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Group trend of lanthanides binding to DNA and DNAzymes with a complex but symmetric pattern

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Using sensitized Tb^{3+} luminescence spectroscopy as a tool, binding of 14 lanthanides to a lanthanide-dependent DNAzyme is studied, where the binding affinity is symmetric cross the series and the tightest binding occurs with Nd^{3+} and Ho^{3+}. This trend does not correlate with DNAzyme activity, suggesting that metal binding may not be the rate-limiting step of the DNAzyme catalysis.

Lanthanides refer to the elements in the periodic table from La to Lu. Beyond their critical roles in modern technologies, lanthanides also emerge as important probes for biology and medicine.[1,2] In particular, they have been extensively used to study the structure and function of nucleic acids. These applications take advantage of a few of their unique properties. First, lanthanides and their complexes can efficiently cleave nucleic acids,[3] and thus are used as RNA structural probes[4] and DNA cleaving agents.[5] In addition, a number of *in vitro* selection experiments were carried out using lanthanides as metal cofactors to obtain DNA-based catalysts (so called DNAzymes[6-12]) for RNA or DNA cleavage.[13,14] Second, a few lanthanides (especially Tb^{3+}) are luminescent and DNA can act as an antenna to increase their light absorption and thus emission intensity. This is useful for probing metal binding sites,[15] and for developing biosensors.[16] Third, lanthanides are hard Lewis acids and some have a similar size as Ca^{2+}. Lanthanides can compete with other metal ions in enzymes and act as enzyme inhibitors. For example, both the 17E DNAzyme and the hammerhead ribozyme are inhibited by lanthanides.[15,17] On the other hand, the Leadzyme and a DNA-based ligase are accelerated by lanthanides.[18-20] All these examples suggest strong interactions between lanthanides and nucleic acids. Finally, nucleotides and lanthanides can form coordination complexes with useful luminescence and DNA binding properties.[21-24]

Given these progresses, few studies explored the binding of the whole lanthanide series with DNA or correlated metal binding with enzyme activity. Such studies are important for revealing the coordination chemistry of lanthanides, and for DNA bioinorganic chemistry in general.[25,26] We recently performed an *in vitro* selection experiment using Ce^{4+} as the intended metal cofactor. The selected DNAzyme (named Ce13d) was found to have similar activity with all trivalent lanthanides.[14] Therefore, Ce13d must bind all the lanthanides and this DNAzyme might provide a good scaffold for studying lanthanide binding. Herein, we employed Tb^{3+} luminescence as a tool to study lanthanide binding to DNAzymes and its relation to catalytic activity.

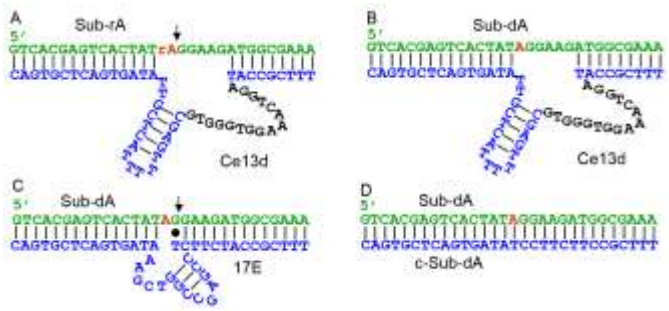

Figure 1. The DNA and DNAzymes used in this work. The secondary structures of the Ce13d DNAzyme with (A) the cleavable and (B) the non-cleavable substrate. They differ only by a single oxygen in the red adenine nucleotide. The catalytically important nucleotides are in the enzyme loop in black. (C) The 17E DNAzyme with the non-cleavable substrate. (D) The non-cleavable substrate and its cDNA forming a duplex.

The secondary structure of the Ce13d DNAzyme complex is shown in Figure 1A. It consists of a substrate strand named Sub-rA with a single RNA linkage (rA, ribo-adenosine), and an enzyme strand named Ce13d. In the presence of a trivalent lanthanide, the substrate is cleaved into two fragments at the position pointed by the arrowhead. Lanthanides alone can catalyze the reaction and no divalent metals are needed. To study metal binding and avoid cleavage, the rA base is replaced by a deoxyadenosine (Sub-dA, Figure 1B). Since the difference is only a single oxygen atom, the perturbation on metal binding should be minimal. For comparison, we also included a classic DNAzyme, 17E (Figure 1C),[7,27,28] which has a lanthanide binding site as well but lanthanides inhibit its Pb^{2+}-dependent activity.[17] Finally, we hybridized the substrate strand with its cDNA forming a duplex, where all the bases are paired and

lanthanide binding takes place only via the backbone phosphate (Figure 1D). This is intended to serve as a negative control. Among the different lanthanides, Tb^{3+} is frequently used because of its luminescence property. When excited at 290 nm, free Tb^{3+} has almost no emission due to its poor light absorption property (Figure 2A, yellow line). When mixed with the Ce13d DNAzyme complex (the construct in Figure 1B), strong emission peaks are observed (black line). The peak at 543 nm has the highest intensity and is used for monitoring subsequent binding assays. Under the same condition, we measured Tb^{3+} emission in the presence of the 17E DNAzyme (red line), where the intensity was only ~55% of that with Ce13d. With the duplex DNA in Figure 1D, the emission was close to the background value (green line). Therefore, the Ce13d DNAzyme binds Tb^{3+} most efficiently and the binding site should reside in the enzyme loop.

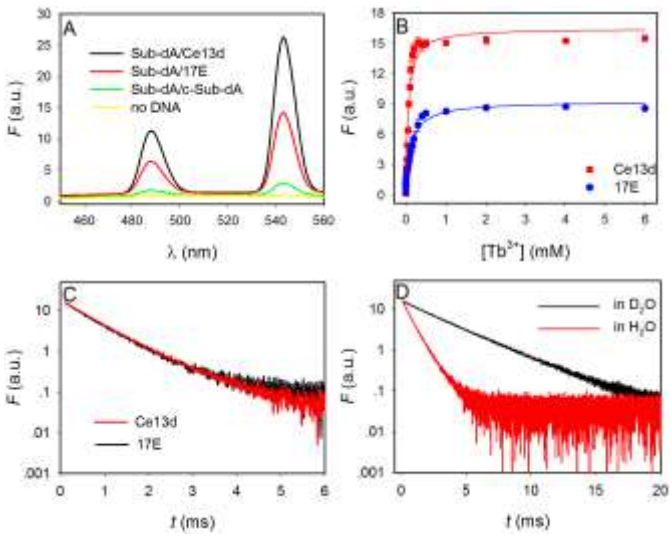

Figure 2. (A) Tb^{3+} luminescence spectra of its free ion (100 μM) and in the presence of various DNA or DNAzymes (1 μM). (B) Binding curve of Tb^{3+} by the DNAzymes measured by its luminescence intensity. The buffer contained 10 mM Mg^{2+} to minimize non-specific interactions. (C) Tb^{3+} emission lifetime follows single exponential decay for both DNAzymes. (D) Comparison of Tb^{3+} luminescence decay with the Ce13d DNAzyme in H_2O and D_2O.

Using the Ce13d and 17E DNAzyme complexes, we next measured binding affinity as a function of Tb^{3+} concentration (Figure 2B). The emission increased with increasing Tb^{3+} concentration, suggesting more complexes were formed and the dissociation constant (K_d) was calculated to be 60 μM for binding to one Tb^{3+} ion for Ce13d. This experiment was carried out in the presence of 10 mM Mg^{2+} to avoid non-specific binding,[15] and binding was slightly stronger in the absence of Mg^{2+} (e.g. K_d = 23 μM for Ce13d). The 17E DNAzyme has a lower final luminescence intensity and the K_d was 147 μM in the presence 10 mM Mg^{2+} (blue dots, Figure 2B). This suggests that both DNAzymes contain a well-defined binding site for Tb^{3+}. We previously probed the metal binding site in Ce13d using phosphorothioate modified DNAzymes, and a phosphate oxygen at the cleavage site is an important ligand for lanthanide binding.[29] The other ligands are believed to be in the nucleobases in the enzyme loop.

To further probe the metal binding sites, we also measured Tb^{3+} luminescence decay time (Figure 2C). Both Ce13d and 17E produced single exponential decay with Tb^{3+} (R^2>0.995), confirming the presence of just one type of metal binding site in both enzymes. This is consistent with the above metal binding curve. The lifetime was 0.672 ms for Ce13d and 0.709 ms for 17E in water, which is similar to that for the Tb^{3+}/hammerhead ribozyme system (0.53 ms).

Lanthanide luminescence lifetime measurement also allows us to probe inner-sphere coordination.[15,17,30] The O-H vibrational oscillation of water directly coordinated to Tb^{3+} is the main route for its non-radiative relaxation. This decay rate is proportional to the number of coordinated water. Since the O-D vibration is less efficient in the relaxation process, the lifetime should be longer. By measuring the lifetime difference due to the isotope effect, we can calculate the number of directly bound water. Indeed, as shown in Figure 2D, the emission decay in D_2O is significantly slower, with a lifetime of 2.95 ms for Ce13d. Based on this, the number of coordinated water is calculated to be 4.5 ± 0.1 for Ce13d and 4.8 ± 0.1 for 17E. Typically Tb^{3+} can be coordinated by nine water molecules. Therefore, about half of the coordination sites are replaced by the DNAzyme. We know that one of them is from the phosphate in the cleavage site,[29] and the rest 3-4 ligands are from the nucleotides in the loop.

With the well-defined metal binding site and its activity with all trivalent lanthanides, Ce13d provides a unique system to compare the binding of different lanthanides. First, we are interested in measuring their relative binding strength. Using Tb^{3+} as the probe, we hypothesize that a stronger metal can displace Tb^{3+} from the DNAzyme and thus decrease the luminescence signal (Figure 3A). Note that none of the other lanthanides can produce the same sensitized emission as Tb^{3+} at 543 nm. Even with Eu^{3+}, its sensitized emission is too weak to be measured under out experimental conditions. For this study, we added an equal concentration of Tb^{3+} and each of the other lanthanides to Ce13d. After overnight incubation, the emission spectra of the samples were measured and the Tb^{3+} luminescence intensities are shown in Figure 3B (black bars). The *x*-axis shows the increased atomic number of the lanthanides. We did not include Pm^{3+} since it is radio-active. The bar labeled with Tb^{3+} has the highest intensity since no other lanthanides were added and this serves as a reference. The pattern of binding strength is quite interesting; it is roughly symmetric centered with Tb^{3+}. Nd^{3+} and Ho^{3+} bind to the DNAzyme the strongest, while the lanthanides at the two ends and in the middle bind quite weakly.

For comparison, we also tested 17E, which is highly active in the presence of Pb^{2+} but is inhibited by lanthanides.[17] Interestingly, it has exactly the same trend as Ce13d (Figure 3B, red bars). These two DNAzymes have very different properties and sequence: one is activated by lanthanides and the other is inhibited by lanthanides. This observation led us to explore the relationship of luminescence intensity and DNA sequence. To have a complete understanding, we respectively measured the emission pattern with the 15-mer DNA homopolymers. We chose 15-mer DNA since it represents the enzyme loop size of Ce13d. The highest luminescence was observed with G_{15} (i.e. a 15-mer all-guanine DNA), which also showed the same trend of lanthanide size dependent emission (Figure 3C, yellow bars), while the other three DNAs produced similarly low emission. Guanine and poly-guanines in both DNA and RNA have been shown to have high affinity towards lanthanides.[31-33] In the hammerhead ribozyme, the binding of Tb^{3+} also points to a single guanine residue.[15] In this work, we compared the relative binding strength of different lanthanides. It is likely that the observed binding is originated from guanine and guanine binding determines both luminescence intensity and affinity. The other three nucleotides have

lower affinity for lanthanides and the emission enhancement factor is also low. Ce13d has a total of eight guanines in the enzyme loop. Since the above lifetime study indicates that maximally 4 ligands might be from nucleobases, it is unlikely that all these guanines are important for metal binding. More structural biology and biochemical experiments are needed to identify the exact binding mechanism.

This binding affinity pattern does not seem to correlate with any simple lanthanide atomic number dependent properties such as atomic or ionic radii, formation constants with common ligands (e.g. EDTA), enthalpy of hydration, entropy of hydration, enthalpy of oxide formation, metal-ligand distance, or polarizability.[34,35] Due to its symmetric pattern, it is however reminiscent of *f* electron filling in their trivalent ions. The tightest binding occurs when the number of unpaired *f* electrons is 3 (total *f* electron being 3 for $Nd^{3+}$and 10 for Ho^{3+}). Since no simple parameters correlate with our observation, multiple factors are likely to be involved to produce the binding pattern. The quantitative understanding of the coordination chemistry of this system will be a subject of future work.

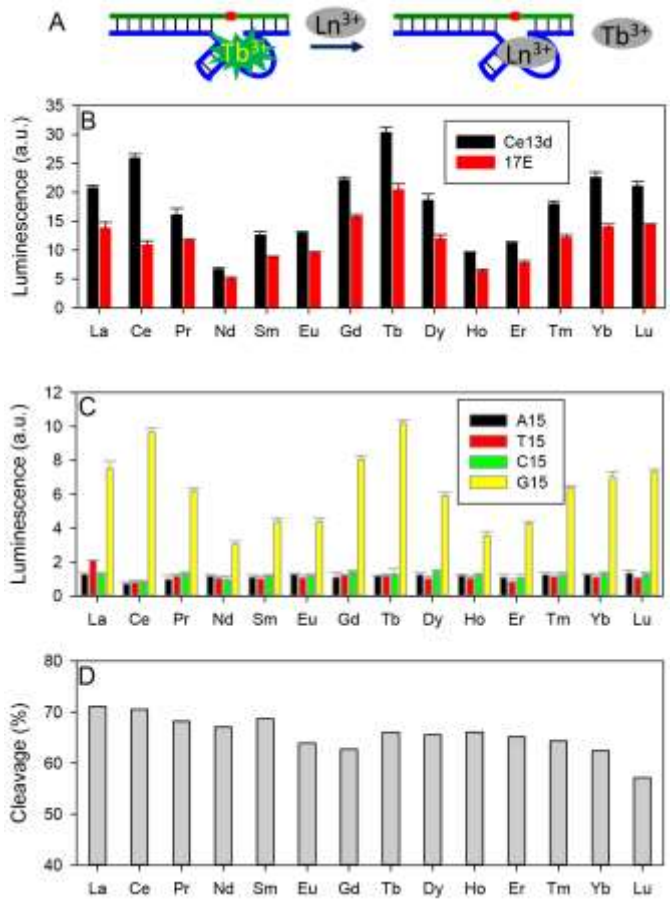

Figure 3. (A) A scheme showing a lanthanide competing with Tb^{3+} for DNAzyme metal binding site. This reaction allows the comparison of metal binding affinity. Ln^{3+} denotes for lanthanides other than Tb^{3+}. Tb^{3+} luminescence intensity when mixed with the DNAzymes (B) or DNA homopolymers (C) and after an equal concentration of another lanthanide was added. (D) Substrate cleavage fraction of Ce13d using the construct in Figure 1A in the presence of 10 μM of lanthanides after 1 h incubation.

Studying the group trend of lanthanides for nucleic acid binding was reported previously with the Leadzyme, which is a Pb^{2+}-dependent small ribozyme.[18,19] Sugimoto and Ohmichi found that lanthanides in general can accelerates the enzyme rate, although Pb^{2+} is still required for activity. Among the 11 lanthanides they tested, Nd^{3+} produced the highest rate enhancement. However, they did not include Ho^{3+} in that study. The reason for assisting cleavage was attributed to Nd^{3+} binding to the leaving group, which is a guanine.[19] This observation is consistent with our binding study. The difference is that we directly probe binding and only lanthanides are involved, which makes analysis more straightforward.

The above assays were carried out overnight to ensure that full equilibrium is reached. Next we performed kinetics studies. When Tb^{3+} was added to Ce13d, a quick initial increase was observed in the first 30 sec, followed by a gradual increase that appeared to plateau in 10 min (Figure 4A). Therefore, we pre-incubate Tb^{3+} and the DNAzymes for most experiments to ensure stable signal. For displacement assays, the kinetics appear to be much faster and the majority of luminescence intensity change occurred in the first 30 sec (Figure 4B). The slow kinetic components might be related to the adjustment of DNA conformation to accommodate lanthanides in the tightest binding site.

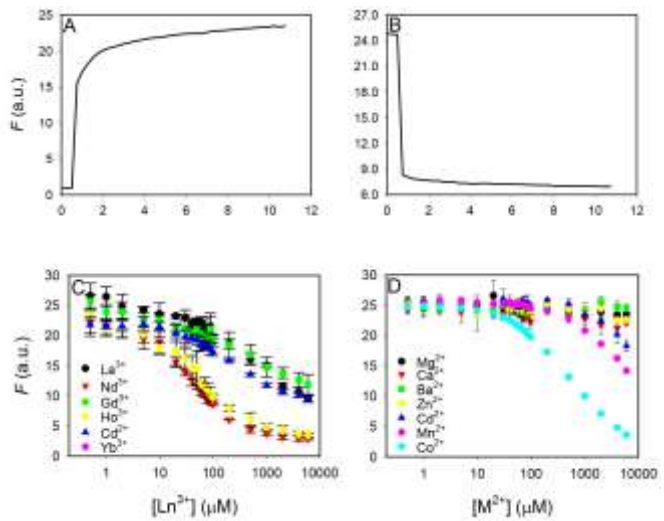

Figure 4. (A) Tb^{3+} luminescence increase kinetics after mixing with Ce13d. Tb^{3+} was added at 30 sec. (B) Kinetics of luminescence change after adding 100 μM Ho^{3+} to the mixture of Tb^{3+} (100 μM) and Ce13d (1 μM). (C, D) Affinity of metal binding to Ce13d measured by the displacement of Tb^{3+}. The system initially contained the Ce13d DNAzyme (1 μM) and Tb^{3+} (100 μM). Other metal ions were titrated and the Tb^{3+} luminescence was measured. (C) Titration of lanthanides. (D) Titration of divalent metal ions.

To have a quantitative comparison, we also measured titration curves to six selected lanthanides (Figure 4C) and other metal ions (Figure 4D). First, Tb^{3+} and Ce13d were mixed and the other metals were gradually added. The luminescence intensity drop was then monitored. Among these, Nd^{3+} and Ho^{3+} showed the strongest luminescence inhibition (inhibition constant K_i = 33.3 μM and 40.4 μM, respectively), which is consistent with the above data. Since we used a Tb^{3+} concentration of 100 μM, these two lanthanides bind to the DNAzyme more tightly. The other lanthanides require higher concentration to displace Tb^{3+}. Alkaline earth metals (Mg^{2+}, Ca^{2+}

and Ba^{2+}) cannot displace Tb^{3+} even at 6 mM concentration (Figure 4D). For the transition metals, Co^{2+} has the strongest inhibition effect with a K_i of 286.5 μM. This experiment further established the binding strength of different metal ions and confirmed the reversibility of lanthanide binding.

Ce13d only requires lanthanide for activity and it is active with all the trivalent lanthanides, which makes it a perfect scaffold for studying lanthanide binding. For example, the Leadzyme or the 17E DNAzyme requires Pb^{2+} for activity, which may complicate analysis. To correlate metal binding with catalysis, we next measured the cleavage activity under the same buffer condition as the above binding assays using 10 μM lanthanides (Figure 3D), where the cleavage only decreased slightly for the larger lanthanides. This trend does not correlate with the binding data. Since binding is a required step for catalysis, our data suggest that binding of lanthanide may not be the rate-limiting step in the whole catalytic process. Subsequent chemical events following metal binding determine the catalytic activity. Another possibility is that in addition to activating the DNAzyme, lanthanides also can inhibit DNAzymes at moderate concentrations (e.g. higher than 10 μM).[14] Therefore, those showing tighter binding may also be stronger inhibitors. In the concentration range we are working with, it is possible that the observed rate is a combined result of activation and inhibition, which is not directly reflected in this Tb^{3+} luminescence binding assay. Systematic biochemical characterizations are needed to gain further quantitative insights.

In summary, we studied the binding affinity of different lanthanides to a lanthanide-dependent DNAzyme, Ce13d. Binding affinity shows a complex lanthanide size dependent trend, where the tightest binding is with Nd^{3+} and Ho^{3+}. This trend correlates with the number of unpaired *f* electrons being 3 (half of the number of total *f* orbitals). Guanine is the main nucleotide responsible for lanthanide binding. This is the first time that lanthanide binding affinity to DNAzyme was compared. Lanthanide binding may not be the rate limiting step of this DNAzyme and subsequent steps after binding are likely to be more important to determine its catalytic rate.

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Notes and references

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