cleaved population may contain catalytically active sequences and they were harvested using gel electrophoresis and amplified by PCR. The incubation time and Dy^{3+} concentration for each round are shown in Table S3. After six rounds of selection, 67% cleavage was achieved (Figure 1A).

Secondary structure analysis. The round 6 library was cloned and sequenced, yielding 40 sequences. The majority of the library (85%) are similar to the previously reported Ce13d and Lu12 DNAzymes (see Table S4 for DNA sequence alignment).[28,29] This is not surprising, since both Ce13d and Lu12 are quite active with Dy^{3+}. For the remaining four sequences, two are identical (Dy1 and Dy17) but inactive (Figure S1). The other two, Dy10 and Dy35, are very similar (Figure 1E). Figure 1B shows the secondary structure of Dy10 predicted by Mfold,[35] and the cleavage site is marked with the arrowhead. This *cis*-cleaving structure can be easily converted to the *trans*-cleaving form by removing the nucleotides in the gray box. We also replaced the nucleotides in the red box by a smaller six-nucleotide loop, generating a truncated DNAzyme named Dy10a (see Figure 1C).

In addition to the hairpin, Dy10a has two loops. The smaller loop is an *AAGG* tetranucleotide. The other sequence (Dy35) has *GAGG* in this part (underlined in Figure 1E). For the larger loop, Dy10 and Dy35 differ only by one nucleotide as well. Therefore, these two highly conserved loops might be important for catalysis. To monitor cleavage, the substrate

strand in Figure 1C was labeled with a FAM (carboxyfluorescein). Indeed, Dy10a is active (Figure 1D, lane 1). We also converted Dy35 into a *trans*-cleaving form and it is also active (the last lane in Figure 1D). Since Dy10a appears to be different from all the previously reported Ln^{3+}-dependent DNAzymes,[28-30] we characterized it in detail.



E

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Dy10 rAGGAAGATGGCGAAACATCTTTATTGCGTAAAGCATCAGTACACTAGATAGTTCTAGTTTGATACAAGGATAGT
Dy35 rAGGAAGATGGCGAAACATCTT-ATTGCGTAAGCCGAAATGGGCGTTAGAGTACGAAACATTCAGCGAGGTTAGT
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Figure 1. (A) Progress of the Dy^{3+}-dependent selection. (B) The secondary structure of the *cis*-cleaving Dy10 predicted by Mfold. For a *trans*-cleaving DNAzyme, the nucleotides in the shaded box are removed and the nucleotides in the red box are replaced by the six nucleotides next to it. (C) The *trans*-cleaving Dy10a. Various mutations/truncations are also shown, where the boxed nucleotides are replaced. (D) A gel image showing activity of the mutants after 1 h reaction with 0.5 μM Sm^{3+}. (E) Sequence alignment of Dy10 and Dy35 starting from the cleavage site rA. The nucleotides in the two loops are shown in boldface; the underlined nucleotides highlight the difference.

With only two active sequences, the information from the DNA library is limited, only supporting the overall secondary structure and two mutable nucleotides. We performed rational mutation studies to test if further truncations are possible. In Dy10a, we assigned the *A*-*C* mismatch to be part of the stem region in the hairpin (see Figure 1C, green box). To confirm this, we mutated the *C* to *T* (named Dy10a1) forming a Watson-Crick base pair, and it is still very active (Figure 1D). Therefore, this *A*-*C* mismatch is indeed part of the hairpin. We next switched the *AAGG* tetranucleotide to *GGAA* (Dy10a3), and the activity was lost (note that *GAGG* is active). It appears that these purines cannot be randomly changed. Next, we gradually truncated a few nucleotides (Dy10a2, 4, 5), and they all inhibited the activity. Therefore, Dy10a appears to be an optimal construct already and further truncations cannot be made.

Dy10a is highly specific for Ln^{3+}. A criterion to confirm a new DNAzyme is to study the Ln^{3+}-dependent activity trend. For example, the Lu12 DNAzyme has descending activity for the last few heavy Ln^{3+}, while the Tm7 is active only with the seven heavy Ln^{3+}. For this purpose, the Dy10a DNAzyme was incubated for 5 min with the 14 Ln^{3+} ions (1 μM each). All the samples induced cleavage (Figure 2A), while the most active ones are in the middle of the series (e.g., from Sm^{3+} to Dy^{3+}). Next, we quantified the cleavage at 1 min and 5 min (Figure 2B). A bell-shaped activity pattern is obtained, which is different from all the previous DNAzymes. In addition, Dy10a is also quite efficient, cleaving >30% substrate in 1 min at pH 6 (1 μM Sm^{3+}).

Next, the activity of Dy10a with 21 other metal ions was studied. With 5 μM metal (see Figure 2C), only Y^{3+} showed activity. Y^{3+} has a size similar to Ho^{3+} and it can activate the previously reported DNAzymes.[28-30] Interestingly, 5 μM Pb^{2+} did not show any cleavage. Based on previous studies, Pb^{2+} is active with many DNAzymes.[1,8,28,36] With 100 μM metal (see Figure

3B), the activity of Y^{3+} dropped significantly due to inhibition, while Pb^{2+} showed moderate cleavage. Overall, Dy10a is highly specific for Ln^{3+}.

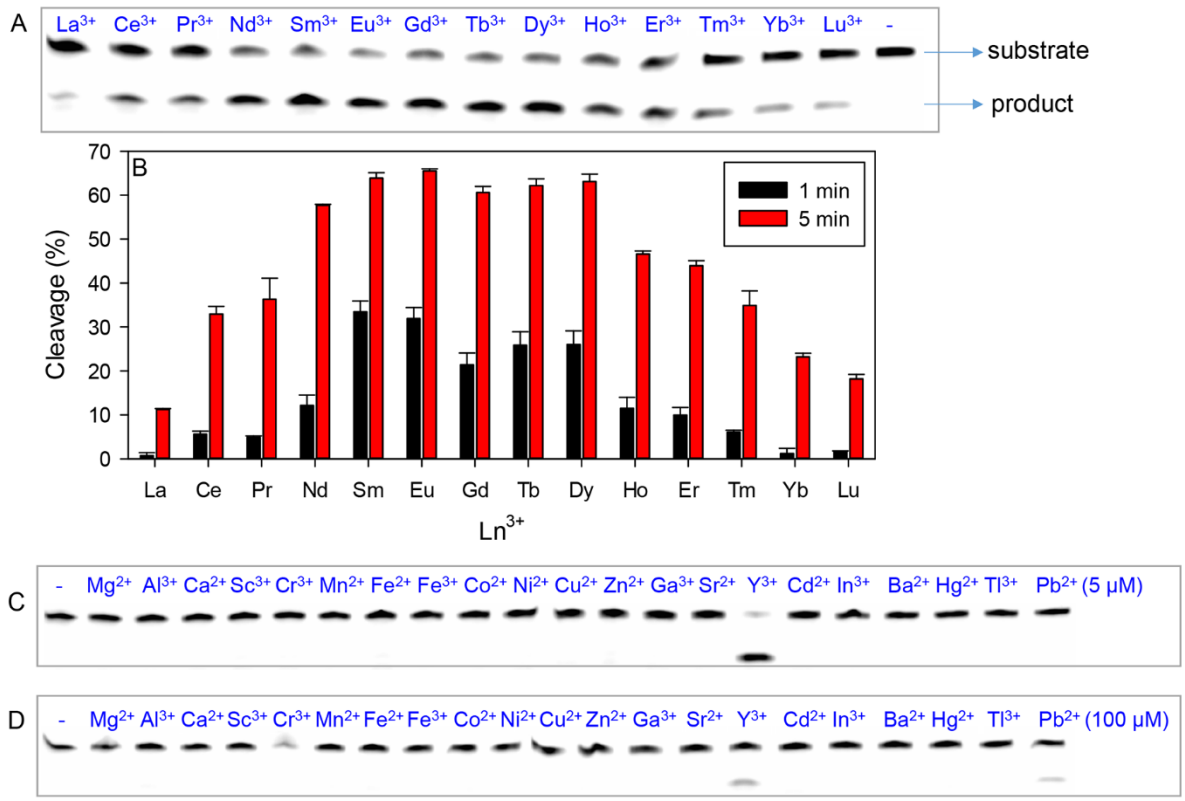

Figure 2. (A) A gel image of Dy10a cleavage in the presence of 1 μM Ln^{3+} for 5 min at pH 6. The last lane is the negative control without Ln^{3+}. (B) The percentage of substrate cleavage in the presence of 1 μM Ln^{3+} after 1 and 5 min reaction. Gel images of Dy10a metal specificity test in the presence of (C) 5 μM and (D) 100 μM of various metal ions at pH 6.0 for 1 h.

Dy10a has strong affinity for Ln^{3+}. After confirming metal specificity, a lanthanide concentration-dependent study was carried out. Sm^{3+} was chosen for this study since it is one of the most active metals for Dy10a (Figure 2B). A few kinetic traces are shown in Figure 3A; each was fitted to a first order kinetics. The rate constants are plotted in Figure 3B, which displays a

sigmoidal trend suggesting metal binding cooperativity. We then plotted the logarithm of rate against the logarithm of Sm^{3+} concentration (see Figure 3C). The highest activity occurs between 0.5 and 1 μM Sm^{3+}. At higher concentrations, Sm^{3+} starts to inhibit the activity. The apparent dissociation constant (K_d) from this gel-based assay is 0.1 μM Sm^{3+}, making it one of the tightest metal binding DNAzymes. The initial linear part was fitted to a straight line with a slope of 1.8, suggesting two metal ions operating cooperatively for catalysis.

Most previously reported RNA-cleaving DNAzymes or ribozymes bind only a single metal ion when probed by enzyme activity.[6,9] Dinuclear Ln^{3+} species for RNA cleavage was previously reported (slope = ~2) using their free metal salts (no DNAzymes).[21,37] However, those rates were very slow even with a high concentration of Ln^{3+} (e.g., a few mM). Cooperativity, using mixtures of Zn^{2+} and Sn^{4+}, was reported but the rate was extremely slow (~0.003 min^{-1}).[38] Some large ribozymes use multiple metal ions but without cooperativity.[39-41] It was proposed that the VS ribozyme binds four Mg^{2+} ions, but the affinity is very low (K_d = 17 mM).[42] We recently reported that the Tm7 DNAzyme employs three Ln^{3+} ions cooperatively.[30] In this study, Dy10a employs two Ln^{3+} ions, further supporting the assertion that it is a new DNAzyme. Dy10a is also a very efficient enzyme; its rate reaches ~0.7 min^{-1} with 0.5 μM Sm^{3+}, the highest among all reported Ln^{3+}-dependent DNAzymes at pH 6.

This Dy10 sequence only appeared twice in the final 40 sequences we obtained. Compared to Ce13d or Lu12 (85% of the final library), Dy10 has much higher activity at low metal concentrations. For example, with 0.5 μM metal, Dy10 is >10 times faster than the other two DNAzymes. The reason that Dy10 failed to dominate the library is attributed to its more easily inhibited by Ln^{3+}. For example, its activity is significantly suppressed with 10 μM Dy^{3+} (see Figure S2). For comparison, Ce13d works optimally with 10 μM Ln^{3+}.[28] We used 10 to 50

μM Dy^{3+} during this selection. Had the selection been carried out with a lower concentration of Dy^{3+}, we speculate that more Dy10 sequences would have been present in the final library.

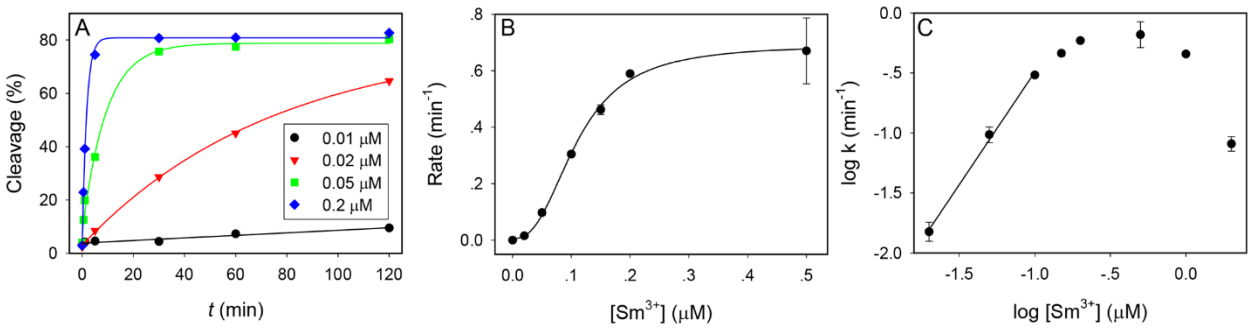

Figure 3. (A) The Dy10a DNAzyme cleavage kinetics at a few Sm^{3+} concentrations at pH 6. (B) Cleavage rate as a function of Sm^{3+} concentration. The trend is sigmoidal suggesting cooperative metal binding. (C) A double log plot with an initial slope of 1.8, suggesting two metal binding. At higher metal concentrations, the rate drops due to DNAzyme inhibition by Sm^{3+}.

pH-dependent activity. To further characterize Dy10a, a pH-dependent study was performed. Figure 4 plots the log of cleavage rate as a function of pH. Overall, the rate increases with increasing pH, and we divided the whole pH range into three distinct segments. From pH 4.2 to pH 5.4, the slope is 0.37. After that, the rate remained essentially constant (slope = 0.03) until pH 7.2. Further increase of pH even decreased the rate. Most RNA-cleaving ribozymes and DNAzymes have a slope of one for the pH-rate profile; this single deprotonation step is often attributed to activation of the 2′-OH nucleophile of the ribose.[43] This is certainly not the case for Dy10a. Such bell-shaped pH-rate profile with the initial slope much less than 1 is reminiscent of the VS ribozyme. In the VS ribozyme, a guanine acts as a general base to assist deprotonation of the 2′-OH.[44,45] At the same time, an adenine was proposed as a general acid to donate a proton to

the oxygen on the leaving group. The pH needs to be high enough to deprotonate the guanine (pK_a = 9.2 normally) but still low enough to protonate the N1 position of the adenine (pK_a = 3.5 normally). The optimal catalysis is achieved when the product of the acidic and basic species is the highest, which explains the bell-shaped pH-rate profile. The role of metal ions is to shift the pK_a of these bases.

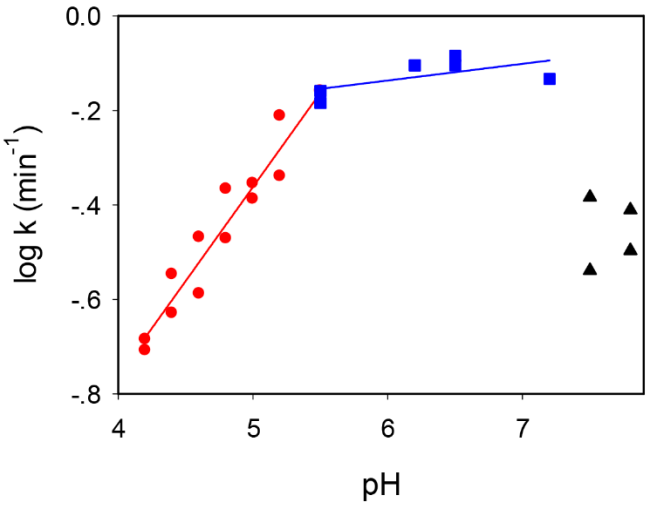

Figure 4. pH-dependent cleavage rate of the Dy10a DNAzyme in the presence of 0.5 μM Sm^{3+}. The pH range was divided into three segments based on the slopes. The buffers used were 50 mM acetate (pH 4.2 to 5.0) MES (pH 5.0 to 6.5) and MOPS (pH 6.5 to 7.8) with 25 mM NaCl.

We hypothesize that similar mechanisms might take place in Dy10a. The pK_a value of water bound to Sm^{3+} is 8.61.[46] In the pH range we studied, this water should remain charge neutral and may serve as a general acid. At present, it is unclear whether the general acid and base roles are carried out by two nucleobases (there are a few guanines and adenines in the enzyme loops), or by Ln^{3+}. To confirm the importance of the 2′-OH group, we also tested substrates with 2′-F (fluorine modified) and 2′-OCH_3 at the ribose ring, and neither can be

cleaved by Dy10a in the presence of Sm^{3+} (see Figure S3). Therefore, the reaction still relies on the 2′-OH. To confirm the cleavage product, we carried out further mass spectrometry studies. The 5′-fragment of the substrate has a cyclic phosphate, which is the standard product from the 2′-OH nucleophilic attack (see Figure S4).

Using 2 mM La^{3+} at pH 8.7 (no DNA), Chin and co-workers suggested a dimeric La^{3+} species that cleaves RNA at a rate of ~0.6 min^{-1}.[37] The pH-rate slope was 5, which was explained by metal ion hydrolysis and formation of the dimeric species required for catalysis. In another report, Matsumura and Komiyama used 5 mM Ln^{3+}. They also proposed a dinuclear Nd^{3+} complex, and the pH-rate slope was ~2 from pH 7 to 9.[21,47] Although the Dy10a DNAzyme also employs two Sm^{3+} ions, our results are quite different from those using free Ln^{3+} ions in a few aspects. First, our metal concentration was only 0.5 μM Sm^{3+}, which was 4,000 to 10,000 times lower than the previous work using free Ln^{3+}. Second, our studies were mainly carried out from pH 4.2 to 7 instead of above pH 7 by the others. Note that in our pH range, the free Ln^{3+} ions are essentially inactive for RNA cleavage. Due to the lower pH and Ln^{3+} concentration, it is less likely for Sm^{3+} to dimerize on its own to account for the two metal ion catalysis we observed. Instead, the DNAzyme scaffold must play a critical role to assist binding of the two metals. This may explain the difference in the pH trend. For free lanthanide ions, high pH and high metal concentration are required to form dimers.

Dy10a has a very large thio effect. Lanthanide ions are hard Lewis acids that like to bind to phosphate. Thus, phosphorothioate (PS) modification is a powerful method to probe such metal binding.[48-50] We replaced one of the non-bridging oxygen atoms at the cleavage junction phosphate by sulfur. By doing so, two diastereoiosmers were produced (referred to as R_p and S_p in Figure 5A). We separated them by HPLC following a previously reported method.[17] Most

known RNA-cleaving enzymes bind metal using the pro-R_p oxygen; when this oxygen is replaced by sulfur, the activity is significantly suppressed (typically by 30-200 fold).[32,48-51] On the other hand, if the pro-S_p oxygen is replaced by sulfur, the enzyme remains quite active (with only a several fold drop in activity).

The Dy10a rate with the R_p PS substrate is 0.013 h^{-1} (see Figure 5B, solid dots), which is >2500 times slower than the normal PO substrate. Intriguingly, the R_p rate is similarly slow (only approximately two-fold faster than the S_p rate). This is very different from most previously reported enzymes, where the S_p is typically faster by two orders of magnitude than the R_p using hard metal ions. This suggests that both non-bridging oxygen atoms might be used equally to bind metal, which is also consistent with Dy10a binding two metals. If both oxygen atoms are involved, replacing either one by sulfur will have a large effect on the rate.

Cd^{2+}, a thiophilic metal, is commonly used rescue enzyme activity after the PS modification. When Cd^{2+} was used, the activity was still quite slow (Figure 5D). Interestingly, activity with the S_p form became slightly faster than with the R_p form. The fact that Cd^{2+} also works but at similarly slow rates further suggests that both non-bridging oxygen atoms are involved.

This non-typical PS behavior might be related to the binding of two metal ions for catalysis. For comparison, the Tm7 DNAzyme binds three lanthanide ions and it is completely inactive with the PS modification, regardless of the R_p or S_p form, and regardless of the type of metal ion tested.[30] On the other hand, most DNAzymes/ribozymes binding one metal have partially retained activity with the PS substrate and the isomer can often be fully rescued by Cd^{2+}.

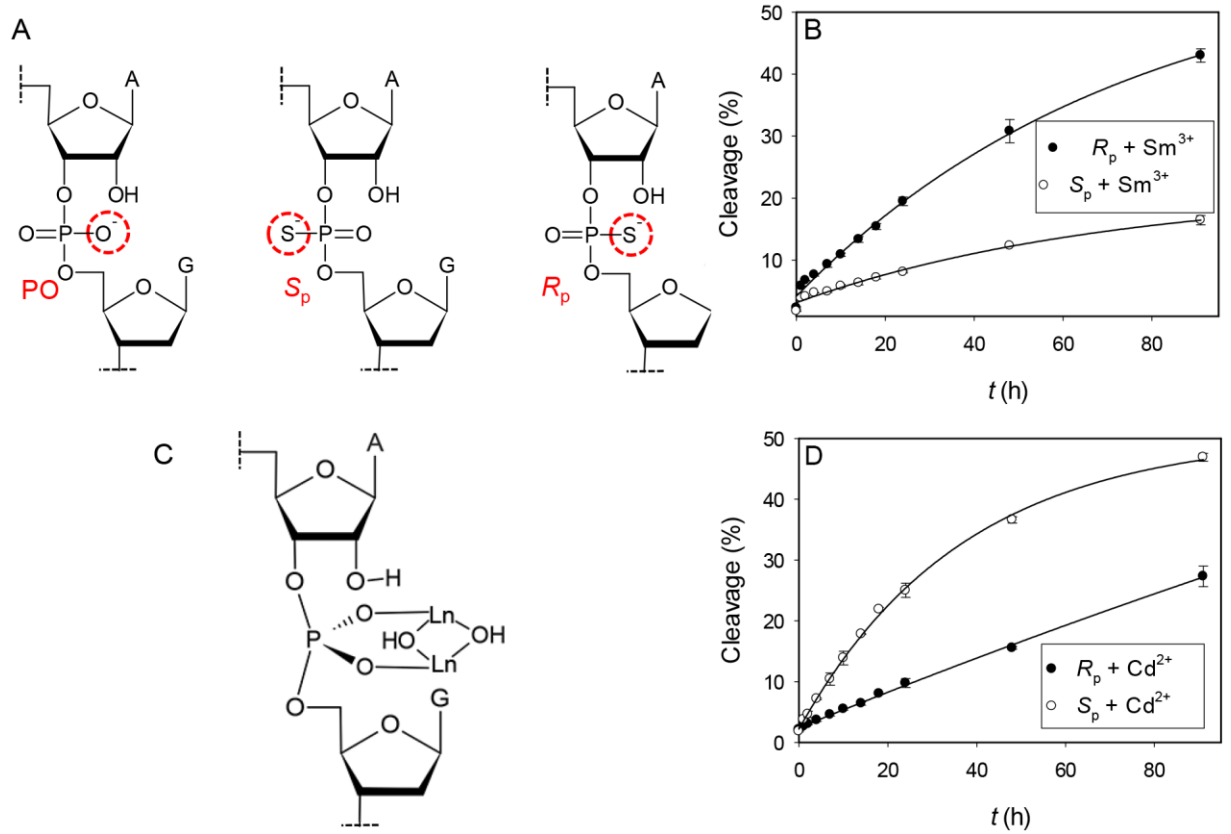

Figure 5. (A) A scheme of the normal PO, R_p and S_p cleavage junctions. Cleavage kinetics of the R_p and S_p PS substrate by (B) 0.5 μM Sm^{3+} or (D) 10 μM Cd^{2+} in 50 mM MES (pH 6.0) with 25 mM NaCl. Note these reactions were carried out up to 4 days. (C) A proposed scheme of a dinuclear lanthanide complex binding the cleavage site phosphate in Dy10a.

Based on the above biochemical studies, we proposed a metal binding scheme in Figure 5C. This scheme is inspired by the mechanism proposed by Komiyama and co-workers for free lanthanide ions.[21] Two Sm^{3+} ions are involved in the catalysis, and the PS-substitution experiment suggests that each Sm^{3+} binds to a non-bridging oxygen at the scissile phosphate.

Lanthanide sensing. The Dy10a DNAzyme is specific towards lanthanide ions as a group, and in particular, it is the most active with the five Ln^{3+} in the middle of the series. This trend is different from any known DNAzyme. We already reported four other lanthanide-specific DNAzymes,[28-31] and this is the fifth one. By accumulating sufficient number of such DNAzymes, we may be able to distinct each lanthanide by designing a sensor array, mimicking the human tongue.[52] To achieve this goal, we need to characterize the analytical performance of each DNAzyme. For this purpose, we designed a catalytic beacon sensor. The 3′-end of the substrate was labeled with a FAM fluorophore and the 5′-end of the enzyme labeled with a dark quencher (Figure 6A and Figure S5). In the initial hybridized state, the beacon has low fluorescence since the fluorophore is close to the quencher. Upon cleavage and release of the cleaved fragment, increased fluorescence is expected.[53]

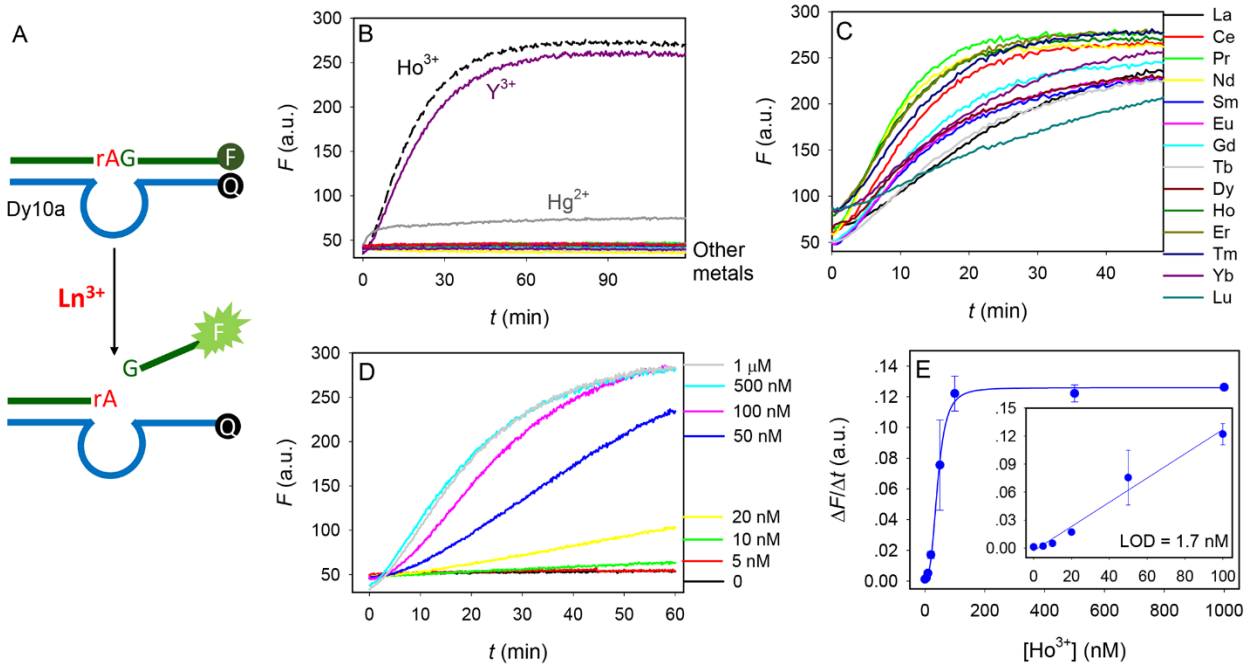

Figure 6. (A) A scheme of the Dy10a-based sensor. After cleavage and releasing of the cleaved fragment, fluorescence enhancement is observed. F = fluorophore; Q = dark quencher. (B) The

Dy10a-based sensor (50 nM) response to 0.5 μM of various metal ions. The list of the metals is in Figure 3. Sensor response to (C) 0.5 μM of various Ln^{3+}, and to (D) various concentrations of Ho^{3+}. (E) Sensor calibration curve based on the initial rate of sensor fluorescence increase. Inset: the initial linear response at low concentrations of Ho^{3+}.

In this experiment, we chose Ho^{3+} as a target, which has the highest magnetic strength among all known elements. Increased fluorescence was observed in the presence of Ho^{3+} and Y^{3+}, which can cleave the substrate (Figure 6B). While most other metal ions were silent as expected, we also observed a moderate signal increase in the presence of Hg^{2+}. A careful activity assay indicated no cleavage over a wide concentration range of Hg^{2+} (see Figure S6). Therefore, the Hg^{2+} signal might be due to other reasons such as DNAzyme folding induced by Hg^{2+}. For example, the enzyme strand contains quite a few thymines, which may form base pairs mediated by Hg^{2+}.[54] Similar artifacts were observed also for an Ag^{+}-specific DNAzyme.[20] Overall, this sensor has excellent specificity consistent with the gel-based assays. We then tested all of the Ln^{3+} ions (Figure 6C). While signal increase was observed in each case, La^{3+} and Lu^{3+} were among the slowest, also consistent with the gel-based assay.

Ho^{3+} was then chosen for measuring sensitivity (Figure 6D). Interestingly, almost no signal was observed with 5 nM Ho^{3+} and a significant enhancement was achieved with 10 and 20 nM Ho^{3+}. This observation also correlates well with the cooperative metal binding observed in the gel-based assays. Figure 6E illustrates the Ho^{3+}-concentration dependent rate of signal increase. The detection limit is 1.7 nM Ho^{3+} based on 3σ/slope, where σ is the standard deviation of background.

Conclusions.

In summary, using a library with 50 random nucleotides, we performed *in vitro* selection in the presence of Dy^{3+} to search for new RNA-cleaving DNAzymes. While the final library was dominated by two previously reported DNAzymes (Ce13d and Lu12), we also noticed a new sequence (represented by Dy10a) that is the most active in the presence of the middle five Ln^{3+} in the series. Further characterizations indicate that Dy10a binds two Ln^{3+} ions cooperatively with a strong thio effect; the cleavage rate is suppressed by over 2500-fold and they cannot be rescued by thiophilic Cd^{2+}. The pH-rate profile has a bell shape with the rising slope of 0.37, which is different from a slope of ~1 for most known DNAzymes. Compared to previously reported Ce13d (binding one metal with a normal PS effect), Tm7 (binding three metals with completely abolished activity with the PS substrate), the property of Dy10a fits in between (binding two metals with strongly suppressed PS activity). Thus, it is an interesting new member of the lanthanide-dependent DNAzyme family. Finally, a biosensor was designed with an ability to detect Ho^{3+} down to 1.7 nM.

Supporting Information. DNAzyme sequences, sequence alignment, mass spectrometry data, and further biochemical characterizations. "This material is available free of charge via the Internet at http://pubs.acs.org."

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