論文の内容の要旨

Enzymatic Synthesis of Cu^{II}-responsive Deoxyribozymes through Incorporation of Artificial Ligand-type Nucleotides

（金属配位子型人工ヌクレオチドを導入した Cu^{II} 応答性デオキシリボザイムの酵素合成）

氏名　　中間 貴寛

1. Introduction

Metal-mediated base pairs, which consist of ligand-bearing nucleotides and a bridging metal ion, have gained much attention as functional units of DNA-based supramolecules and nanoarchitectures[1]. For example, hydroxypyridone (**H**) nucleotides form a 2:1 complex with a Cu^{II} ion (**H**–Cu^{II}–**H**) in a DNA duplex[2] and **H**–Cu^{II}–**H** base pairing was utilized for the DNA-templated Cu^{II} assembly[3] and the control of DNA conductivity[4]. In spite of the great potential for metal-dependent regulation of DNA functions, metal-mediated unnatural base pairs have scarcely been applied to metal-responsive DNA materials. This is mainly because chemical synthesis of artificial ligand-bearing oligonucleotides is often cumbersome and time-consuming.

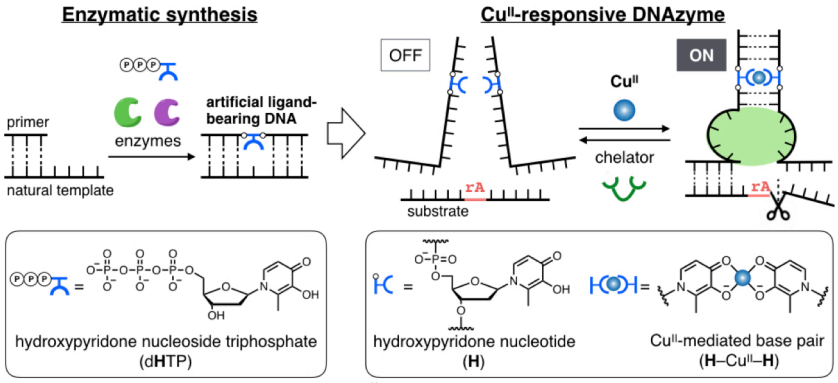

Figure 1. Enzymatic synthesis of Cu^{II}-responsive DNAzymes through incorporation of hydroxypyridone (**H**) nucleotides. rA: adenosine ribonucleotide.

Herein, I report efficient enzymatic methods to synthesize artificial DNA strands containing **H** nucleotides (Figure 1). Enzymatic reactions were expected to incorporate hydroxypyridone nucleoside triphosphate (d**H**TP) under mild conditions without any protecting group of the ligand moiety. I subsequently applied the enzymatic synthesis to develop Cu^{II}-responsive deoxyribozymes (DNAzymes). **H** nucleotides were incorporated into reported DNAzymes so that the formation of one **H**–Cu^{II}–**H** base pair can regulate their catalytic activities.

2. Enzymatic synthesis of artificial ligand-bearing DNA strands

Polymerase incorporation of d**H**TP was first investigated using a natural DNA template. I expected that unnatural **H** nucleotides can be introduced into an opposite site of natural nucleobases through misincorporation by a DNA polymerase. An exonuclease-deficient Klenow fragment (KF exo–) was found to incorporate one d**H**TP in a

quantitative manner when the opposite base (X) was A or T.

Next, the subsequent primer extension with natural nucleoside triphosphates (dNTPs) was examined to obtain a fully-elongated strand (Figure 2a). However, KF exo– hardly incorporated any dNTPs presumably due to the inhibition of primer elongation by the **H**–X mismatch. In contrast, a lesion bypass DNA polymerase, Dpo4 incorporated dNTPs after the **H** to afford a full-length product in over 90% yield. The product was characterized by MALDI-TOF mass spectrometry and an exonuclease-digestion experiment. This two-step primer extension allowed for the incorporation of a single **H** nucleotide into any sequences to afford **H**-containing DNA strands.

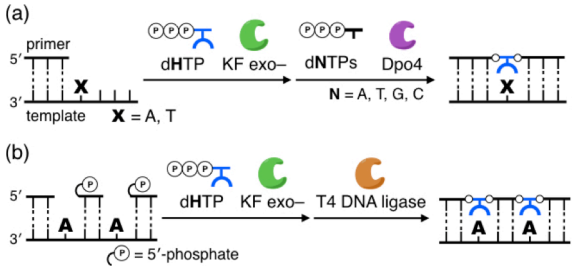

Figure 2. Enzymatic synthesis of artificial DNA strands bearing **H** nucleotides utilizing a natural DNA template. (a) Two-step primer extension using two DNA polymerases. (b) Incorporation of multiple **H** nucleotides using a polymerase and a ligase.

The incorporation of multiple **H** nucleotides was also accomplished using a polymerase and a ligase (Figure 2b). **H** nucleotides were appended to the 3′-ends of DNA strands by KF exo– and the **H**-bearing strands were subsequently connected through ligation by T4 DNA ligase.

In addition, I investigated the enzymatic synthesis of DNA strands containing consecutive **H** nucleotides. Dpo4 polymerase was found to incorporate three consecutive **H** nucleotides with the aid of Mn^{II} ions as a cofactor. **H**-oligomers (e.g. 5′-G**HHH**C-3′) were also enzymatically incorporated into a DNA strand by T4 DNA ligase.

These enzymatic methods enabled the incorporation of **H** nucleotides at internal positions without any protecting groups by using commercially available enzymes.

3. Development of Cu^{II}-responsive split DNAzymes

DNAzymes are catalytically active DNA molecules and applied to biosensors, logic gates, and molecular machines. Thus, regulation of DNAzyme activities by external stimuli is highly demanded for developing more sophisticated DNA-based systems.

The first Cu^{II}-responsive DNAzyme was developed based on a split design. A known RNA-cleaving DNAzyme (E5 DNAzyme[5], Figure 3a) was divided into two strands, and **H** nucleotides were incorporated into its stem site. The split DNAzyme was expected to restore its catalytic activity upon addition of Cu^{II} ions through the formation of a **H**–Cu^{II}–**H** base pair (Figure 3b).

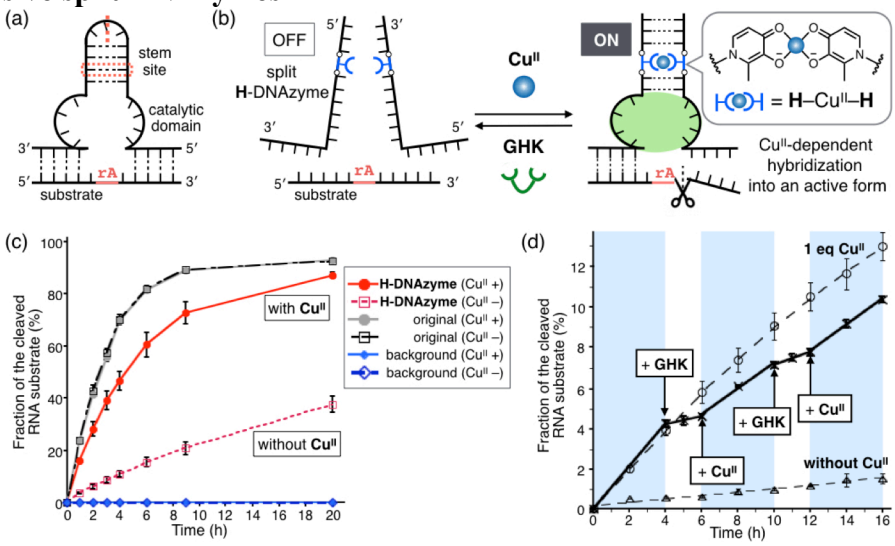

Figure 3. Development of a split Cu^{II}-responsive DNAzyme (**H**-DNAzyme). (a) Original E5 RNA-cleaving DNAzyme. (b) Reversible Cu^{II}-dependent regulation of the DNAzyme activity by the formation of a **H**–Cu^{II}–**H** base pair. (c) RNA-cleaving activity of the split **H**-DNAzyme and the original E5 DNAzyme in the absence and in the presence of one equivalent of Cu^{II} ions. (d) Iterative switching of the activity of the **H**-DNAzyme by the alternate addition of one equivalent of Cu^{II} ions and a Cu^{II}-binding peptide (GHK). [DNAzyme] = 1.0 μM, [substrate] = 10 μM, [$CuSO_4$] = 1.0 μM in 10 mM HEPES (pH 7.0), 1 M NaCl, 10 mM $MgCl_2$, 25 °C. *N* = 3. Error bars indicate standard errors.

I prepared 11 **H**-bearing DNA stands by the two-step primer extension (Figure 2a) to investigate the optimal position of the **H**–**H** pair. The Cu^{II}-dependent catalytic activity of each DNAzyme was evaluated with an equimolar substrate in the absence and in the presence of one equivalent of Cu^{II} ions. As a result, the highest on/off ratio (~5) was observed with a DNAzyme containing a **H**–**H** at the third position, which is referred to as "split **H**-DNAzyme".

The RNA-cleaving activity of the split **H**-DNAzyme was examined with 10 equivalents of the substrate (Figure 3c). The time-course analysis clarified that the split **H**-DNAzyme catalytically cleaved the substrate in the presence of Cu^{II} ions at the 5.5-times higher initial rate than that under the Cu^{II}-free condition. No substrate cleavage was observed with Cu^{II} ions but without any DNAzymes, which evidenced that the DNAzyme activity itself was enhanced by Cu^{II}.

The metal specificity of the split **H**-DNAzyme was examined with 11 kinds of transition metal ions. Among them, only Cu^{II} ions enhanced the activity while the other ions had no effect on the RNA-cleaving reaction.

The reversible regulation of the DNAzyme activity was demonstrated by the addition and removal of Cu^{II} ions. The addition Cu^{II} ions in the course of the reaction immediately increased the RNA-cleaving activity. When a Cu^{II}-binding peptide (GHK) was added as a chelator, the activity was immediately diminished to almost the same level as that in the absence of Cu^{II} ions. Moreover, the two-cycle switching of the DNAzyme activity was demonstrated by the alternate addition of Cu^{II} ions and the Cu^{II}-binding peptide (Figure 3d).

4. Development of Cu^{II}-responsive single-stranded DNAzymes

Next, a Cu^{II}-responsive single-stranded DNAzyme (ss **H**-DNAzyme) was developed from the same E5 DNAzyme. The DNAzyme strand was synthesized by incorporating two **H** nucleotides into a DNA stand using the enzymatic ligation (Figure 2b). The sequence of ss **H**-DNAzyme was designed so as to form a catalytically inactive secondary structure in the absence of Cu^{II} ions (Figure 4a). The formation of one **H**–Cu^{II}–**H** base pair could stabilize its active form and induce the structure transformation to restore the RNA-cleaving activity.

The Cu^{II}-dependent structure conversion was verified by fluorescent assay (Figure 4b). A fluorescent cytosine analogue, pyrrolo-C (PC)[6], was introduced into a stem duplex of the inactive structure, where the PC forms a base pair with a guanine and emits weaker fluorescence. When Cu^{II} ions were added, the fluorescent intensity was significantly enhanced. This indicates the dissociation of the PC–G pair caused by the Cu^{II}-induced structure conversion to the active form.

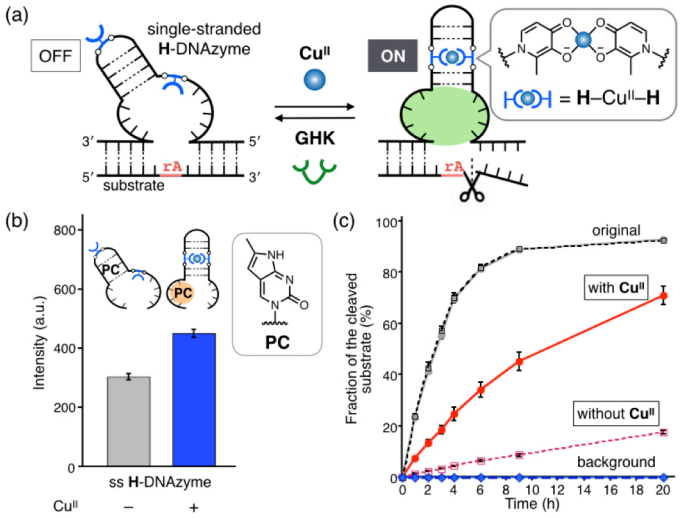

Figure 4. Development of a single-stranded Cu^{II}-responsive DNAzyme (ss **H**-DNAzyme). (a) Regulation of the DNAzyme activity through the Cu^{II}-dependent structure conversion. (b) Fluorescent assay of the structure conversion using a pyrrolo-C. λ_{ex} = 345 nm, λ_{em} = 450 nm. (c) RNA-cleaving activity of ss **H**-DNAzyme and the original E5 DNAzyme with and without Cu^{II} ions. [DNAzyme] = 1.0 μM, [substrate] = 10 μM, [$CuSO_4$] = 1.0 μM in 10 mM HEPES (pH 7.0), 1 M NaCl, 10 mM $MgCl_2$, 25 °C. *N* = 3. Error bars indicate standard errors.

The RNA-cleaving activity of ss **H**-DNAzyme was monitored under the same condition as that of split **H**-DNAzyme (Figure 4c). Upon addition of one equivalent of Cu^{II} ions, the initial reaction rate was enhanced by 6.8-fold, which was comparable to the split DNAzyme. The activity of ss **H**-DNAzyme was also reversibly controlled by the addition and removal of Cu^{II} ions. Furthermore, I succeeded in developing other Cu^{II}-responsive DNAzymes from different types of DNAzymes but based on the same design principle. Thus, the incorporation of **H** nucleotides was proven to be a promising method to develop metal-responsive DNAzymes.

5. Multimetal-dependent regulation of DNAzyme activities

Based on its high metal specificity, the split **H**-DNAzyme was applied to metal-dependent orthogonal regulation of DNAzyme activities using well-known Hg^{II}-mediated thymine base pairs (T–Hg^{II}–T) as well as **H**–Cu^{II}–**H** (Figure 5a). A novel Hg^{II}-responsive DNAzyme (T-DNAzyme) was developed by introducing four T–T mismatches into E5

DNAzyme. The metal-dependent activation of **H**-DNAzyme and T-DNAzyme was examined with a mixture of both DNAzymes and their substrates. When one equivalent of Cu^{II} ions was added, the activity of **H**-DNAzyme was enhanced eight-fold while T-DNAzyme showed the same activity (Fig. 5b). On the other hand, the addition of Hg^{II} ions selectively increased the activity of T-DNAzyme. In the presence of both Cu^{II} and Hg^{II} ions, both DNAzymes were activated by two- to three-fold. Furthermore, the selective deactivation of the DNAzymes was also demonstrated by the removal of Cu^{II} or Hg^{II} ions using corresponding chelating agents. In this way, the orthogonal regulation of the DNAzymes was achieved through the metal-specific formation of **H**–Cu^{II}–**H** and T–Hg^{II}–T base pairs.

A DNAzyme that responds to two metal ions was also developed through the incorporation **H** nucleotides into a reported Ag^{I}-dependent RNA-cleaving DNAzyme[7]. In the absence of Ag^{I} ions, no substrate cleavage was observed. The addition of only Ag^{I} ions but without Cu^{II} ions yielded little cleaved substrate. The activity of the modified DNAzyme was restored only when both Cu^{II} and Ag^{I} ions were added. Therefore, it displayed an AND-gate behavior using the two metal ions as inputs, which is applicable to DNA-based logic gates, multiplexer, and computing circuits.

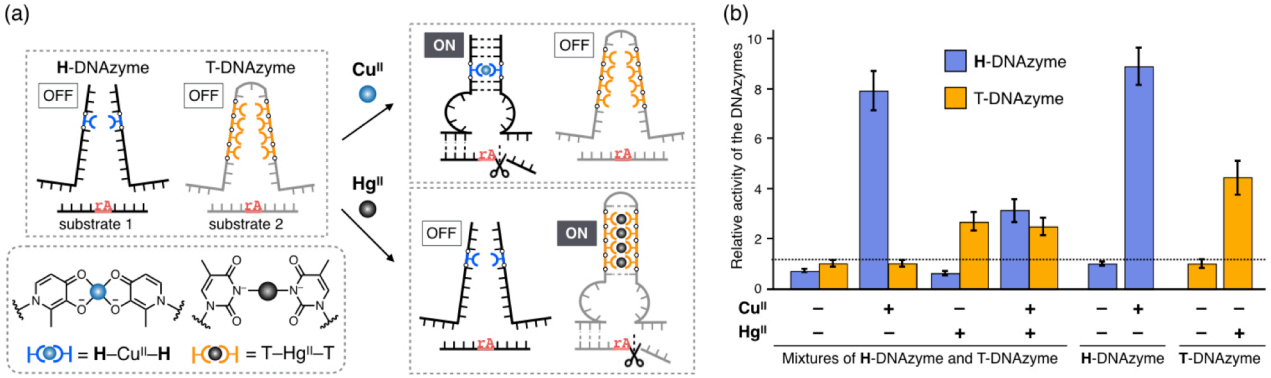

Figure 5. Orthogonal activation of a Cu^{II}-responsive **H**-DNAzyme and a Hg^{II}-responsive T-DNAzyme. (a) Design of the orthogonal activation. (b) Metal-dependent activation of **H**-DNAzyme and T-DNAzyme. Cu^{II} and/or Hg^{II} ions were added to a mixture of **H**-DNAzyme and T-DNAzyme. [**H**-DNAzyme] = [T-DNAzyme] = 1.0 μM, [substrate 1] = [substrate 2] = 2.0 μM, [$CuSO_4$] = 0 or 1.0 μM (1 equiv for **H**–**H**), [$Hg(ClO_4)_2$] = 0 or 4.0 μM (1 equiv for T–T) in 10 mM HEPES (pH 7.0), 1 M $NaNO_3$, 10 mM $Mg(NO_3)_2$, 25 °C, 3 h. $N \geq 3$. Error bars indicate standard errors.

6. Conclusion

In my doctorial study, I have established the enzymatic synthesis of artificial ligand-bearing DNA strands and developed Cu^{II}-responsive DNAzymes. Compared to the conventional chemical synthesis, the enzymatic methods allowed for the protection-free incorporation of hydroxypyridone (**H**) nucleotides without any specialized equipment. Cu^{II}-responsive DNAzymes were synthesized by the enzymatic incorporation of **H** nucleotides. Because Cu^{II} ions rarely interfere with natural nucleotides and selectively bind to **H** nucleotides, the rational designs of metal-responsive DNAzymes were accomplished. The RNA-cleaving activities of both split and single-stranded DNAzymes were reversibly regulated by the formation of one **H**–Cu^{II}–**H** base pair. Moreover, multimetal-dependent regulation of DNAzyme activities was also demonstrated. The Cu^{II}-responsive DNAzymes developed here will be utilized for the metal-triggered control of DNA-based molecular machines and logic gates.

The facile synthesis and design concepts would be applicable to other artificial ligand-bearing nucleotides. Accordingly, this study would provide a powerful tool to develop DNA materials that respond to diverse metal ions.

References

[1] Y. Takezawa *et al.*, *Chem. Lett.* **2017**, *46*, 622–633. [2] K. Tanaka *et al.*, *J. Am. Chem. Soc.* **2002**, *124*, 12494–12498. [3] K. Tanaka *et al.*, *Science* **2003**, *299*, 1212–1213. [4] S. Liu *et al.*, *Angew. Chem. Int. Ed.* **2011**, *50*, 8886–8890. [5] R. R. Breaker *et al.*, *Chem. Biol.* **1995**, *2*, 655–660. [6] D. A. Berry *et al.*, *Tetrahedron Lett.* **2004**, *45*, 2457–2461. [7] J. Liu *et al.*, *Anal. Chem.* **2016**, *88*, 4014–4020.