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Two Pb^{2+}-specific DNAzymes with opposite trends in split-site-dependent activity

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By splitting the catalytic core of DNAzymes into two halves, two Pb^{2+}-specific DNAzymes retain partial activity, while they show opposite trends of activity as a function of split site, revealing important nucleotides for catalysis and metal binding.

DNAzymes (DNA with catalytic activity) are highly useful for a diverse range of applications including anti-virus, sensing, and nanotechnology.[1] Compared to other types of biocatalysts, DNAzymes are attractive for their stability, ease of synthesis and modification, and excellent programmability. Currently, all DNAzymes are obtained using in vitro selection. By controlling the selection condition, a number of RNA-cleaving DNAzymes have been isolated, where they employ various metal ions as cofactors, including Pb^{2+},[2,3] Zn^{2+},[4] UO_2^{2+},[5] Hg^{2+},[6,7] trivalent lanthanides[8] and other metals.[9]

Among these, the Pb^{2+}-dependent DNAzymes have tremendously fueled the growth of this field since its inception. The first DNAzyme ever reported (called GR5) was selected in the presence of Pb^{2+},[2] and it was indeed highly specific for Pb^{2+}.[10] Since this DNAzyme cannot cleave all-RNA substrates and it uses a toxic heavy metal, its chemical biology applications are limited. In 1997, two general purpose RNA-cleavage DNAzymes were reported,[11] namely the 10-23 and 8-17 DNAzymes, which have since become model systems for both fundamental studies and applications. These two small DNAzymes cleave both RNA/DNA chimera and all-RNA substrates. The 10-23 DNAzyme is often used for in vivo applications since it is quite active under physiological Mg^{2+} concentrations. The 8-17 DNAzyme has occurred many times from in vitro selections carried out under different conditions in different labs.[4,11-13] It has the highest activity in the presence of low concentrations of Pb^{2+} and was first proposed to be a Pb^{2+} biosensor component.[3] The GR5 DNAzyme was recently re-visited as a Pb^{2+} biosensor and it has even better specificity compared to the 8-17 DNAzyme.[10]

To reach the full potential of the DNAzyme technology, fundamental studies are needed to understand metal binding, reaction mechanism, and folding.[14] While most previous studies employed full-length enzymes, we reason that new mechanistic insights can be achieved by splitting DNAzymes in the catalytic core region. Split DNAzymes have been reported in a few cases. For example, Sen and co-workers split several enzymes in the substrate binding arms to modulate substrate/enzyme binding.[15-17] Maxizyme[18] and MNAzyme[19] are two ways to split enzymes in the catalytic core.[20] However, the split junction was rigidified by forming extra base pairs. Alternatively, aptamers have been incorporated.[21] All these designs are under the notion that the split point needs to be rigidified by forming certain stable secondary structures. Only the 10-23 DNAzyme was directly split in the catalytic core.[22,23] In this work, we report splitting the two Pb^{2+}-specific DNAzymes: GR5 and 8-17. Interestingly, we observed a completely different pattern of enzyme activity as a function of splitting site. This indicates two different modes of binding the same metal cofactor for cleaving the same bond.

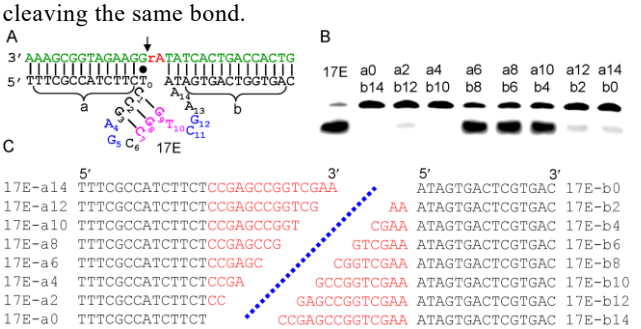

Figure 1. (A) Secondary structure of the 8-17 DNAzyme complex. Cleavage site is indicated by the arrowhead. The important nucleotides are in blue. Splitting around the pink nucleotide has less effect on activity. (B) A gel image showing the split enzyme activity. The enzyme combinations are on the top of each lane. The low band is the cleaved product. (C) Split DNAzyme sequences. The sequences in the catalytic core are in red.

The 8-17 DNAzyme complex contains a substrate strand (Figure 1A, in green) and an enzyme strand (called 17E). The substrate is a DNA/RNA chimera with a single RNA linkage (rA) serving as the cleavage site (indicated by the arrowhead). The substrate was labeled at its 3′-terminus with a FAM fluorophore. The full 17E DNAzyme is highly active and almost completely cleaves the substrate in 30 min with 10 μM Pb^{2+} (lane 1 in Figure 1B). We then tested the feasibility of splitting the enzyme strand. To facilitate discussion, the catalytic core is numbered from T_0 to A_{14}, For a systematic study, splitting was performed at every other nucleotide, giving a total of eight split enzyme pairs. The sequences of the split enzymes are in Figure 1C. For example, the 17E-a0 and 17E-b14 combination gives splitting between T_0 and C_1.

In this case, the enzyme is inactive as indicated by the second lane of the gel. Moderate cleavage (~8%) was observed by splitting between C_2 and G_3, while splitting between A_4 and G_5 abolished the activity. Interestingly, high activity was observed for the next three splitting positions, suggesting that the nucleotides between C_7 and T_{10} (in pink) are less important for catalysis, or they do not comprise the metal binding pocket. The activity again dropped significantly for the next two splitting sites. The cleavage after 30 min as a function of cleavage site is plotted in Figure 2A (blue squares). Since splitting between C_6 and C_7 gives the highest amount of cleavage, we further measured its cleavage rate to be 0.13 min^{-1} (Figure 2B, black dots). The rate of the original 17E was very fast under the same condition, where more than 80% of the substrate was cleaved in 10 sec (red triangles). The rate should be greater than 10 min^{-1}. If we extend the incubation time to 18 h, the substrate alone sample (no enzyme strand) showed 26% cleavage. The samples with split DNAzymes after this background subtraction are shown in Figure 2A (red dots), where all the DNAzymes are active to some extend. Therefore, splitting is generally tolerated but some sites are more favored than the others.

The 17E DNAzyme is an important model for studying DNA catalysis. This is partly due to its recurrence in many different selections.[4,11-13] In addition, its application for Pb^{2+} detection also promotes fundamental studies.[24] The Li group has performed extensive studies to understand its conserved nucleotides.[13,14,25,26] For example, A_4 and G_5 are absolutely conserved, and C_{11}, G_{12} are also very important. Most other nucleotides can be mutated while still retain a fraction of the activity.[13] This also agrees with our splitting DNAzyme data: almost no activity was observed upon splitting around these nucleotides.

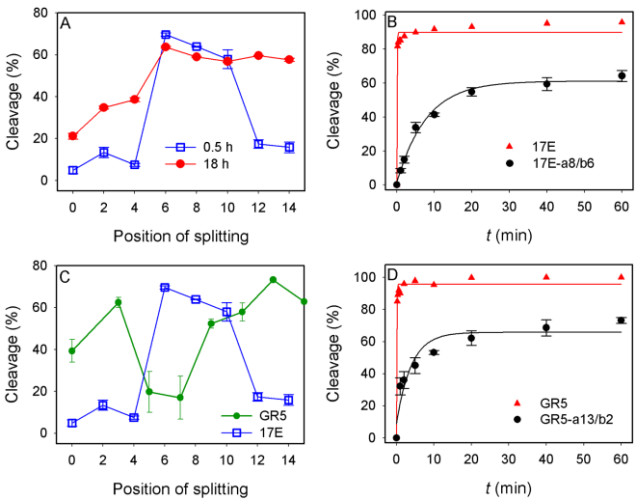

Figure 2. Quantification of substrate cleavage as a function of 17E split site in 0.5 h or 18 h. Cleavage kinetics with the full 17E DNAzyme and one of its split pairs (B); and full GR5 and one of its split pairs (D). (C) Quantification of substrate cleavage as a function of GR5 and 17E split site in 0.5 h.

The GR5 DNAzyme has a simple loop structure since this enzyme loop cannot be predicted into a stable secondary structure (Figure 3A). Using the same method, we constructed a series of split DNAzyme pairs and the cleavage pattern is shown in Figure 3B; cleavage was observed for each split DNAzyme pair, although their activities are lower compared to the full enzyme. We quantified the cleavage in Figure 2C (green dots). Interestingly, it shows an opposite trend compared to the 17E DNAzyme (blue squares): splitting GR5 in the middle part is more detrimental. Therefore, the nucleotides important for catalysis should be around that region. Indeed, the sequence alignment in the original paper shows that the highly conserved nucleotides are from T_5 to G_9,[2] which overlaps nicely with our more sensitive splitting sites. On the other hand, the length and contents of the nucleotides spanning these conserved ones can be varied. We next measured the cleavage rate of GR5-a13/b2 to be 0.25 min^{-1}. Of note, we obtained a much higher rate (>10 min^{-1}) for the full enzyme compared to the previous literature reports (~1 min^{-1}).[2,10] This difference is attributed to buffer conditions, where we used a much lower salt concentration and higher pH.

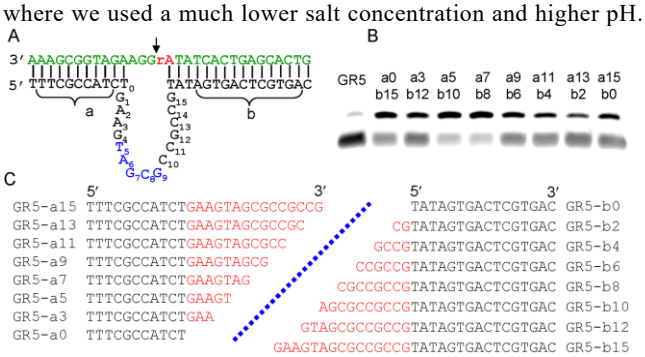

Figure 3. (A) Secondary structure of the GR5 DNAzyme complex. The important nucleotides are in blue. (B) Gel image showing the split enzyme activity (0.5 h). The enzyme combinations are on the top of each lane. (C) Split DNAzyme sequences. The sequences in the catalytic core are in red.

After knowing the important nucleotides are in the middle of the GR5 loop, we next tested making active partzymes with just one truncated half. We first blocked the 5′-half of the substrate strand with GR5-b0 and systematically shortened the other half (Figure 4A). No activity was observed in 1 h except with GR-a15, where effectively the full enzyme was formed. On the other hand, when the 3′-half of the substrate was blocked by GR5-a0, we observed activity for both GR5-b12 and b15; the former was even more active. In the GR5-a0/b12 sample, three nucleotides ($G_1A_2A_3$) were truncated. This suggests that the nucleotides for Pb^{2+} binding (Figure 3A, in blue) can be positioned close to the cleavage site via using C_{10} to G_{15} alone. We next repeated the experiment without using any blocking strands and similar activity patterns were also observed with an overall lower activity (Figure 4C, D).

Since the nucleotides important for the 17E are distributed on both sides (e.g. optimal split sites are in the middle part), we asked another question: can we truncate or insert nucleotides in the split enzymes. To test this, we generated a few split enzyme combinations (Figure 4E). When the numbers add up to 14, it means untruncated enzyme. From the gel, most truncations and insertions abolished the activity, while only in one insertion case (a8b8) did we observe moderate activity. This indicates that each nucleotide in 17E is important. Even though splits can take place at various locations, nucleotides cannot be removed. Insertion might be

slightly more tolerable.

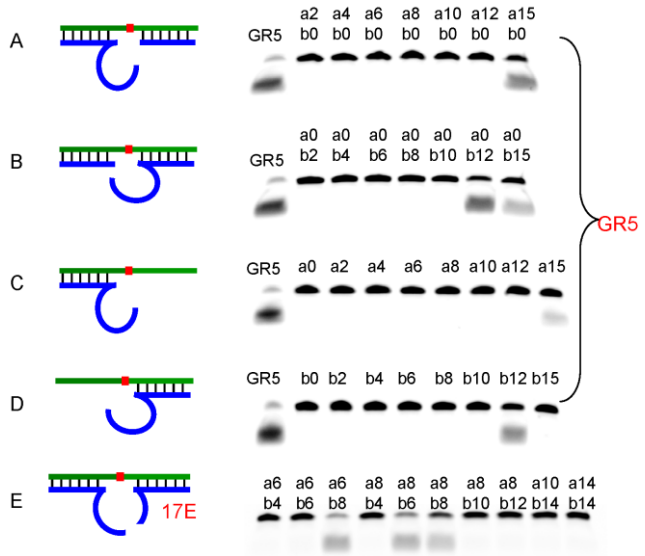

Figure 4. (A-D) Gel images and schematics of GR5 partzymes with only half of the enzyme loop. (E) Removing or inserting nucleotides to the split 17E DNAzyme. The original lengths of the two halves add up to 14.

A number of DNAzymes' catalytic cores contain a stem-loop,[5,8] which only serve a structural role. It is not surprising that those enzymes can split in the stem. These two Pb^{2+}-specific enzymes are different in that they only contained a small and essentially structureless loop. The fact that they can split enhances our understanding on metal binding and catalysis. This study indicates that there are at least two ways to arrange catalytically important nucleotides. For 17E, those nucleotides are distributed on the two sides; while for GR5, they are in the middle part. Splitting has significantly reduced the DNAzyme activity, which is likely due to flexibility in the metal binding pocket.

For the 17E DNAzyme, drawing the three base pairs in the enzyme loop is a common practice. However, our split result poses a question mark on this secondary structure. The DNAzyme is more active when split is made in the pink stem region (Figure 1A), which make it difficult to justify such a stem loop to form during catalysis, especially considering it only has three base pairs. Little fundamental work was performed on GR5 previously. Our results indicate that the catalytically important nucleotides are in the middle part.

Aside from the fundamental insights, split DNAzymes and partzymes will allow for more versatile designs of the DNAzyme-based sensors and devices as well. Therefore, they are likely to find new applications in analytical chemistry and bionanotechnology.

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Notes and references

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