

In vitro selection and biochemical characterization of trivalent metal ion dependent RNA-cleaving DNazymes

by

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A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Chemistry (Nanoscience)

Waterloo, Ontario, Canada, 2016

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

The work presented in this thesis is the result of work performed by the author and several scientific collaborations. Contributions from each scientist and the resulting publications are listed in detail below.

The work in Chapter 2 has been published as: Vazin, M.; Huang, P. J.; Matuszek, Z.; Liu, J. Biochemical Characterization of a Lanthanide-Dependent DNAzyme with Normal and Phosphorothioate-Modified Substrates. *Biochemistry*. 2015, 54, 6132–6138. The R_p and S_p diastereomers analysis was performed by Po-Jung Jimmy Huang. Żaneta Matuszek performed the pH-dependent enzyme activity. All other works in this chapter were performed by the author.

The R_p and S_p diastereomers analysis presented in chapter 3 was performed by Po-Jung Jimmy Huang. All other works presented in this chapter were performed by the author. Part of the results in Chapter 3 were included in the published paper: Huang, P. J.; Vazin, M.; Liu, J. “*In Vitro* Selection of a New Lanthanide-Dependent DNAzyme for Ratiometric Sensing Lanthanides”. *Anal. Chem.* 2014, 86, 9993-9999.

All the results presented in chapter 4 were performed by the author. Part of the results were included in the published paper; Huang, P. J.; Vazin, M.; Liu, J. "Desulfurization Activated Phosphorothioate DNAzyme for the Detection of Thallium". *Anal. Chem.* 2015, 87, 10443-10449.

The author performed all the results presented in chapter 5. Part of the results were included in the submitted paper; Zhou, W., Vazin, M., Yu, T., Ding, J., and Liu J. "In Vitro Selection of Chromium-Dependent DNAzymes for Sensing Cr(III) and Cr(VI)", *Chem. Eur. J.* 2016, DOI: 10.1002/chem.201601426.

Abstract

DNAzymes are DNA sequences with catalytic activity. So far, all known DNAzymes have been isolated using the high-throughput *in vitro* selection method. DNAzymes have been used for analytical, biomedical, and nanotechnology applications. All known DNAzymes require metal ions for activity. Therefore, a particularly interesting direction is the isolation of DNAzymes that function only in the presence of specific metal ion cofactors. Metal-specific DNAzymes can be used for developing metal ion biosensors and also provide insights into the interaction between metal ions and DNA.

Since the first DNAzyme was reported in 1994, most metal dependent-DNAzymes have been isolated using divalent metal ions, such as Pb^{2+} , Zn^{2+} , Hg^{2+} , UO_2^{2+} , Cu^{2+} and Cd^{2+} . Recently, a few monovalent metal dependent DNAzymes were also reported. However, relatively little is known about trivalent metal ions. Compared to DNAzymes using monovalent metal ions, those using divalent metal ions are usually more efficient. Therefore, we suspect that trivalent metal ions may result in even more efficient DNAzymes. At the same time, trivalent metal ions are also very important for technological applications. Hence, the main goal of this thesis is to select and characterize DNAzymes using trivalent metal ions as cofactors, in the hope of developing biosensors for this category of metal ions. There are three types of trivalent metal ions used in this work, including trivalent lanthanide ions (Ce^{3+} , Yb^{3+} and Lu^{3+}), Group 3A metal ions (Al^{3+} , Ga^{3+} , In^{3+} and Tl^{3+}), and Cr^{3+} . Different selection strategies were employed to fulfill each metal ion criteria. Among the various types of DNAzymes, this thesis is focused on those cleaving RNA. In each chapter, the conditions and processes of the *in vitro* selection with the target metal ion are described and the results are discussed. Biochemical studies of the selected DNAzymes are also presented.

The first chapter of this thesis gives a general introduction to DNA and DNAzymes, as well as some of their applications. In chapter two, the *in vitro* selection with Ce^{3+} was described, which resulted in the reselection of the Ce13d DNAzyme. Ce13d was previously reported in a Ce^{4+} -dependent selection carried out by another member of the Liu lab. This DNAzyme appears to be an optimal sequence for Ce^{3+} , but it is also highly active with all the trivalent lanthanides, Y^{3+} , and to a lesser extent with Pb^{2+} . Interestingly, by changing the cleavage junction from the normal phosphodiester to phosphorothioate (PS), the enzyme has a decreased activity with lanthanide but shows a high activity with all thiophilic metals. Since Ce13d is an interesting DNAzyme, a careful biochemical study of the enzyme was performed.

In chapter three, in order to find more specific DNAzymes that distinguish each lanthanide, two new *in vitro* selections were conducted with Yb^{3+} and Lu^{3+} , respectively. The new Lu12 DNAzyme was selected. Lu12 is more active with smaller lanthanides and has the lowest activity with the largest lanthanide, Lu^{3+} . Lu12 was extensively studied and some interesting characters of the enzymes were found, such as a pH-rate slope of 2, using pro- S_p for metal binding at the cleavage site, and acceptance of a diverse range of cleavage junctions. Such properties were never previously reported for any known RNA-cleaving DNAzymes.

In chapter four, efforts toward the isolation of DNAzymes specific for the group 3A metal ions using *in vitro* selection were described. Four independent selections were carried out with Ga^{3+} , In^{3+} , Al^{3+} and Tl^{3+} ; however, no specific DNAzymes were identified. The failure in the selection with this group of metal ions was probably due to the very low pK_a of these metal ions in aqueous solution, and also their inability to tightly bind to phosphate group of the DNA molecule. The Tl^{3+} -dependent selection was also repeated with PS-modified library, but the selection still did not work, because Tl^{3+} can desulfurize the substrate back to the normal PO

substrate. While no new DNAzymes were isolated in this work, this study has enhanced our understanding of the interaction between Group 3A metals with DNA, and this information is useful for future in vitro selection works using these metals.

In chapter five, the cleavage of the previously selected DNAzyme, Ce13d, by Cr^{3+} was studied, initially. This preliminary study gives us information about the condition for the efficient activity of Cr^{3+} . Then, two new Cr^{3+} -dependent selections were conducted to isolate a specific DNAzyme. To discourage a Ce13d type of sequence, a blocking DNA and a smaller N35 library were tested separately. However, the Cr^{3+} selections resulted in obtaining a non-specific cleaving DNAzyme as the major product, and accompanied with a small fraction of 17E, suggesting the Ce13d as an optimal sequence for Cr^{3+} . Cr^{3+} is a highly important metal and is also an environmental contaminant. This study suggests the possibility of using DNAzyme for Cr^{3+} detection.

Acknowledgements

I would like to express my gratitude to my supervisor, Dr. Juewen Liu, for his seemingly never-ending patience and support. His knowledge and guidance has been appreciated throughout the entirety of my graduate career. I would like to thank my committee members, Dr. John Honek, Dr. Guy Guillemette, Dr. Richard Manderville, and Dr. Frank Gu for their knowledgeable advice and kindness. I would like to thank my external examiner, Dr. Maxim V. Berezovski, for taking the time to attend my examination. Additionally, I would like to thank them for their thorough feedback on my thesis. Also, I would like to acknowledge Cathy van Esch and Kim Rawson for their support and guidance.

I would like to thank all the members of the Liu lab, both past and present, for their support and helpful advice; Dr. Feng Wang, Dr. Po-Jung Jimmy Huang, Biwu Liu, Wenhui Zhou, Zijie Zhang, Runjhun Saran, Ziyi Sun, Howard Tsai, Michael Hoang, Qingyun Chen, Zhicheng Huang, James Yu, Chang Lu, and Lingzi Ma. I would also like to thank the undergraduate volunteers and co-op researchers who worked with me: Żaneta Matuszek, Jenny Lin, and David Lin.

I would like to acknowledge my family and friends for their unconditional love and support. Also, this work would not be possible by the contributions of the funding agencies, so I would like to thank the financial supports from the University of Waterloo, the Natural Sciences and Engineering Research Council (NSERC) of Canada, and Canadian Institutes of Health Research (CIHR). Finally, I would use this opportunity to show my gratitude to the University of Waterloo and the Department of Chemistry for financial support and for providing access to research facilities.

Dedication

*I would like to dedicate this thesis to my husband and my family for their love,
encouragement and support.*

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List of Abbreviations

A	adenine
AES	atomic emission spectrometry
bp	base pairs
C	cytosine
CE	capillary electrophoresis
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dPAGE	denaturing polyacrylamide gel electrophoresis
dsDNA	double-stranded DNA
EPA	Environmental Protection Agency
FAAS	flame atomic absorption spectrometry
FAM	carboxyfluorescein
FRET	fluorescence resonance energy transfer
G	guanine
G-quartet	guanine quartet
HDV	hepatitis delta virus

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
ICP-MS	inductively coupled plasma mass spectroscopy
k_{cat}	turnover number
K_{d}	dissociation constant
K_{m}	Michaelis constant
Ln^{3+}	trivalent lanthanides ions
MES	2-(N-morpholio)ethanesulfonic acid
μl	micro-liter
ml	milliliter
μM	micro-molar
MOPS	3-(N-morpholino)propanesulfonic acid
nM	nano-molar
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
PO	phosphodiester
ppb	parts-per-billion
ppm	parts-per-million

ppt	parts-per-trillion
PS	phosphorothioate
Q	fluorescence quencher
rA	ribo-adenosine
RNA	ribonucleic acid
ssDNA	single-stranded DNA
Tris	tris(hydroxymethyl)aminomethane
PCR	polymerase chain reaction
pmol	pico-mole
T	thymine
U	uracil
VS	Varkud satellite

Chapter 1. Introduction

1.1 Nucleic acids (DNA/RNA)

Nucleic acids are biopolymers of nucleotide units and are named due to their existence in the nucleolus of the cells. Each nucleotide, itself, is composed of a nitrogenous base linked to a ribose (for RNA) or deoxyribose (for DNA) joined by a phosphate backbone (Figure 1). DNA (also called deoxyribonucleic acid) is composed of four bases, adenine (A), cytosine (C), thymine (T), and guanine (G), while RNA (or ribonucleic acid) has uracil (U) instead of thymine. Adenine base pairs with thymine and guanine base pairs with cytosine, via hydrogen bonding. The base pairing usually refers to DNA strands that form a double-stranded helix structure. The double-stranded DNA is also stabilized by base stacking in addition to hydrogen bonding.¹ RNA is naturally in a single-stranded form. The 2'-OH makes the ribose to adopt a different conformation (RNA forms A-form helix, while DNA generally forms a B-form helix under physiological conditions), which allows it to form more complex and complicated structures.² At the same time, the 2'-OH also makes RNA much less stable compared to DNA, since it can act as an internal nucleophile.³

In terms of their functions, DNA is more responsible for carrying the genetic information and transcribing this information to RNA form. While RNA can carry genetic information in some species such as viruses, it is mostly important for transferring the genetic information and protein synthesis.

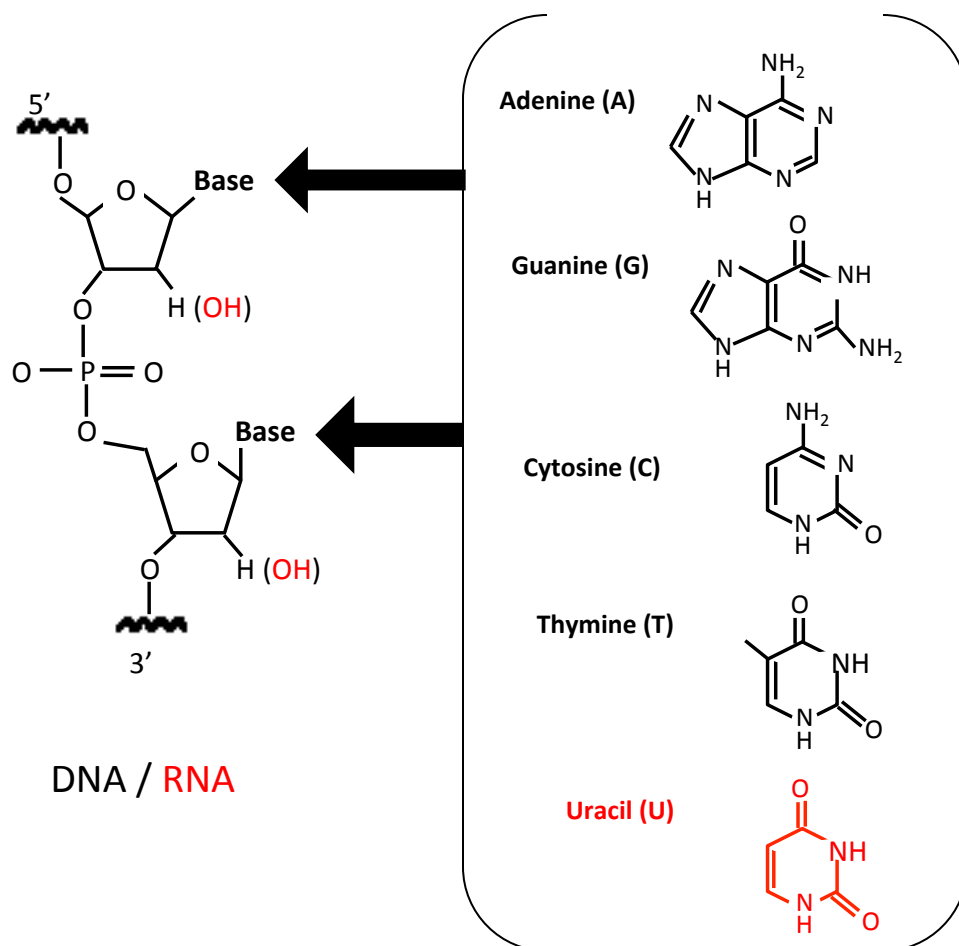


Figure 1.1 The structure of nucleic acids (DNA and RNA) and the bases.

1.2 Functional nucleic acids

As mentioned earlier, the RNA molecule is assumed to form very diverse and complicated secondary structures. This assumption directed researchers in 1960s and 1970s to hypothesize that RNA might have other important roles beyond the well-known genetic functions.^{4,5} In the following years, the hypothesis was validated by the discovery of the first ribozymes and later by the discovery of the riboswitches.⁶⁻¹¹ These two terms, ribozyme and riboswitch, are defined as the naturally occurring or *in vitro*-evolved RNAs with enzymatic activities or affinities toward specific targets.

Soon after, a novel method called “*in vitro* selection” was proposed,¹²⁻¹⁹ which led to the discovery of not only more ribozymes but also DNA aptamers and DNAzymes²⁰⁻³¹ (also refer to DNAs with catalytic activities). Today, the term “functional nucleic acids” is used to address a wide range of nucleic acids with different functions beyond the traditional genetic roles of RNAs/DNAs. Functional nucleic acids have different important applications in biochemical research, medicine, and analytical chemistry and are widely used.

The focus of this thesis is mostly on newly selected DNAzymes. Each of the terms “ribozymes” and “DNAzymes” will be discussed in detail using different examples.

1.2.1 Ribozymes

Ribozymes are RNA-based functional nucleic acids that can facilitate catalysis in different biochemical reactions, and are comparable to protein enzymes in function. Cech and co-workers discovered the first naturally occurring ribozyme in 1982.⁶ The first ribozyme was found in the intron of an RNA transcript of *Tetrahymena thermophila* to catalyze a transesterification reaction.⁶ At the same time, Sidney Altman and Norman Pace, who were studying the bacterial RNase P complex, have also found a ribozyme involved in the maturation of pre-tRNAs.⁸ These initial discoveries and the later findings about the ribozymes contributed to the RNA world hypothesis, which emphasizes on the role of RNA in the evolution of self-replicating systems.

After introducing the naturally occurring ribozymes, the discovery of laboratory-evolved ribozymes (or artificial ribozymes), using the *in vitro* selection method, opened a new chapter for the investigators in this field.³² (details about the *in vitro* selection process will be discussed in section 1.4). RNA ligase was one of the first ribozymes to be isolated through this method.³³

However, today, the ribozymes that can catalyze other types of biochemical reactions are also known. Reactions such as, RNA cleavage,³⁴⁻³⁷ RNA capping,³⁸⁻⁴⁰ Diels-Alder reactions,⁴¹⁻⁴⁴ Michael addition,⁴⁵ aminoacylation⁴⁶⁻⁴⁸ and porphyrin metalation⁴⁹ can all be catalyzed by ribozymes. Researchers studying the “origin of life” have even selected an RNA polymerase ribozyme with the ability to catalyze their own reaction under a very specific condition.⁵⁰

Among the various ribozymes, those catalyzing RNA cleavage have been most extensively studied. Based on the identity of the nucleophile attacking the phosphodiester linkage, the ribozymes are classified into two categories, small self-cleaving ribozymes and large ribozymes.⁵¹⁻⁵⁴ In the first group, the 2'-OH of the ribose moiety attacks the 3'-phosphodiester linkage of its own, while in the large ribozymes an exogenous nucleophile, such as water or the 2' or 3'-OH of another nucleotide is also involved in the system and attacks the phosphodiester linkage.⁵¹⁻⁵⁴ The hammerhead,⁵⁵⁻⁵⁷ hairpin,⁵⁸⁻⁶⁰ hepatitis delta virus (HDV),⁶¹⁻⁶³ and leadzyme⁶⁴⁻⁶⁶ ribozymes are classified as “small” self-cleaving ribozymes, while, Group I⁶⁷⁻⁶⁹ and Group II introns,⁷⁰⁻⁷³ *Neurospora* VS,⁷⁴⁻⁷⁶ and RNaseP systems,^{77,78} are classified as the “large” ribozymes.⁵¹⁻⁵⁴

1.2.2 DNazymes (also called deoxyribozymes, DNA enzymes, or catalytic DNAs)

DNazymes are DNA molecules with the ability to catalyze chemical reactions.⁷⁹ In contrast to ribozymes, there are no naturally occurring DNazymes and all the known DNazymes are laboratory-evolved. DNA lacks the 2'-hydroxyl groups of RNA, known as the potential internal nucleophile, which diminishes its chemical reactivity and reduces its ability to form complex tertiary structures. On the other hand, this difference also makes DNA much more chemically stable. Almost all the biological DNAs are found in the double helix conformation

and only the artificial DNazymes obtained though *in vitro* can form active structures. Therefore, unlike ribozymes, no naturally occurring DNazymes are known.

Comparing to different types of proteins, which are built up from 20 different monomers, RNA and DNA molecules have only four monomers (A, G, C and T/U) to choose from, which limits the construction of interesting catalytic sites. Despite this, DNazymes and ribozymes can still perform a diverse range of chemical reactions.

Breaker and Joyce reported the first DNzyme in 1994.²⁰ This DNzyme catalyzes the Pb²⁺-dependent RNA cleaving reaction. The amplification by this enzyme is about 10⁵ fold compared to the uncatalysed reaction. Many other DNzyme have since been developed that catalyze a variety of chemical reaction, catalytic reactions such as, RNA cleavage,^{20, 80-91} DNA cleavage,^{21, 22} DNA hydrolysis,⁹² RNA ligation,⁹³⁻⁹⁷ DNA ligation,^{98,99} DNA phosphorylation,¹⁰⁰ DNA depurination,¹⁰¹ DNA adenylation,¹⁰² thymine dimer cleavage,¹⁰³ phosphoramidate bond cleavage,¹⁰⁴ DNA deglycosylation,¹⁰⁵ porphyrin metalation,¹⁰⁶ and carbon-carbon bond formation.¹⁰⁷

Nucleic acids are highly negatively charged and metal ions are needed to stabilize their duplex structures. In addition, metal ions may assist their catalysis just like in protein metalloenzymes. In fact, most of the reported DNazymes require metal ions for their functions. In addition to the biochemical aspect of studying the role of metal ions, these DNazymes can be used as probes for detecting different metal ions. Some of the selected DNazymes have already found applications in metal biosensors.¹⁰⁸ In comparison with RNA, DNA is more stable toward hydrolysis, has much easier chemical synthesis procedure and is also easily amenable to

chemical modifications. These features have made DNazymes ideal choices for practical applications.

Some of the reactions catalyzed by DNazymes and the co-factors required for the chemical reactions are listed in Table 1.1. As shown in the table, there are many types of DNazymes, and this thesis is focused on RNA-cleaving DNazymes.

Reaction	Cofactor	$k_{\max}$ (min ⁻¹)	$k_{\text{cat}}/k_{\text{uncat}}$	Ref.
RNA cleavage	Pb ²⁺	1	10 ⁵	20
	Mg ²⁺	0.01	10 ⁵	80
	Ca ²⁺	0.1	10 ⁴	84
	Mg ²⁺	10	>10 ⁵	83
	None	0.01	10 ⁸	82
	L-histidine	0.2	10 ⁶	85
	Zn ²⁺	~40	>10 ⁵	86
	Mg ²⁺	1.7		88
	Co ²⁺	7		90
	Cd ²⁺ , Mn ²⁺ , Ni ²⁺	~1		91
	UO ₂ ²⁺	~1.2		81
	Na ⁺	0.1		112
DNA cleavage (oxidative)	Cu ²⁺	0.2	>10 ⁶	21,22
DNA hydrolysis	Mn ²⁺ , Zn ²⁺	2.7 h ⁻¹	10 ¹²	92
RNA ligation	Mn ²⁺	~2.2	>10 ⁶	93
	Mg ²⁺	0.5	10 ⁵	94
	Mg ²⁺	0.013	1.9*10 ⁴	95
	Mg ²⁺	0.1	450	96
	Zn ²⁺	0.5	1.7*10 ⁴	97
	Zn ²⁺ or Cu ²⁺	0.07	10 ⁵	98
DNA ligation	Mn ²⁺	10 ⁻⁴	>10 ⁵	99
DNA phosphorylation	Ca ²⁺	0.01	10 ⁹	100
DNA depurination	IO ₄ ⁻			101
DNA adenylation	Cu ²⁺	0.003	>10 ¹⁰	102
Thymine dimer cleavage	None	4.5	2.5*10 ⁴	103
Phosphoramidate bond cleavage	Mg ²⁺	~5*10 ⁻⁴	>10 ³	104
DNA deglycosylation	Ca ²⁺	0.5	10 ⁶	105
Porphyrin metallation	None	1.3	10 ³	106
C-C bond formation	Ca ²⁺	3.0	4*10 ⁵	107

Table 1.1 Some of the reactions catalyzed by DNazymes, and the co-factors required for the reactions.

1.2.3 Examples of metal independent and monovalent dependent DNazymes

Since metal ions play a particularly important role in DNzyme catalysis, the following sections will introduce this aspect in more detail. The discussion is organized by the valence of the metal ions. Although most of the known ribozymes and DNazymes were shown to require a metal co-factor for their activities, researchers have found DNA catalysts that can be activated in the absence of any co-factors. The first effort on this direction was reported by Geyer and Sen, who have also used an *in vitro* selection strategy to isolate DNA molecules with the ability to cleave their internal phosphodiester bond at rates of $\sim 0.01 \text{ min}^{-1}$ (a rate acceleration of $\sim 10^8$ over the un-catalyzed rate of hydrolysis) without any divalent metal ions, and this enzyme is named G3.⁸² They reported that the selected G3 self-cleaving DNzyme or its relatives can catalyze the cleavage of an RNA phosphodiester in the absence of divalent or higher-valent metal ions. Intriguingly, adding cations such as Mg^{2+} , Ca^{2+} , Pb^{2+} , Zn^{2+} and Cu^{2+} did not significantly influence the rate of G3 self-cleavage.⁸² There are also some examples of ribozymes that do not require divalent metal ions for their activities. For example, PSSpl, which is a self-cleaving RNA hairpin of T4-infected *Escherichia coli* cells, has been reported to cleavage in the absence of divalent metal ions and ribonucleases.¹⁰⁹ There are also a number of RNA molecules that are found to be cleaved preferentially at UA (uracil-adenine) sites, in the presence of a variety of proteins and also in the presence of polyvinylpyrrolidone.¹¹⁰

In addition to the DNazymes activate in the absence of divalent ions, monovalent metal ions, such as Na^+ and Li^+ , were also reported to activate DNazymes.⁸² However, higher concentrations ($\geq 1 \text{ M}$) of these metal ions are required to activate a specific DNzyme, which suggests that folding of the DNA instead of the specific binding of the metal ions activates the DNazymes.⁸² For instance, the 8 -17 DNzyme, which is a small RNA-cleaving DNzyme, can

be activated with a variety of divalent metal ions, such as Pb^{2+} , Mg^{2+} and Zn^{2+} . In addition to these metal ions, high concentrations of monovalent metal ions can also activate the 8-17 DNAzyme and fold it to the Z-form that is the essential form for the activity of the DNAzyme.¹¹¹

Recently, a new RNA-cleaving DNAzyme has been reported with specific activity toward Na^+ monovalent.¹¹² This DNAzyme is suggested to be the first Na^+ -dependent RNA-cleaving DNAzyme selected through *in vitro* selection method, with a fast reaction rate ($\sim 0.1 \text{ min}^{-1}$), remarkable selectivity for Na^+ over other competing metal ions ($>10,000$ -fold), and a detection limit of $135 \mu\text{M}$ (3.1 ppm).¹¹² The selected DNAzyme has been developed into a biosensor for Na^+ monovalent, which can even function as an intracellular sensor to detect Na^+ in cells.¹¹²

1.2.4 Examples of divalent metal dependent DNAzymes

While monovalent metal ions alone can activate some DNAzymes, divalent metal ions can achieve much more efficient catalysis. As mentioned in the previous sections, almost all the DNAzymes require metal ions for their functions. There are many examples of DNAzymes that are activated in the presence of different divalent metal ions. In this section, the most-characterized divalent-dependent RNA-cleaving DNAzymes will be discussed.

Starting with the GR-5 DNAzyme, which is the first DNAzyme obtained through *in vitro* selection method.^{20, 23} The GR-5 enzyme is a Pb^{2+} -dependent DNAzyme with the ability to cleave a single ribo-adenosine (rA). It has a $k_{\text{cat}} = 1 \text{ min}^{-1}$ and the ability to catalyze the RNA hydrolysis with a rate that is about 10^5 times higher than the un-catalyzed reaction. As shown in Figure 1.2, the GR-5 enzyme has a catalytic core containing fifteen nucleotides flanked by the base-pairing regions. The conserved nucleotides in the catalytic core of the DNAzyme are

highlighted in blue. The substrate is a DNA strand with a single rA in the middle. There is an unpaired region (AGG) next to the single rA cleavage site, which possibly acts as the binding pocket for a metal ion, since it is required for the activity of the DNAzyme. Although the GR-5 DNAzyme is selected in the buffer containing 50 mM MgCl_2 , it can also work in the absence of Mg^{2+} . Surprisingly, the GR-5 enzyme appears to activate specifically with Pb^{2+} . The activity of GR-5 DNAzyme is shown to be 40,000 folds more with Pb^{2+} compared to other competing metal ions.¹¹³

The initial proposed application of RNA-cleaving DNAzymes is to cleave viral RNA. Since this DNAzyme cannot cleave full RNA substrate (it cleaves only RNA/DNA chimeric substrate), and Pb^{2+} is a required metal, which does not exist in physiological conditions, this DNAzyme had not attracted much attention until the report by the Lu group was made, indicating that it has an excellent specificity for Pb^{2+} .¹¹³ Since then, it had been considered more as a biosensor for Pb^{2+} .

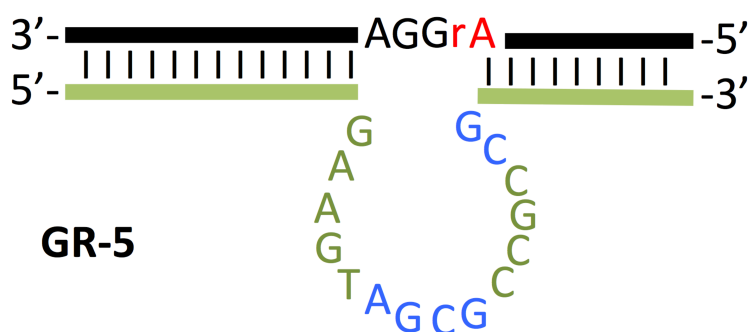
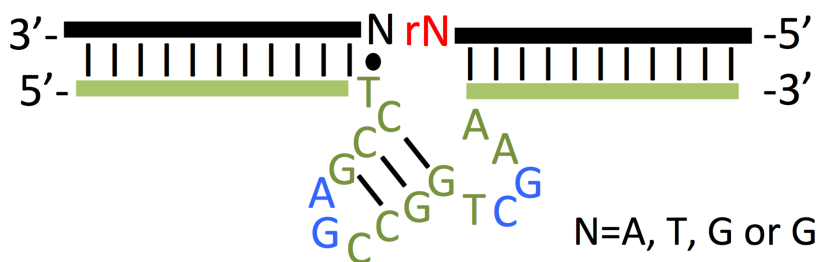


Figure 1.2 Secondary structure of GR-5 DNAzyme. The substrate strand is indicated in black, the enzyme is shown in green, and the conserved nucleotides are marked in blue. The cleavage site (rA) is also marked with red.

To search for DNAzymes that can cleave full RNA sequences, the Joyce group changed the selection strategy. Instead of using a chimeric substrate region in the selection, a full RNA region was incorporated. In 1997, the seminal paper by Santoro and Joyce reported two general purpose RNA-cleaving DNAzymes, named the 8-17 and 10-23 DNAzymes.^{114, 115} The 8-17 DNAzyme is another example of RNA-cleaving DNAzyme with a remarkable Pb^{2+} -dependent activity. This enzyme was also isolated by *in vitro* selection strategy, under biological conditions (150 mM KCl, 2 mM MgCl_2 , 50 mM Tris buffer, at pH 7.5 and 37°C).¹¹⁴ The 8-17 and its variants are probably the most widely studied RNA-cleaving deoxyribozymes. The structure of the enzyme is shown in Figure 1.3, the important nucleotides in the catalytic core are marked in blue. However, a recent study suggested that only four nucleotides are conserved, which are A, G in the stem loop and C, G in the unpaired region.¹¹⁶ There is also a G.T “wobble” pair located immediately downstream from the cleavage site essential for the activity of the enzyme, and replacing it with a Watson-Crick pair eliminates the enzyme catalytic activity.¹¹⁴ The 8-17 DNAzyme was recently shown to be able to cleavage all the possible sixteen dinucleotide junctions (NrN , $\text{N}=\text{A, G, T and C}$), at different rates.¹¹⁷⁻¹²⁰

The 8-17 DNAzyme is a versatile enzyme, and its discovery has provided researchers with extensive insights into the unique structural and catalytic properties of the RNA-cleaving DNA catalyst. In comparison to other RNA-cleaving DNAzymes, 8-17 has a very small catalytic core, has a relatively fast reaction rate, and has the ability to catalyze the RNA-cleavage reaction with both DNA and RNA substrates. Interestingly, more than ten times, the 8-17 DNAzyme has been selected independently by different research groups, from random-sequence DNA libraries using *in vitro* selection.¹¹⁶ Even though the 8-17 DNAzyme is most active with Pb^{2+} , it is also active with Zn^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} , and Ca^{2+} , and does not have

selectivity toward the Pb^{2+} divalent.¹¹⁸ The RNA-based leadzyme and the GR-5 DNzyme are much more specific for Pb^{2+} .¹²¹⁻¹²³

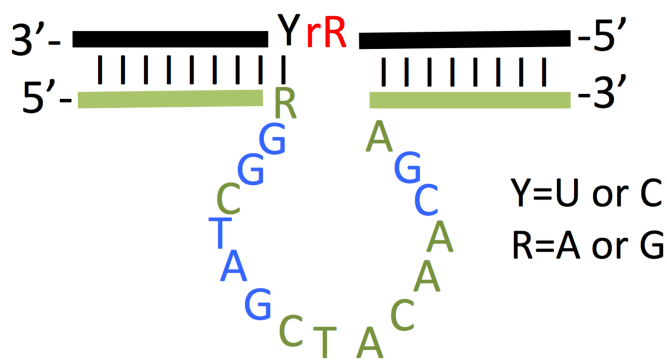


8-17

Figure 1.3 Secondary structure of 8-17 DNzyme. The cleavage site (rA) is indicated with red. The substrate is in black, the enzyme is in green, and the conserved nucleotides are marked in blue.

The 10-23 DNzyme is a special variant of the 8-17 DNzyme, which was obtained in the same selection as 8-17.^{114, 115} The catalytic core of this enzyme comprises fifteen important nucleotides. Nucleotides in the middle of the catalytic core appear to be more tolerated to mutagenesis, while those on the border sides are essentials for the efficient activity. For example, either of the two bases in the bottom of the core, C or T, can be deleted without having significant effects on the activity of the DNzyme. The 10-23 DNzyme can cleave any purine-pyrimidine junctions.¹¹⁴ This enzyme has many important applications in medicine; it can be used as a therapeutic agent to decrease the RNA levels in different systems^{124, 125}, and is also used to degrade viral RNA.¹²⁶⁻¹²⁸

Two other variants of 8-17 DNAzyme are the Mg5 enzyme, which has been selected in the presence of both magnesium and histidin,¹²⁹ and the 17E enzyme, which is highly active in the presence of zinc.¹³⁰



10-23

Figure 1.4 Secondary structure of the 10-23 RNA-cleaving DNAzyme. The substrate strand is indicated in black, the enzyme is shown in green, and the conserved nucleotides are marked in blue. The cleavage site (rA) is also marked with red.

The 39E DNAzyme is another example of a divalent metal-dependent DNAzyme. This RNA-cleaving DNAzyme is preferentially active in the presence of UO_2^{2+} , which can detect UO_2^{2+} down to 45 pM.^{81, 131} While no activity of the 39E is observed in the absence of uranyl or in the presence of other metal ions, the 39E enzyme was reported to have >1 million-fold selectivity for UO_2^{2+} over other metal ions,⁸¹ and has a $k_{\text{cat}} = \sim 1.2 \text{ min}^{-1}$.⁸¹ These features of 39E have promoted its utility for the uranium sensor with high selectivity. The conserved nucleotides of the 39E DNAzyme are shown in Figure as marked in blue. As emphasized in Figure 1.5, the bulge region contains the nucleotides responsible for the binding and the activity of the enzyme.

Despite the fact that there are very limited numbers of chemical functionalities between the nucleic acid and metal ions, their interaction is very complex, it continues to be a hot topic of research that is studied by many researchers in various fields of science. An example of this type of interaction is the role of metal ions in the reaction catalyzed by ribozyme, which has been suggested as an origin of life by the theory of “RNA world”.¹³³ Studying the binding of platinum complexes to DNA (most importantly, cisplatin and its analogs), which leads to the development of new anticancer drugs, is another important example that helps to produce practical applications.¹³⁴⁻¹³⁷

Even though some ribozymes or DNAzymes do not show metal ion-dependent activities, most of them require metal ions for their function. Metal ions can be directly involved in reactions catalyzed by ribozymes and DNAzymes, or they can have an indirect role. The direct and indirect roles of metal ions in ribozyme or DNAzyme catalysis will be discussed in two separate sections.

1.3.1 Direct role of metal ions in ribozyme/DNAzyme catalysis

As shown in Figure 1.6, a metal ion can directly participate in the catalysis in different ways: a) It can act as a general acid, donate a proton, and stabilize a developing negative charge on the leaving group; b) it can act as a general base catalyst and abstract the proton from the 2'-OH; c) it acts as a Lewis acid catalyst and stabilizes the leaving group; d) it can act as Lewis acid and promote deprotonation of the attacking nucleophile; and also e) it acts as an electrophilic catalyst and increases the electrophilicity of the phosphorus atom at phosphodiester bond.¹³⁸ What has been mentioned here are the potential roles of the metal ions, however, their exact role

is still not very well understood in most cases. In the space below, the progress made regarding this aspect are reviewed.

For example, for the hammerhead, a single metal ion mechanism is proposed, in which a Mg^{2+} , in the hydroxide ion form, acts as a general base to deprotonate the 2'-OH.^{139, 140, 141} A different mechanism has been suggested for the group I intron. For this group, a two-metal ion mechanism has been suggested, in which one metal ion acts as general acid and helps to deprotonate the 2'-hydroxyl, while another metal ion acts as a general base to stabilize the 5'-oxygen leaving group.¹⁴²⁻¹⁴⁴ Accordingly, the crystal structure of the group I intron revealed a highly coordinated cluster of two Mg^{2+} ions, and interestingly this structure clearly shows that five of the water ligands of one of the Mg^{2+} ions are replaced with oxygen atoms from the RNA.^{143, 144}

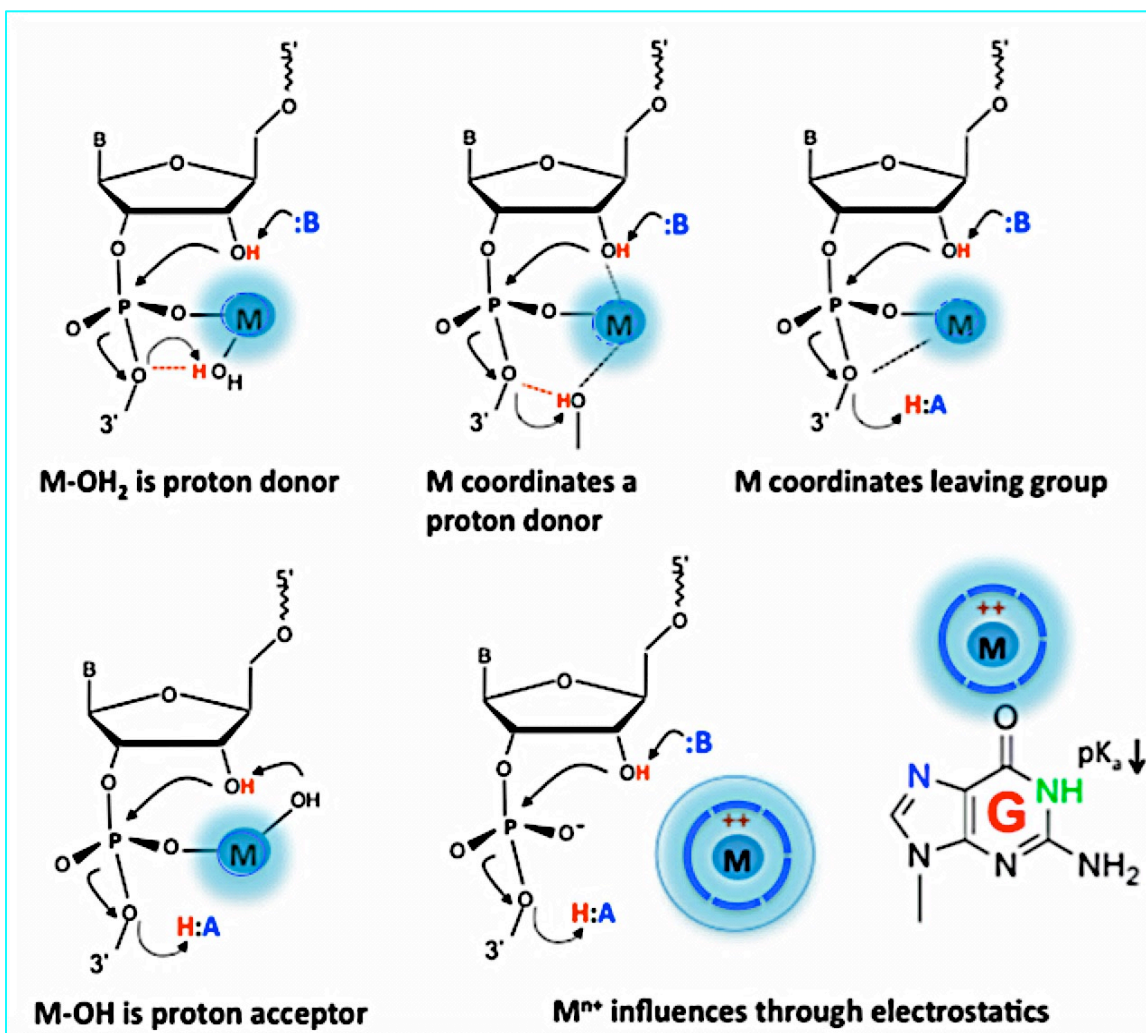


Figure 1.6 Potential roles of metal ion in RNA-cleaving reactions catalyzed by ribozymes or DNazymes. (Figure adapted from reference 145).

1.3.2 Indirect role of metal ions in the ribozyme/DNAzyme catalysis

Some of the metal ion co-factors participate in the reactions catalyzed by ribozyme/DNAzyme through their hydrated forms and do not have direct role in the catalysis. For instance, Mg^{2+} was demonstrated to be involved in the hairpin ribozyme catalyzed reactions through the out-sphere interaction, and replacing it with the coordinatively saturated metal complex, $\text{Co}(\text{NH}_3)_6^{3+}$ did not destroy the ribozyme's activity.¹⁴⁶ A similar result was observed

for the Mg^{2+} -dependent activities of an acyl-transferase ribozyme. It seems that in these cases, the Mg^{2+} metal ion is involved via out-sphere interactions through its fully hydrated form and has an indirect role in the hydrolysis.¹⁴⁷

Moreover, the electrostatics effects caused by metal ions can also indirectly activate the functional sites of DNAzymes or ribozymes and facilitate their catalysis. More especially, divalent metal ions due to the high charge density and relatively well-defined coordination geometry can have important roles.¹⁴⁸ These features of divalent metal ions give them the ability to induce or perturb the acidity of various nucleobases where necessary. For example, the presence of Mg^{2+} ion near the guanosine N_7 nitrogen will change the pK_a of the N_1 .¹⁴⁹ This is an important interaction in the hairpin ribozyme, since the N_1 -deprotonated site on an active site guanosine is involved in catalysis.^{150, 151}

Another aspects of electrostatics effects caused by metal ions can be demonstrated by the fact that some small ribozymes such as the hairpin, hammerhead, and VS ribozymes can be catalytically activated by molar concentrations of monovalent.¹⁵²⁻¹⁵⁵ Another example is the hepatitis delta virus (HDV) ribozyme that requires divalent metal ions for its functional and/or structural roles.¹⁵³⁻¹⁵⁶ In addition to $\text{Co}(\text{NH}_3)_6^{3+}$, polyamines and aminoglycoside antibiotics can replace Mg^{2+} ions to induce cleavage activity by the hairpin ribozyme.¹⁵⁷ These observations suggest that most of the these ribozymes do not have a specific binding sites for a particular metal ion, however, their requirement for the metal ions are for essential structural formation or conformational changes.¹⁵²

1.4 *In vitro* selection and characterizations of functional nucleic acids

1.4.1 *In vitro* selection

Even though enzymes composed of DNA have not been found in nature, *in vitro* selection has been applied to isolate the DNA that can form complicated secondary structures and impart catalytic ability.¹⁵⁸⁻¹⁶¹

The *in vitro* selection is a combinatorial biology technique with the ability to isolate the DNA with a specific activity or affinity toward a specific target. The function of this technique is similar to that of natural evolution. It starts with a pool of nucleic acids, which may include the sequences with favorable property that are also called winners. The goal is to select and enrich the winners from the rest of the DNAs. The generalized schematic of the various stages of *in vitro* selection is demonstrated in Figure 1.7.

The DNA pool usually contains about 10^{12} - 10^{14} different nucleic acid sequences, while the population of the winners is present at a much lower abundance. Therefore, polymerase chain reaction (PCR) has to be used to enrich the selected winners. This procedure is called a round of selection, and the amplifications produced by PCR are the pool for the next round of selection. Normally, 5-20 rounds of selection are required to select for functional DNA.¹⁵⁸⁻¹⁶¹ This is because after each round of selection, the desired DNAs may not completely be separated from the rest of DNA library. In addition, non-specifically cleaved or bound DNA may be selected as well, thus increasing the background signal. These factors contribute to the less-than-ideal separation efficiency. Thus, many rounds of selection are needed to enrich the winner population.

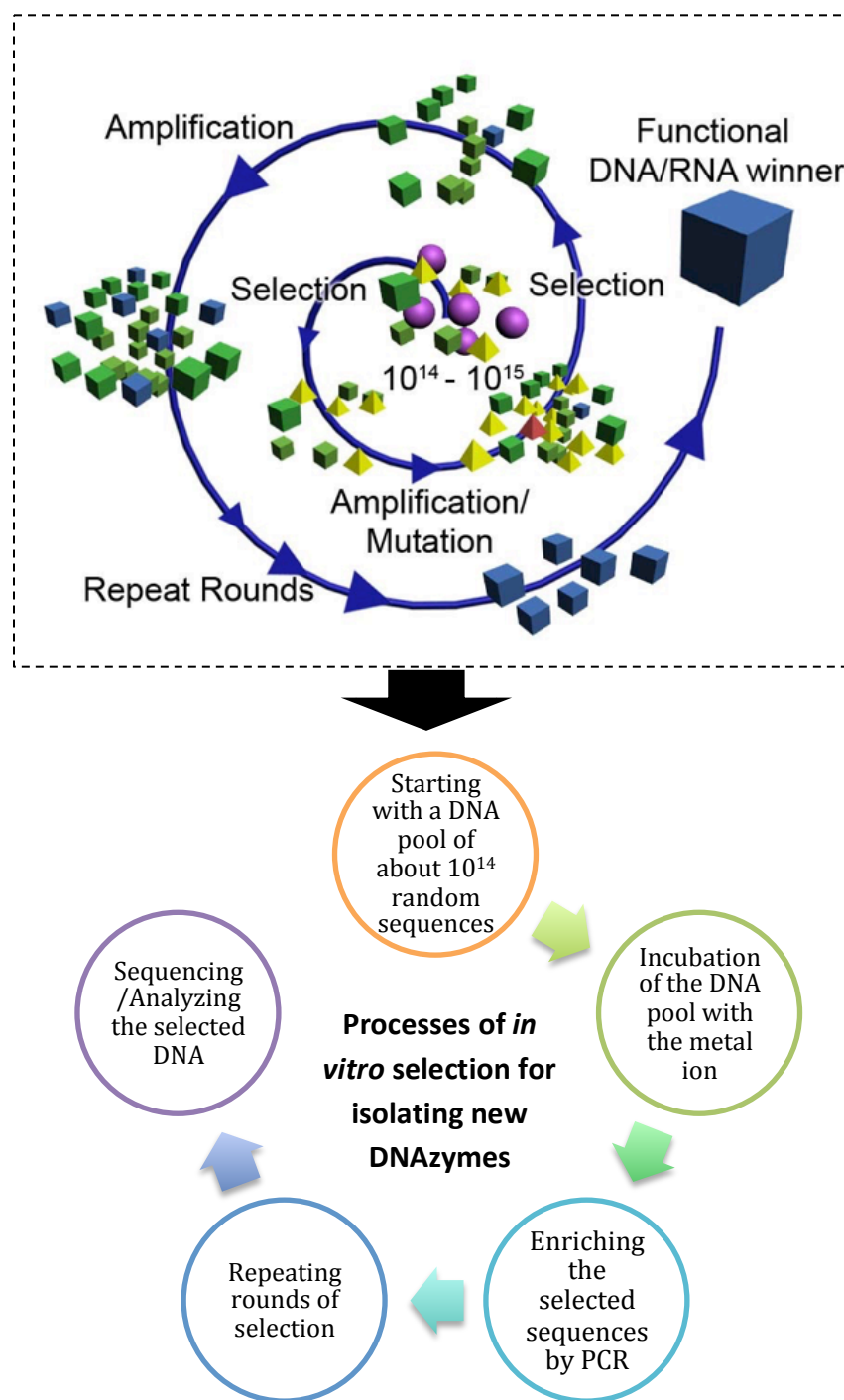


Figure 1.7 Schematic representing the general processes of the *in vitro* selection. The selection start with a random pool and after different rounds of selection, amplification, and mutation, the target sequence will be isolated, which has the desired catalytic activity. Since the PCR is not an error free technique, it can produce mutations in the sequences that are carrying to the next rounds of selection. The upper Figure is adapted from WEICHEN XU's thesis, University of Illinois at Urbana-Champaign, 2011.

Sometimes the selected sequence shows unfavorable enzymatic activity, and in addition to the metal used as a co-factor for the selection, they activate with other metal ions. In order to eliminate this problem, negative selection was developed to increase the selectivity of selected DNA.¹⁶² Negative selection is the same as the normal selection, except the library is first incubated with competing metal ions and the active sequences are discarded. For the remaining inactive sequences, the normal positive selection is carried out to select for winners that have better specificity.

After many rounds of selection the selected DNA pool are sequences and then analyzed. The sequence with the highest activity will be choose as a new functional nucleic acid sequence.

1.4.2 Biochemical characterization

The three-dimensional structure of deoxyribozymes is still not known and, thus, it is very important to elucidate the mechanism of these catalytic nucleotides by employing various kinetic and thermodynamic studies. The phosphodiester cleavage reaction of the DNAzymes was proposed to adopt the same mechanism as the small self-cleaving ribozymes.^{35, 43, 163, 164} As shown in Figure 1.8, RNA-cleaving DNAzymes are involved in a two step hydrolysis mechanism, which initiates with a nucleophile attack of 2'-OH to the phosphorus atom, forming a pentacoordinated intermediate (or transition state 1), and in the following step, the 5' oxygen is eliminated from the intermediate to complete the cleavage of phosphodiester bond at transition state 2 (TS2). TS1 and TS2 can be stabilized by a general base catalyst and a general acid catalyst, respectively, or they can be stabilized by the direct binding of Lewis acids to the 2'-attacking oxygen and the 5'-leaving oxygen.¹⁶⁵ This is the generally accepted mechanism for most DNAzymes, however, full details of the mechanisms of action remain to be elucidated.

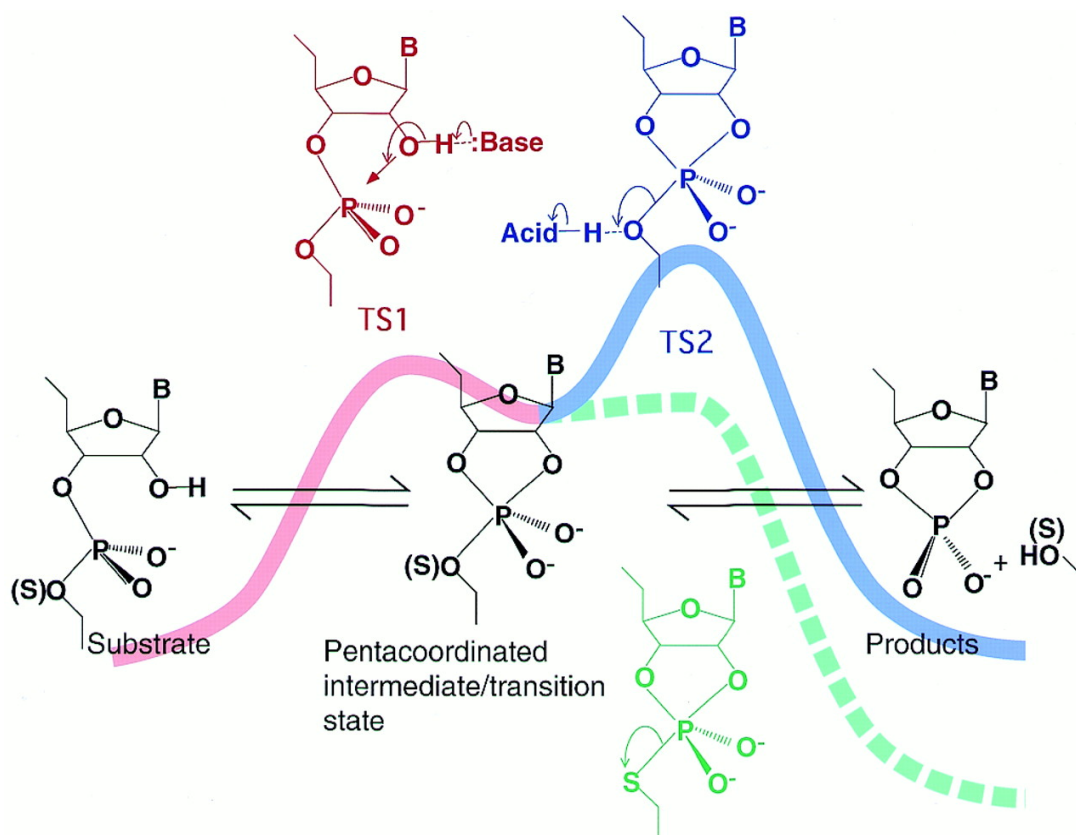


Figure 1.8 Proposed mechanism of RNA hydrolysis catalyzed by small self-cleaving ribozymes and DNAzymes. The nucleophile attaches the phosphorus atom to form pentacoordinated intermediate and then the 5' oxygen is leaving the intermediate. (The Figure adapted from reference 165).

Different biochemical experiments including Michaelis–Menten kinetics, the pH profile analysis, and metal concentration-dependent studies, can each be applied to uncover different aspects of the DNAzyme mechanism, and may demonstrate the role of metal ions in the catalysis. Systematic modifications can be applied to address the functional structural basis of ribozymes or DNAzymes.¹⁶⁶⁻¹⁷⁴ These types of modifications will allow to identify the important nucleotides or chemical groups, whose removal or replacement impair catalysis, thereby providing useful information on the mechanism of DNA enzymes. These types of mutagenesis can also show which groups can be removed or modified safely, and thus increase the stability of

the DNA to nuclease degradation.¹⁷⁰ In addition to what has been mentioned, mutational analyses can also lead to the identification of DNAzyme variants with more activity.¹⁷⁵

Phosphorothioate (PS) modification is another biochemical tool which has been used extensively to identify the important catalytic metal ion binding sites.¹⁷⁶ A phosphorothioate is basically an iso-structural mutation in which a non-bridging backbone oxygen atom is modified to sulfur atom (Figure 1.9). Accordingly, based on the “Hard Soft Acid Base” theory,¹⁷⁷ upon introducing the PS modification the affinity of metal changes toward this binding site, for example, the activity with the “hard” metal ion (such as lanthanides), which prefers oxygen over sulfur, will be lost if metal ion binding at the PS-mutated site is catalytically important. The PS modifications have been used to identify possible direct metal-coordination sites in the Group I¹⁷⁸ and Group II introns.¹⁷⁹ On the other hand, the absence of a thio-affect on the activity of the hairpin ribozyme could have shown that no direct coordination of a Mg^{2+} ion with any of the non-bridging oxygen atoms at the cleavage site is necessary for catalytic activity.^{180, 181}

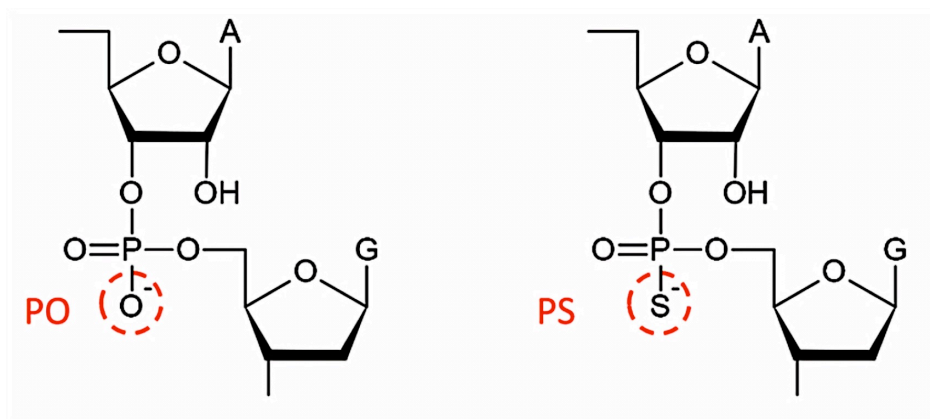


Figure 1.9 Figure represents the structure of the normal phosphate (PO) and phosphorothioate (PS) in the DNA backbone. (The Figure adapted from reference 182).

1.4.3 Biophysical characterization

Biophysical methods provide powerful tools to characterize the interaction of ribozyme and DNAzyme with metal ions and the corresponding conformational changes prior to or at the catalysis. The relationship between structure and function of ribozymes¹⁸³ and DNAzymes¹⁸⁴⁻¹⁸⁷ was widely studied using fluorescence spectroscopy, or more specifically Fluorescence Resonance Energy Transfer (or FRET).¹⁸⁸ Using the FRET technique, the global folding of the hairpin ribozyme has been well characterized and shown to have a good correlation between the extent of ion-induced folding and cleavage activity.¹⁸⁹ Ion-induced studies of the hammerhead ribozyme have also shown that the folding made by sequential binding of Mg^{2+} is essential for the formation of the catalytic domain and the catalytic activity of the ribozyme.¹⁹⁰⁻¹⁹³ The conformational changes produced in the 8-17 DNAzyme by different metal cations have been also assessed by FRET. Intriguingly, the conformational change induced by Zn^{2+} or Mg^{2+} was found to be different from that of Pb^{2+} .^{188, 194, 195} The folding made by Zn^{2+} or Mg^{2+} followed by the catalytic cleavage, while Pb^{2+} -induced cleavage did not involve the folding.^{188, 194, 195} Other biophysical characterization techniques include isothermal titration calorimetry (ITC), NMR, and X-ray crystallography.

1.5 Bio-sensing applications of RNA-cleaving DNAzymes

One of the most important applications of DNAzymes, more specifically RNA-cleaving DNAzymes, is the ability to detect a specific target. On other words, RNA-cleaving DNAzymes can be converted into biosensors, as they require co-factors, most often metal ions, for their activities. The desired selectivity and sensitivity toward a specific target required for a sensor is given to the DNAzyme during the *in vitro* selection method. Different techniques can be applied

to convert the DNzyme target binding or metal-ion cleavage event to a signal transduction event, such as fluorescence, color, electrochemical detection, magnetic resonance imaging, surface acoustic wave-based detection, microfabricated cantilever-based detection, mass spectrometry, and surface plasmon resonance-based detection.¹⁹⁶

The focus of this research is on fluorescence-based, RNA-cleaving DNzymes. Functional nucleic acids can be easily labeled with fluorophores and quenchers and converted into fluorescent sensors due to the sensitivity offered by this detection method.^{196, 197} A common strategy utilized in sensing applications of RNA-cleaving DNzymes is based on the catalytic beacon method¹⁹⁷ (Figure 1.10, left). In this method, the biosensor is composed of the enzyme strand labeled with a single quencher and a substrate strand containing an RNA base (as a cleavage site) labeled with a fluorophore and/or quencher. In the absence of a metal ion, the sensor is not activated, thus, the fluorophore is quenched due to the close proximity to a quencher. While in the presence of a specific metal ion when the enzyme is active, the cleavage takes place at the RNA site, the substrate arm containing the fluorophore releases and results in an overall increasing the fluorescence intensity. An example of using this method is the 39E DNzyme that can specifically detect UO_2^{2+} with a detection limit of 45 pM, which is considerably lower than the U.S. EPA (Environment Protection Agency) defined limit of 126 nM.¹⁹⁸ In addition, this sensor has a more than one million-fold selectivity over the other metal ions tested.¹⁹⁸ Biosensors based on this method have also been developed for some other metal ions such as Pb^{2+} and Hg^{2+} (shown in Figure 1.10, right)¹⁹⁸⁻²⁰³, and researchers are still looking to isolate new DNzymes specific for other metal ions to utilize them for metal sensing.

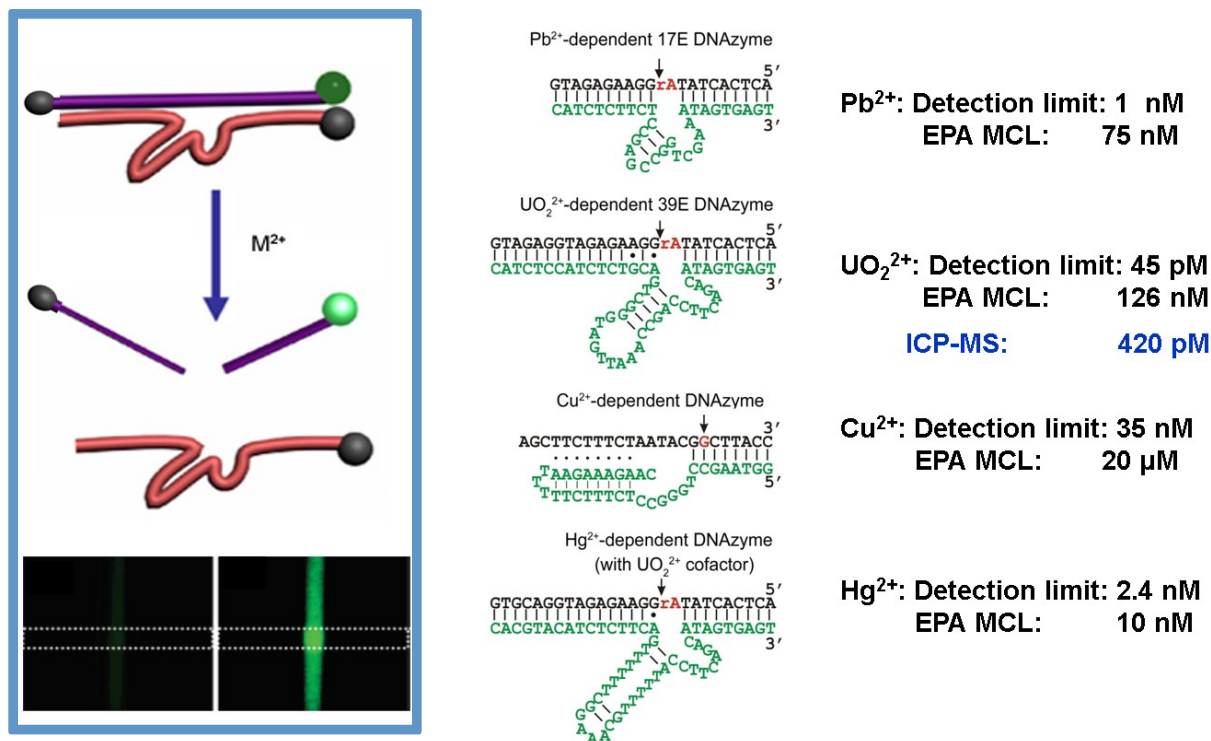


Figure 1.10 The catalytic beacon method (left) and the biosensors were developed based on this method with their detection limits for specific metal ions (right). The substrate strand containing fluorophor and quencher is shown in purple, and the enzyme strand labeled with a single quencher is shown in red. In the presence of a specific metal ion, the enzyme is being activated and cleaves the substrate, which leads to increase of florescence signal.¹⁹⁸⁻²⁰³

1.6 Trivalent metal ions

The majority of the previous work was carried out with divalent metal ions and a few monovalent dependent enzymes, however, unfortunately little is known about trivalent metal ion dependent DNAzymes. Since divalent metal ions provide much better catalysts than monovalent ions, we suspect that trivalent metal ions may result in even more efficient DNAzymes. However, such a topic has not been systematically explored. Therefore, the main goal of this thesis is to select and characterize trivalent metal ion as cofactors, in the hope of developing biosensors for this category of metal ion. There are three types of trivalent metal ions used in this work: trivalent lanthanide ions, Group 3A metal ions, and Cr³⁺. Our lab has already published a

few lanthanide dependent DNAzymes, but we are still searching for more specific DNAzymes for this class of metal ions.

1.6.1 Lanthanide ions and their interaction with nucleic acids

Lanthanide (Ln) is comprised of the fifteen metallic chemical elements, from lanthanum through lutetium, with an atomic number of 57 to 71, which are also known as the rare earth elements. They can all form trivalent cations, Ln^{3+} , although particularly stable 4f configurations can also form Ln^{+4} (Ce, Tb) or Ln^{+2} (Eu, Yb). All these three forms are strongly electropositive, thus, lanthanide ions are considered as hard Lewis acids. Lanthanides are unique in their ionic radii and the first pK_a values of bound water molecules. The chemistry of these elements is largely determined by the ionic radius, which decreases regularly from lanthanum (La) to lutetium (Lu) (1.17 to 1.0 Å). However, the similarity in ionic radius between adjacent lanthanide elements makes it difficult to separate them from each other in naturally occurring ores and other mixtures. Lanthanides aqua ions are weak acids with pK_a range from 9.4 (La^{3+}) to 8.2 (Lu^{3+}). Due to their low aqueous solubility, the lanthanides have a low availability in the biosphere, and are not known to naturally form part of any biological molecules. These chemical elements are mostly non-radioactive and are classified as low toxic.

Lanthanide elements and their compounds have many important applications, and are particularly useful in technologies that take advantage of their reactivity to specific wavelengths of light.²⁰⁴ They are used as the active ions in luminescent materials used in optoelectronics applications, the most important one is the ND:YAG laser.²⁰⁴ Lanthanides are consumed as catalysts in different reactions, used in the production of glasses, and also have different applications in phosphors and magnets.²⁰⁵ In addition, they are used in different devices such as

superconductors, samarium-cobalt and neodymium-iron-boron high-flux rare-earth magnets, magnesium alloys, electronic polishers, refining catalysts, and hybrid car components.²⁰⁶

1.6.1.1 Lanthanides for RNA cleavage

RNA molecules, due to having the 2'-OH nucleophile group, are much more susceptible to hydrolysis than DNAs ($10^5 - 10^6$ fold); even so, the uncatalysed hydrolysis of RNAs under physiological conditions is not easy. A few years ago, the remarkable catalytic activity of lanthanides for RNA hydrolysis was discovered.²⁰⁷⁻²¹⁴ These metal ions seem to be very efficient for catalyzing the cleavage of RNA, as they have the capability of completing the hydrolysis in a few minutes, at pH 7 and 30 °C. At the aforementioned pH, the catalytic activity of the lanthanide (Ln^{3+}) ions gradually increases with the increasing atomic number, and thus the last three lanthanides (Tm^{3+} , Yb^{3+} , and Lu^{3+}) have the most activity.²¹⁵ Interestingly, Ce^{4+} , which is the most effective lanthanide for DNA hydrolysis, is not so active for the hydrolysis of RNA.²¹⁶⁻

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The bimetallic cluster is the active species of lanthanides required for an efficient RNA hydrolysis.^{214, 219, 220} The formation of active species, $[\text{Ln}^{3+}_2(\text{OH})_2]^{4+}$, is accomplished by the release of two protons. Lanthanides share the same pH-rate profile, which consists of an increasing straight line at a lower pH and a plateau at higher pH.²¹⁴ The straight line is when the active species form and the plateau corresponds to the stage that the formation is completed. As the atomic number increases, the plateau gradually shifts to the lower pH side and the concentration of the active form increases at a lower pH.

The mechanism for RNA hydrolysis catalyzed by lanthanide ions is indicated in Figure 1.11. As previously mentioned, the RNA hydrolysis is completed in two steps. The lanthanide

ion (in its active form) cooperates with the phosphodiester linkage of RNA to facilitate each step of the hydrolysis.²¹⁴ In the first step, the lanthanide ion bound to the attacking hydroxide ion functions as the general base catalyst. In the following step, the metal-bound water acts as an acid catalyst to catalyze the elimination of 5'-OH. The water bound to the lanthanide ions has a pK_a of around 8-9, which is further decreased in the bimetallic clusters. This pK_a is ideal for the acid catalysis, since the RNA hydrolysis also occurs at around pH 7.²²¹ Hence, the acid catalysis cooperates with the base catalysis in the hydroxo clusters to promote the hydrolysis of RNA.

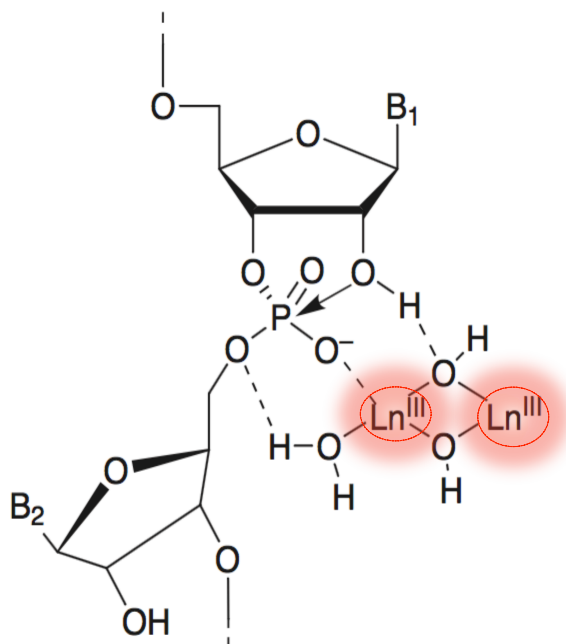


Figure 1.11 Proposed mechanism of RNA hydrolysis induced by Lanthanide (III) ions. Figure adapted from reference 214.

The energy diagram for the hydrolysis of RNA and DNA is indicated in Figure 1.12. As shown in the diagram, in the catalyzed hydrolysis of both RNA and DNA (shown by black solid line), the second step (the removal of the 5'-OH of the ribose from the phosphorus atom) is the

rate-limiting step.^{215, 221}; however, since the first barrier in the alkaline hydrolysis (uncatalysed) of RNA is much lower than that in DNA hydrolysis, then the main role of the catalyst is to lower the energy of the second barrier of RNA hydrolysis. The trivalent lanthanide ions are suitable to serve as the acid catalysis in the second step to lower the energy, because they have many coordinated water molecules (8 to 9) and also, as mentioned before, their pK_a values are close to the pH of the reaction mixture.²²² Accordingly, the lanthanide ions accelerate the rate-limiting removal of the 5'-OH, and at the same time, the first step is also promoted to some extent, so that the second-step remains rate limiting. Unlike RNA, in the uncatalysed hydrolysis of DNA the heights of the first barrier and the second barrier are almost the same. Therefore, the catalysis should have the capability of efficiently lowering the energy of the first step, while the lanthanides (III) are poor for the DNA hydrolysis, and ions such as Ce^{4+} are more efficient. The Ce^{4+} accelerates both steps of DNA hydrolysis and also help to stabilize the penta-coordinated intermediate.²¹⁴

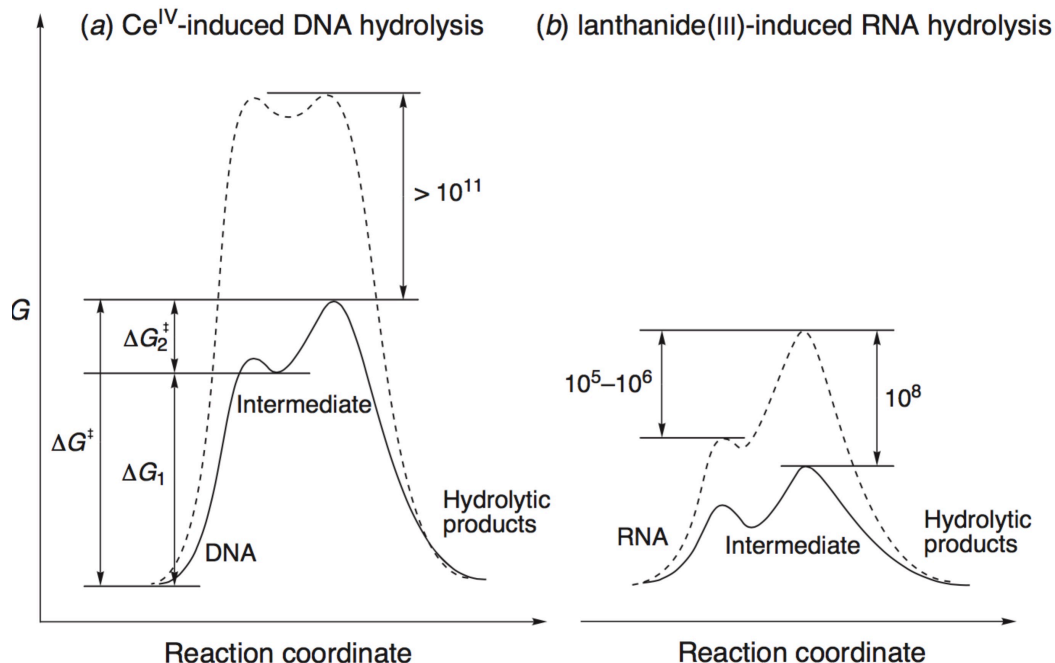


Figure 1.12 Energy diagram for the DNA hydrolysis (a), and RNA hydrolysis (b), induced by Ce (IV) and lanthanides (III), respectively. The dotted lines correspond to the alkaline hydrolysis, and the solid lines show the catalyzed hydrolysis. (Figure taken from reference 214).

1.6.1.2 Lanthanides as nucleic acid probes

Lanthanides have been extensively used to study the structure and function of nucleic acid. The unique physical and chemical properties of some lanthanides, such as terbium (Tb^{3+}) and europium (Eu^{3+}) and their chelates, have provided important tools to investigate various biological assays due to their luminescence.²²¹ The enhanced emission of Tb^{3+} has been explored as a potential tool in the detection of single base mismatches in DNA duplexes.²²⁴ This is based on the fact that the Tb^{3+} emission is greatly enhanced by single-stranded oligonucleotides, while double strands have no effect on the emission.²²⁴ Thus, in the presence of Tb^{3+} , single mismatches in the sequence of a duplex lead to selective luminescence enhancement. Interestingly, different mismatches have different effects on the Tb^{3+} emission. The GG

mismatch has the largest enhancement of the emission, CA, GA, and CC mismatches have a moderate effect, and the smallest emission intensity are caused by TT and TG mismatches. Conclusively, the enhanced emission of lanthanides can selectively and efficiently detect single mismatches in duplex.²²⁴

In addition, lanthanide metals, more particularly Tb^{3+} and Eu^{3+} , are important probes for mapping metal-binding sites in RNA.²²⁵⁻²³⁰ For example, Tb^{3+} can bind to the same sites on RNA as magnesium, but with a much higher affinity.²³¹⁻²³³ Therefore, low concentrations of it can displace magnesium and promote phosphodiester cleavage. However, at higher concentrations, terbium induces non-specific cleavage in the RNA backbone, with a preference for single-stranded and non-Watson–Crick base-paired regions.²³⁴

Recently, the Tb^{3+} -induced RNA cleavage has been introduced as a technique to study dynamics in RNA. Using this technique, the structural changes in different RNAs such as tRNA²³⁴ and the trans-acting version of the HDV ribozyme²³⁵, upon binding to their specific ligands or substrates, were monitored. The cleavage pattern obtained by this method is consistent with the observations proposed by the complex techniques such as fluorescence resonance energy transfer,²³⁶ fluorescence quenching,²³⁷ and NMR spectroscopy,²³⁸⁻²⁴⁰ while this strategy can also show details about the structural changes associated with binding event and conditions such as, pH, and concentration of monovalent cations.

1.6.1.3 Lanthanides inhibits the hammerhead ribozyme and the 17E DNzyme

In addition to the previous information about lanthanides, they can compete with other metal ions in enzymes and act as enzyme inhibitors. For instance, both the hammerhead ribozyme²⁴¹ and the 17E DNzyme are inhibited by the Tb^{3+} ion.^{241, 240} In these cases, the

sensitized Tb^{3+} luminescence spectroscopy can be used to provide information about the metal-binding site in enzymes, such as possible locations and number of functional groups involved in the metal binding in each of the enzymes.

In the hammerhead ribozyme catalysis, terbium (Tb^{3+}) competes with the single magnesium ion (Mg^{2+}) to inhibit the cleaving reaction.²⁴¹ It has been suggested that the inhibition of cleavage by Tb^{3+} results from metal binding to the site adjacent to G5, which is an essential guanosine in the catalytic core of the ribozyme.²⁴¹ There are two explanations for terbium inhibiting the hammerhead ribozyme, with each suggesting different roles of the Mg^{2+} ion during hammerhead catalysis.²⁴¹ The first hypothesis suggests that the Mg^{2+} ion helps to form a proper transition state structure and maintain a particular orientation between G5 and the nearby stem III; thus, Mg^{2+} has to be positioned correctly by the functional groups of G5. While the Tb^{3+} ion is not a perfect substitute for Mg^{2+} (due to different coordination preferences and size), and may not be able to maintain this structure properly. The second hypothesis proposes that the Mg^{2+} ion should be released from G5 and be allowed to participate in the transition-state structure, while Tb^{3+} binds at this site much more tightly than Mg^{2+} and locks the ribozyme. The synthetic modification assays also confirmed the second hypothesis and introduced a modified version of this ribozyme that is resistant to Tb^{2+} inhibition, most likely by sterically blocking the binding site.²⁴¹

A similar reversible competitive Tb^{3+} inhibition has been suggested for the 8–17 DNAzyme catalysis.²⁴² The Tb^{3+} ion probably binds either to the same or close to the active metal-binding site(s) in the DNAzyme, and inhibits the DNAzyme activity. Similar to hammerhead inhibition, Tb^{3+} can also inactivate the DNAzyme by preventing the binding of active metal ions or changing the conformation of the DNAzyme.^{241, 242} However, Pb^{2+} (the most

active metal ion for this enzyme) was suggested to bind to a different metal-binding site from Tb^{3+} and other less active metal ions.²⁴² Thus, Tb^{3+} does not directly compete with Pb^{2+} for the same site, but its binding may cause indirect conformational changes that disturb the perfect coordination of Pb^{2+} in the DNAzyme, resulting in the inhibition of the enzyme activity.²⁴²

1.6.1.4 Lanthanides promote the leadzyme and activate the GR-5 DNAzyme

Although lanthanides inhibit the activity of some enzymes, they may promote or activate some other enzymes, such as the leadzyme and the GR-5.²⁴³⁻²⁴⁵ The RNA-based leadzyme and GR5 DNAzyme are two phosphodiester-cleaving Pb^{2+} -dependent enzymes with high selectivity toward the Pb^{2+} .^{113, 120, 121} While, in addition to Pb^{2+} , lanthanides can function as effective co-factors for these enzymes to accelerate their catalytic activities. The proper steric coordination, perfect first pK_a of bound water molecules, and strong acidities are the main features of lanthanides being involved in the mechanism catalyzed by these enzymes, as effective co-factors.

Kinetic studies indicated that the RNA-cleaving activity of leadzyme increases significantly when Nd^{3+} cation is present in the reaction, in addition to Pb^{2+} .^{243, 244} While other divalent ions (Fe^{2+} , Ni^{2+} , Cu^{2+} , and Cd^{2+}) or the trivalent ions (Al^{3+} , Cr^{3+} , and Fe^{3+}) have no effect on the catalysis, and may even inhibit the enzyme activity.²⁴⁴ It is probably that the Nd^{3+} ion promotes the RNA hydrolysis by increasing the stability of the complex between the leadzyme and the substrate.²⁴⁴ Moreover, Nd^{3+} was suggested to involve in a two-metal-ion mechanism and accelerate the catalysis (mechanism is shown in Figure 1. 13).²⁴⁴ The two-metal ion mechanism has been also proposed for some other ribozymes such as DNA polymerase I and hammerhead ribozyme.²⁴⁶⁻²⁵⁰ In these cases, the reaction is assumed to be accelerated by the action of two ions. In the mechanism catalyzed by the leadzyme, the contribution of Nd^{3+} and

Pb^{2+} on the mechanism seems not to be independent, and once they are equally present in the reaction (1:1 ratio), then the enzyme has the optimal activity.²⁴⁴ Accordingly, in the proposed two-metal-ion mechanism, Pb^{2+} may act as a base catalyst and activate the attacking water, while Nd^{3+} ion is probably the acid catalyst and coordinates or stabilizes the leaving oxygen group.²⁴⁴ However, in the presence of Pb^{2+} only, Pb^{2+} has to act as both acid and base, and thus, the cleavage rate is slow because Pb^{2+} is not a good acid. On the other hand, due to having a lower pKa value, the Pb^{2+} is a better base catalyst and could serve this role more efficiently (Pb^{2+} -bound water molecule has pKa of 7.2 and the pKa value of Nd^{3+} is 8.6).

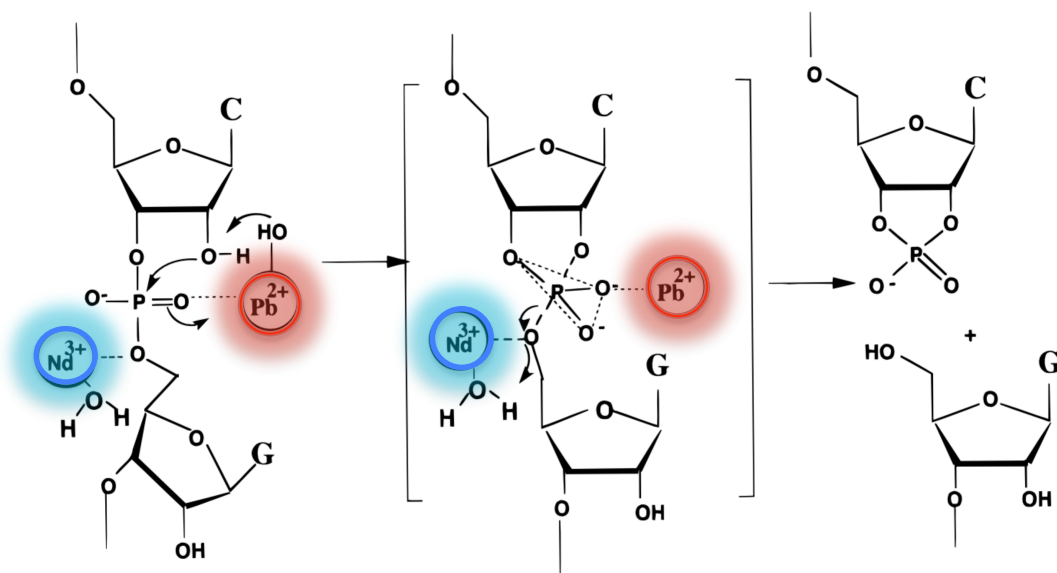


Figure. 1.13 The two-metal ion mechanism of the RNA cleavage catalyzed by leadzyme, in the presence of Pb^{2+} and Nd^{3+} . (Figure adapted from reference 244). Pb^{2+} and Nd^{3+} act as the base and the acid catalyst in the mechanism, respectively.

A range of lanthanide ions are also able to function as co-factors for GR-5 DNAzyme, with the smaller lanthanides, such as Tb, Tm, and Lu being the most effective ones.²⁴⁵ Due to having a smaller size, these ions are probably more suited for coordination of the metal binding

site in the DNAzyme than those of the larger lanthanides. The leadzyme has a catalytically important binding site for a single metal ion, which seems to be the same for both the lanthanide ion and the lead ion.²⁴⁵ However, the ion has to be positioned appropriately in this site to be able to catalyze the phosphodiester bond. The hydrolysis is suggested to be a single deprotonation event in this enzyme, which is probably a deprotonation of water coordinated to the bound lanthanide cation.²⁴⁵ The lanthanide-bounded water acts as a nucleophile and facilitates the polarization of the 2' hydroxyl group in the substrate strand. Removal of this group has a significant effect on the enzyme activity, which can indicate the direct coordination of this group with the lanthanide, while the two non-ester phosphate oxygen atoms seem not to make inner-sphere coordination contacts with the bound lanthanide. Interestingly, the substrate alone seems to be mostly responsible for the binding of the lanthanide, and the enzyme is probably enhancing the rate by a means other than binding, such as introducing the selectivity to the ion-binding site.²⁴⁵

1.6.2 Group 13 (3A) metal ions and their possible interaction with nucleic acids

Group 13 (3A), sometimes referred to as the boron group, includes boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), and ununtrium (Uut). However, ununtrium is only created in a laboratory and is not found in nature. Boron is the only metalloid in this family, and the rest of the elements (Al, Ga, In and Tl) are considered as poor metals. The atomic number and ionic radii increase from boron to thallium. Most of the elements in the boron group show increasing reactivity as the elements get heavier in atomic mass and higher in atomic number, while boron, the first element in the group, is generally unreactive with many elements except at high temperatures.

These elements all have three valence electrons with the valence electron configuration ns^2np^1 . They adopt oxidation states +3 or +1. Most of the elements in this group tend to form a 3+-oxidation state, but heavier elements like Tl prefer a +1 oxidation state due to their stability. Intriguingly, Tl^+ (140 pm) has an ionic radius intermediate between K^+ (133 pm) and Rb^+ (148 pm), resulting in a very similar ionic chemistry. The Tl^+ salts are quite toxic, and can enter the body via potassium uptake pathways and replace K^+ in the cells.

In an aqueous solution, all M^{3+} ions are acidic, except $B^{3+}(aq)$ that does not exist. The other four elements, Al, Ga, In, and Tl have pK_a values of 4.95, 2.6, 3.7 and 1.15, respectively. Heavier ions in the group are assumed to have stronger acidity, due to poor shielding by underlying d subshells (Ga^{3+} , In^{3+} , Tl^{3+}) and $4f$ subshell (Tl^{3+}).

The focus of this study is on the heavier elements of this group including Al, Ga, In, and Tl. Aluminum is the third most abundant element and the most abundant metal. While, Ga, In, and Tl can be classified as rare elements, all these elements can be toxic at a high concentration. Some of them are only toxic to animals, but some are toxic to both animals and plants. Aluminum, gallium, and indium are moderately toxic and may have some minor effects if they are present in large doses. Thallium is the most hazardous element in the group. It is extremely toxic, and has caused many poisoning deaths. Thallium compounds are usually colorless, odorless, and tasteless; thus, they should be sensed in the environment by recognizable techniques. Techniques such as neutron activation analysis, atomic absorption spectrophotometry, spectrochemical analysis, and spectrophotometry can successfully detect thallium.²⁵¹⁻²⁵³ However, these techniques are usually so expensive, time consuming, and also require sophisticated laboratory equipment. A simple method of detection for all the four elements (Al^{3+} , Ga^{3+} , In^{3+} , and Tl^{3+}), and more importantly for thallium, would be very useful. In

this research, attempts were made to select specific DNazymes for group 3A metal ions, using the *in vitro* selection strategy. As discussed previously, the functional nucleic acids have the ability to easily and quickly detect different metal ions. However, despite the importance of these elements, no specific DNzyme has been reported for group 3A trivalent metal ions. Selecting specific DNzyme for group 3A metal ions is very important, and can then be established as DNA-based biosensor for these ions.

Group 3A elements have numerous uses and applications. Their alkyl compounds, particularly those of aluminum, are widely used in industrial catalysis, organic synthesis, and the electronics industry. Aluminum has many different uses in everyday life, including the construction materials, electrical devices, and tools and vessels for cooking and preserving food.^{254, 255} Gallium and its derivatives have recently found important applications, and are used in semiconductors, amplifiers, solar cells (for example, in satellites) and dental equipment. However, its major application is LED lighting.²⁵⁶ The largest part of indium production (70%) is used for coatings (combined as indium tin oxide), and it is also used in electrical components and semiconductors.²⁵⁷ Thallium also has many uses, though it is mostly applied in the elemental form. It is used in low-melting glasses, photoelectric cells, switches, thallium salts, and can also be found in lamps and electronics.²⁵⁸

1.6.2.1 Group 13 (3A) metal ions interactions with nucleic acids

The monovalent thallium, Tl^+ , was suggested to directly interact with DNA, since it was shown to bind to the major and minor grooves of B-DNA. The structural analysis of Tl^+ -DNA complexes indicated that the Tl^+ cations are mainly bounded at guanine N7, O6, and thymine O2 as well as O2P groups, while they showed larger contact distances with sugar donor atoms.²⁵⁹

The Tl^+ metal ion was seen to bind to both nitrogen atoms of the purine and pyrimidine rings as well as to the phosphate groups.²⁶⁰⁻²⁶²

Interestingly, Tl^{3+} was indicated to adsorb protein at neutral or alkaline pH, and acts as “soft” acids, like the transition metal ions such as Zn^{2+} , Ni^{2+} , and Cu^{2+} .²⁶³ There seems to be a direct relationship between the toxicity of thallium and its ability to form coordination bonds with amino-nitrogen and the corresponding ability to adsorb proteins.²⁶³ This can suggest the possibility of an interaction between these trivalent metals and other nitrogen-containing substances, like nucleic acids.²⁶³

Studying the interactions between each of Al^{3+} and Ga^{3+} metal ions with DNA has demonstrated that at higher concentration of these cations, base binding occurs which results in the alteration of the helical structure. However, at low concentrations, these trivalent metal ions preferably bind to the backbone phosphate groups, which results in an increase of DNA helix stability.²⁶⁴⁻²⁶⁶ Between the two ions, Al^{3+} was suggested to produce larger perturbations of DNA bases, whereas gallium induces larger alterations of the backbone phosphate groups.²⁶⁷

1.6.3 Trivalent chromium (Cr^{3+}) metal ion and its interaction with nucleic acids

Chromium is a chemical element with the symbol Cr and atomic number 24. It is the first element in Group six, and is considered as a transition metal. Cr has an electronic configuration of $4s^1 3d^5$. It can be present in several forms (I to VI), while the Cr^{3+} is the most stable form that is naturally found in nature in rocks, animals, plants, soil, volcanic dust, and gases. The Cr^{3+} ion has a similar radius (63pm) to the radius of Al^{3+} ion (50pm), so the two metal ions can replace each other in some compounds, such as chrome alum and alum. Cr^{3+} is acidic, with a tendency to

hydrolyse and form poly-nuclear complexes containing OH⁻ bridges in a process known as “olation”.

Although in some studies Cr³⁺ has been classified as non-essential for mammals,²⁶⁸ there are other reviews that have mentioned Cr³⁺ as a nutrient and an essential trace element in humans.²⁶⁹ The Cr³⁺ ion is assumed to be required in trace amounts for sugar and lipid metabolism. While larger amounts of Cr³⁺ can be toxic and carcinogenic. The recommended daily dose of Cr³⁺ is 50-200 µg. At higher concentrations, Cr³⁺ binds to the DNA, negatively affecting cellular structures and damaging the cellular components. However, compared to Cr⁶⁺, the toxicity of Cr³⁺ is less serious. Cr⁶⁺ is the most hazardous form of chromium and can be very toxic, even in small amounts. Unfortunately, these two oxidation states, Cr³⁺ and Cr⁶⁺, are interchangeable. At a high pH, the Cr³⁺ can be oxidized and form the more toxic Cr⁶⁺. Hence, it is necessary to develop a sensitive and selective method to detect the Cr³⁺ level in the environment.

There are different analytical methods of detecting Cr³⁺ in the environment, including inductively coupled plasma (ICP), atomic emission spectrometry (AES),²⁷⁰ flame atomic absorption spectrometry (FAAS),²⁷¹ chromatography,²⁷² fluorometry,²⁷³⁻²⁷⁶ and electrochemical methods.^{277, 278} Even though these methods are accurate and sensitive, they are very expensive, time-consuming, and also require sophisticated laboratory equipment. DNazymes can be a suitable substitute for these traditional methods of detection. DNazymes are not expensive, and they function simple and quickly. Accordingly, in this research, our efforts were made to isolate a DNzyme specific for Cr³⁺ to determine the Chromium (III) contamination in the environment. The process of the Cr³⁺-dependent DNzyme selection is described in chapter 5.

Chromium metal has a high value because of its high corrosion resistance and hardness, and is involved in many applications. It is an important alloying material for steel, and can be used for the formation of high-speed tool steels, stainless steel, and jet engines. Chromium (III) and its compounds are used for chrome plating, dyes and pigments, the tanning of leather, and wood preserving. The high heat resistivity and high melting point makes chromite and chromium (III) oxide a material for high temperature refractory applications, like blast furnaces or cement kilns. In addition, several chromium compounds are used as catalysts for processing hydrocarbons. For instance, the Phillips catalyst, which is made from chromium oxides, is used for the production of about 50% the world's polyethylene.²⁷⁹ Fe-Cr mixed oxides are used as high-temperature catalysts for the water gas shift reaction.²⁸⁰ Copper chromite is a practical hydrogenation catalyst.²⁸¹

1.6.3.1 The Cr^{3+} and nucleic acid interactions

The Cr^{3+} metal has been indicated to directly interact with nucleic acids. Cr^{3+} can bind to any kind of nucleic acid, but it is suggested that it mostly interacts with the G and C bases, as its binding activity increases with the increasing G + C content of DNA/RNA strands.²⁸² However, the Cr^{3+} ions appear to mainly interact with phosphate groups of nucleic acids. Intriguingly, the interaction of Cr^{3+} produces a destabilization of the DNA structure, while also stabilizing the RNA structure.^{282, 283} Cr^{3+} produces chelation between bases and phosphate groups of the same DNA strand, and results in destabilization of the DNA molecule,²⁸³ while the Cr^{3+} -RNA interaction induces intramolecular crosslinking, involving phosphate groups and sandwich bonds between neighbouring guanine residues, which stabilize the RNA molecule.²⁸³ The interaction of Cr^{3+} with DNA molecules, which leads to DNA-DNA or DNA-protein cross-links, can also severely affect the function of DNA-polymerase.²⁸⁴

The Cr^{3+} metal was shown to be able to catalyze the removal of pyrophosphate from the nucleoside triphosphate (NTP) molecules.²⁸⁵ In this reaction, the pyrophosphate group is removed from nucleoside triphosphates. NDP and NMP are the degradation products of NTP. Studies show that the incubation of NTP with Cr^{3+} ions for several hours leads to a distinct increase of NMP and decrease of NTP concentrations, while the amount of NDP remains unchanged.²⁸⁵ It has been suggested that Cr^{3+} ions that form stable bidentate complexes with NTP ligands can promote the removal of a Cr^{3+} -pyrophosphate unit and the subsequent formation of NMP molecules.²⁸⁵ On the other hand, the NMP molecules are poor ligand for Cr^{3+} and cannot make such stable complexes. In this reaction, Cr^{3+} was suggested to mainly interact with DNA through phosphate groups, while, the guanosine moieties may also be involved.²⁸⁵ The ability of other metal ions for catalyzing the dephosphorylation of nucleoside 5'-triphosphate has been also indicated.²⁸⁶ However, those metal ions catalyze monophosphate removal during the hydrolysis process, but Cr^{3+} ions are able to catalyze the removal of a pyrophosphate group from ligands, which is a unique fact about Cr^{3+} metal ions.²⁸⁵

1.7 Overview of the thesis

DNAzymes are promising tools for detecting different metal ions, with their particular ability to easily convert to selective and sensitive bio-sensors. Since the discovery of the first DNAzymes, many types of catalytic DNAs have been introduced. RNA-cleaving DNAzymes are indeed one of the most-studied types of catalytic DNAs. However, most of the previous studies have been done on monovalent- and divalent- dependent RNA-cleaving DNAzymes. In this research, the focus is on selecting and characterizing new RNA-cleaving DNAzymes specific for trivalent metal ions - including some lanthanides (Ln^{3+}), group 3A metal ions (M^{3+}), and Cr^{3+} , in

the hope of discovering new biosensors for this group of metal ion, and elucidating some of the unknown details about the RNA-cleaving DNAzymes and their hydrolysis mechanism.

Chapter one gave a brief introduction to metal ion dependent-DNAzymes, their applications, and the potential roles of metal ions in the mechanism catalyzed by functional nucleic acids, with overviews on the lanthanides, group 3A metal ions, and chromium. In addition, the *in vitro* selection strategy, a method of selecting new DNAzymes, was also explained. The starting point for the development of new biosensors based on functional nucleic acids is their isolation, which is achieved by this simple method of selection. **Chapter two** describes our efforts to use this method of selection to find new DNAzymes for Ce^{3+} , followed by analyzing the selected sequences and characterizing a lanthanide-dependent DNAzyme, named Ce13d. A very unique activity of the Ce13d DNAzyme in the presence of different metal ions is indicated by introducing a PS-modification at the cleavage site. **Chapter three** focuses on our efforts to use *in vitro* selection to identify DNAzymes specific for the last two lanthanides in the periodic table, Yb^{3+} and Lu^{3+} . The biochemical characterization studies of Lu12 DNAzyme, a newly selected DNAzyme also included in this chapter, which collectively allowed us to suggest a unique RNA hydrolysis mechanism for this DNAzyme. **Chapter four** summarizes our efforts on selecting DNAzymes specific for group 3A metal ions, using N35 and N50 DNA libraries. Data for Tl^{3+} -dependent DNAzymes isolation using the PS-modified library is also included, and **Chapter five** describes our works on studying the interaction between the DNA and Cr^{3+} , using the previously selected Ce13d DNAzyme. It also describes our efforts to select specific Cr^{3+} -DNAzymes, using a different *in vitro* method called blocked selection. Negative selections to overcome non-specific interference at different stages of selection are explained as well.

Chapter 2. *In vitro* Selection of Ce³⁺-dependent DNazymes and Characterization of Ce13d, a General Lanthanide or Soft Metal Dependent RNA-Cleaving DNzyme

2.1 Introduction

Lanthanides are an extremely important category of metal ions, and have very unique catalytic, magnetic, optical and electronic properties.^{287, 288} There are many lanthanide-containing products for different applications. Well-known examples include superconductors, lasers, TV displays, luminescent probes, and imaging contrast agents. Their widespread applications highlight the importance of accurately measuring these elements. Lanthanides are typically analyzed by instrumentation methods such as inductively coupled plasma-mass spectrometry (ICP-MS), capillary electrophoresis (CE), and vibration spectroscopy.²⁸⁹ These methods are sensitive and selective, but they often require multiple steps of sample preparation and working in centralized laboratories at a high cost. On-site and real-time detection methods like sensors are more desirable. DNazymes can make a unique platform for designing metal biosensors and serve as suitable alternatives of sophisticated instrumentation methods. DNzyme-based detections do not need expensive, time-consuming, or sophisticated laboratory equipment. They are preferred for sensing applications because of their high stability as well as their solubility in aqueous solutions. They can act as good sensors to selectively detect many metal ions.

DNazymes are functional nucleic acids with the ability to catalyze different reactions. They typically require metal ions for their function, and only perform efficient activity in the presence of specific metal ions.²⁹⁰ DNazymes are selected using *in vitro* selection method. DNazymes specific for a few divalent metal ions have already been reported, including Mg²⁺,

Zn^{2+} , Mn^{2+} , UO_2^{2+} , Pb^{2+} , Hg^{2+} , and Co^{2+} .^{6, 291-298} Lanthanides, particularly trivalent lanthanides, are also known to be very effective for RNA hydrolysis, and thus can be considered as good cofactors for isolating RNA-cleaving DNazymes.²⁹⁹⁻³⁰¹ They were used for probing nucleic acids.³⁰²⁻³⁰⁶ There are also examples of lanthanides that can inhibit or promote ribozyme or DNazymes.³⁰⁷⁻³⁰⁹ Recently, our lab has successfully selected very interesting new DNazymes by employing various lanthanides.²⁹⁹⁻³⁰¹ In this chapter and the next chapter, my work on isolating new lanthanide-dependent DNazymes using *in vitro* selection is described. The biochemical characterization to gain insight into the structure and the hydrolysis mechanism of the selected DNazymes is also stated.

In this Chapter, Ce^{3+} was explored as the target metal ion for the selection, and a careful biochemical characterization was performed on the selected Ce13d DNzyme. The Ce13d activity with the normal phosphate and the PS-modified substrates were studied and compared with different metals. New insights are gained by the side-by-side comparison of the DNzyme cleaving the normal substrate and cleaving the PS-substrate to explore the effect of metal concentration, pH, and mutations.

2.1 Results and discussion

2.2.1 Ce^{3+} -dependent *in vitro* selection

Previously, the Liu lab performed an *in vitro* selection with Ce^{4+} as the intended cofactor, which resulted in the isolation of a new DNzyme (named Ce13) with a remarkable activity toward Ce^{3+} .²⁹⁹ Interestingly, the Ce13 DNzyme has almost no activity with Ce^{4+} , but is highly active with all trivalent lanthanides (Ln^{3+}) and Y^{3+} .²⁹⁹ After converting this Ce13 DNzyme to a biosensor, it has a low detection limit for Ce^{3+} and almost the same sensitivity for other

lanthanides.²⁹⁹ Here, an interesting question arose, that is, whether new DNazymes can be isolated if Ce^{3+} is used.

The selection was carried out using a DNA library containing 50 random nucleotides (N50), as shown in Figure 2.1 (The sequences of N50 DNA library are shown in 2.1B, and the randomized region is marked in blue). In the whole DNA sequence, there is a single RNA linkage (rA). RNA is about one million fold less stable than DNA,³¹⁰ thus it can provide a scissile bond. The initial DNA library contained $\sim 10^{14}$ random DNA sequences. The library is incubated with Ce^{3+} target metal ion to induce cleavages. The cleaved sequences were 28-nucleotide shorter than the original full-length DNA and could be isolated using gel electrophoresis. Two rounds of polymerase chain reactions (PCR) were carried out to amplify the cleaved DNA. In PCR1, the full-length library was regenerated using P1 and P2 primers. In the PCR2, two different primers were used: P3 and P4. P3 has a FAM label on its 5'-end and a riboadenosine (rA) base near its 3'-end. P4 has a polymer spacer that can stop the polymerase reaction. Subsequently, two strands of unequal length were produced after PCR2, allowing isolation of the positive strand containing rA via gel electrophoresis to seed the next round of selection. After conducting five rounds of selection, the activity of library reached $\sim 50\%$ and no further improvement was observed afterwards (Table 2.1). Selection was stopped at round 6, when the library was cloned and sequenced. Summary of the selection is shown in Figure 2.2. In the following step, the selected sequences were aligned and analyzed. Interestingly, we found about one third of the sequences belonged to the Ce13 sequence family, while the rest were the random sequences that did not form properly.

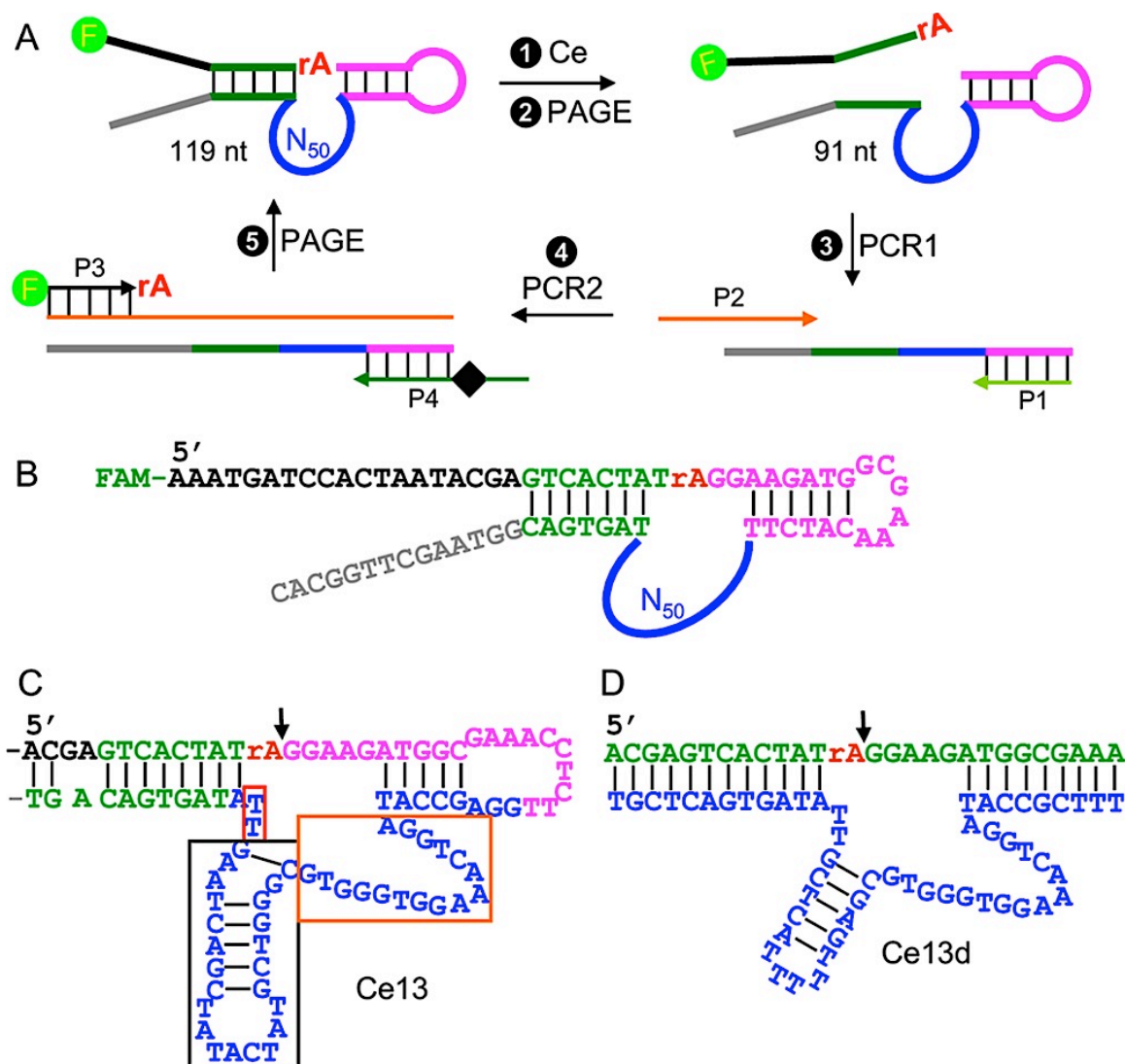


Figure 2.1 (A) Processes of *in vitro* selection method. The DNA library is incubated with the target metal to induce the cleavage. The cleaved fragments are used to regenerate the library for the next round selection, using two rounds of PCR. PCR1 generate a full-length library and PCR2 produce two unequal DNA strands. Unequal DNA strands are separated by dPAGE. DNA strand containing rA is used for the next round. (B) Sequence of DNA library. (C), (D) trans-cleaving structures of the original Ce13 and mutated Ce13d DNAzymes, respectively. Reprinted (adapted) with permission from Vazin. M., Huang. P-J. J., Matuszek. Z., Juewen Liu. *J. Biochemistry*. 2015, 54, 6132-6138).³¹¹

Ce13 was previously selected in the Ce^{4+} selection and was also produced independently as the main sequence. Therefore, Ce13 appears to be an optimal sequence for Ce-dependent selections. The secondary structure of Ce13 is demonstrated in Figure 2.1C. The DNAzyme

contains a bulged hairpin structure. Since the Ce13 DNzyme appeared to be the optimum sequence for Ce^{3+} , an optimum and truncated version of the enzyme named Ce13d was employed for biochemical characterizations. The structure of Ce13d DNzyme is indicated in Figure 2.1D. Preliminary studies indicated some structural features of the DNzyme.²⁹⁹ In Figure 2.1C, the region with the important nucleotides is marked with an orange box and, the hairpin in the black box can be replaced by other hairpin sequences without affecting the DNzyme activity.²⁹⁹ The following biochemical studies were carried out using Ce13d.

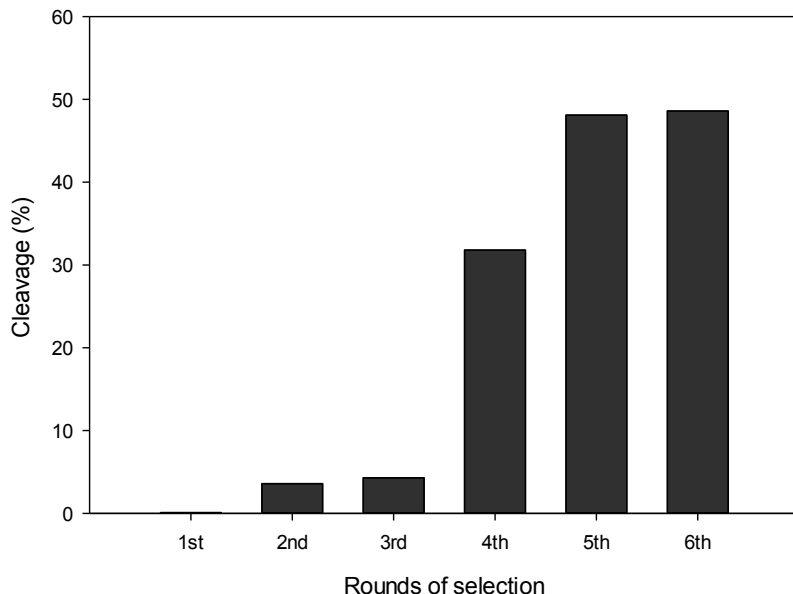


Figure 2.2 Summary of the Ce^{3+} -dependent *in vitro* selection.

Table 2.1 Ce^{3+} selection progress and condition.

round #	$[\text{Ce}^{3+}]$ (μM)	incubation time (min)	cleavage (%)
1	50	60	0.1
2	50	60	3.6
3	50	60	4.3
4	50	60	31.8
5	50	60	48.1
6	20	60	48.6

2.2.2 Biochemical characterization of Ce13d DNzyme

2.2.2.1 Metal-dependent activity of Ce13d enzyme

The detailed trans-cleaving structure of the Ce13d DNzyme is demonstrated in Figure 2.3. In the structure, the enzyme strand is colored blue and red, and the substrate strand is green. The cleavage junction in the substrate strand is indicated with the arrowhead. This cleavage junction can be either a normal phosphate (PO) or a modified phosphorothioate (PS). The PO and PS cleavage junctions are illustrated in Figure 2.3B, and C, respectively. Primary studies of Ce13d indicated the Ce13d enzyme with PO substrate is active with all the lanthanides.²⁹⁹ Interestingly, by introducing a single phosphorothioate (PS) modification at the cleavage site, its activity with lanthanides decreases, while all thiophilic metals (e.g. Cu^{2+} , Hg^{2+} , Cd^{2+} , and Pb^{2+}) can activate the DNzyme.²⁹⁹ Here, more comprehensive and quantitative studies were carried out on Ce13d with PO and PS substrates.

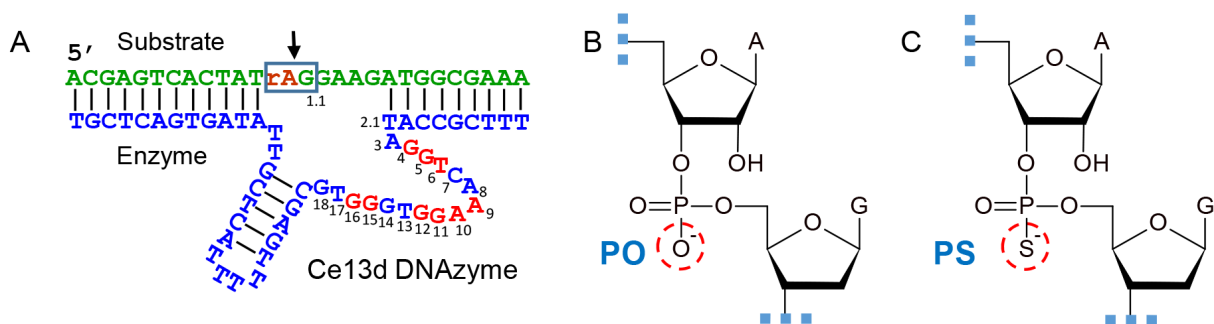


Figure 2.3 (A) The secondary structure of Ce13d DNzyme. The enzyme strand is blue and red, and the substrate is green. The rAG junction cleavage is shown with a green box. (B), (C) Structures of the normal (PO) and the phosphorothioate modified (PS) cleavage junction.³¹¹

First, we measured the Ce13d activity rate with each of the substrates, and also with various metal ions at 10 μM . With the PO substrate, Ce^{3+} had the highest rate (0.18 ± 0.02

min^{-1}), while Pb^{2+} was nearly 20-fold slower, and Cd^{2+} and Cu^{2+} were inactive (result shown in Figure 2.4A). On the other hand, with the PS substrate, Pb^{2+} ($0.11 \pm 0.02 \text{ min}^{-1}$), Cd^{2+} ($0.06 \pm 0.01 \text{ min}^{-1}$), and Cu^{2+} ($0.021 \pm 0.003 \text{ min}^{-1}$) became more efficient, but Ce^{3+} became slower (Figure 2.4D). The PS modification produced a ~ 11 -fold decrease in the activity rate with Ce^{3+} , but rates were faster with other metals (Pb^{2+} by 11-fold, Cu^{2+} by 172-fold, and Cd^{2+} by 196-fold). This is consistent with the soft–hard acid–base theory, where Ce^{3+} has a higher affinity with oxygen, while Cd^{2+} and Cu^{2+} bind sulfur ligands better, and Pb^{2+} can bind both.³¹²

In the next step, Ce^{3+} and Cd^{2+} were used to study the two PO and PS systems, respectively. First, the enzyme activity was studied with PO substrate at various concentrations of Ce^{3+} (Figure 2.4B). No cleavage was detected in the absence of Ce^{3+} in 2hr. At low Ce^{3+} concentration, by increasing the Ce^{3+} concentration, both the cleavage rate and the final cleavage yield increased. The cleavage activity peaked at $10 \text{ }\mu\text{M}$ Ce^{3+} with the final cleavage of $\sim 80\%$. All the data can fit into a first-order reaction kinetics, and the rate constants are plotted in Figure 2.4C. At higher Ce^{3+} concentrations (e.g., $\geq 50 \text{ }\mu\text{M}$), however, the final cleavage yields dropped. In the presence of $50 \text{ }\mu\text{M}$ Ce^{3+} , 40% cleavage was observed and with $100 \text{ }\mu\text{M}$, the final cleavage was only $<20\%$. For these two samples, cleavage occurred only in the first 2 min, and afterwards the enzyme was completely inhibited. Due to 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 calculated to be $27.5 \pm 18 \text{ }\mu\text{M}$ Ce^{3+} for binding a single metal ion (inset of Figure 2C).

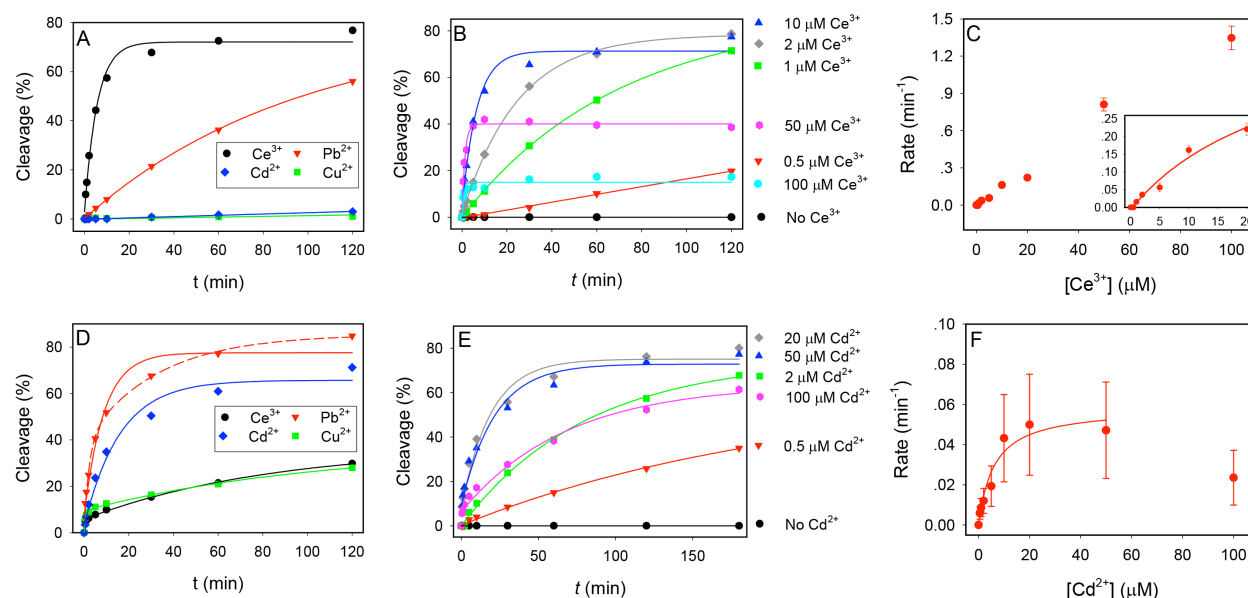


Figure 2.4 Kinetic studies of Ce13d with the normal (PO) and modified (PS) substrates. Ce13d cleavage activity with PO (A) and PS substrates (D), in the presence of different metal ions (10 μM). The dashed red line in panel D is fitted to a double-exponential kinetics, and the rest are single-exponential. Kinetics of Ce13d cleavage with various concentrations of (B) Ce³⁺ for the PO substrate or (E) Cd²⁺ for the PS substrate. Rate constant of cleavage as a function of (C) Ce³⁺ for the PO substrate or (F) Cd²⁺ for the PS substrate. Note that the PS substrate data are from a mixture of *R_p* and *S_p* diastereomers, and the obtained rate constants are averaged values. Copyright 2015, American Chemical Society.³¹¹

DNAzymes can be either activated or inhibited by lanthanide metal ions. Due to the strong affinities of trivalent lanthanides for the DNA phosphate and bases, however, it is not surprising to inhibit the DNAzyme activity. A high concentration of Ln³⁺ may disrupt nucleic acid secondary structures.³¹³ For the above assays, the final DNAzyme concentration is 0.7 μM, and there are 78 phosphates in each DNAzyme complex, leading to a total phosphate concentration of 55 μM. Since strong inhibition occurred with 50 μM Ce³⁺, we suspect that when the ratio between phosphate and Ln³⁺ is ~1:1, Ln³⁺ starts 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. As expected, only a moderate inhibition was observed with 100 μM Ce^{3+} (green dots, Figure 3). On the other hand, if both the substrate and enzyme strand were decreased to 0.1 μM , inhibition occurred at even lower Ce^{3+} concentrations (black dots).

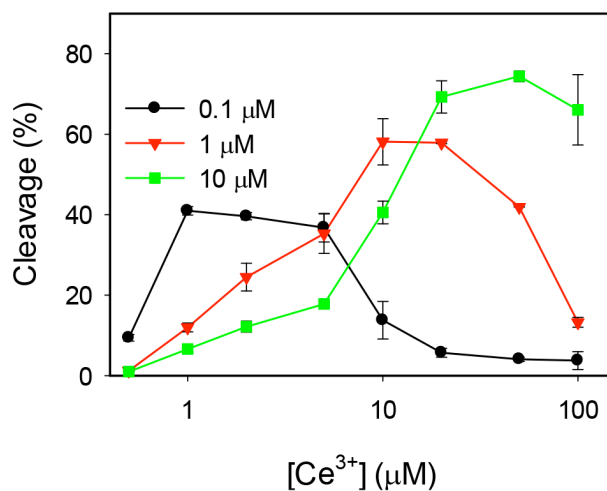


Figure 2.5 Percentage of the PO substrate cleavage by Ce13d in the presence of various concentrations of Ce^{3+} after 1 h. The DNAzyme concentration was 0.1 μM (substrate and enzyme), 1 μM (substrate and enzyme), or 1 μM substrate with 10 μM enzyme strand (green squares).³¹¹

2.2.2.2 Ce13d cleavage activity with PS-modified substrate

For comparison, we studied the Ce13d cleavage activity with the PS-modified substrate with various Cd^{2+} concentrations (Figure 2.4E). In the first glimpse, a similar trend was observed and inhibition occurred with over 50 μM Cd^{2+} . While a careful look declared that the Cd^{2+} -induced inhibition is quite different from the Ce^{3+} inhibition. Even with 100 μM Cd^{2+} concentration, time-dependent cleavage was still observed after 3 h, while with 100 μM Ce^{3+} the DNAzyme was completely inactive after 2 min. Thus, inhibition of the DNAzyme by Cd^{2+} is milder. Unlike a complete disruption of DNAzyme structure by Ce^{3+} , Cd^{2+} might only cause

reversible changes in the DNAzyme. The calculated K_d was $6.0 \pm 2.8 \mu\text{M}$ for binding Cd^{2+} to the PS substrate.

2.2.2.3 R_p and S_p diastereomers

Two different diastereomers are produced by PS modification, R_p and S_p .³¹⁴ In the previous assay, a mixture of the two are used, and the rates obtained are averaged values. The single exponential fittings in Figure 2.4D do not follow all the data points, and the system fits better using a bi-exponential equation. To gain further insights, the two diastereomers were individually analyzed. Each diastereomer was isolated by HPLC and then tested with the two metals.³¹⁵ The results are demonstrated in Figure 2.6A, and B. With Ce^{3+} (Figure 2.6A), the PO substrate is cleaved ($0.18 \pm 0.02 \text{ min}^{-1}$) 15 times faster than the S_p ($0.012 \pm 0.001 \text{ min}^{-1}$), while the S_p substrate is cleaved 8 times faster than the R_p ($0.0016 \pm 0.0002 \text{ min}^{-1}$). Ce^{3+} is a hard metal that likes oxygen-based ligands, thus, it is reasonable to assume that pro- R_p oxygen is responsible for binding to Ce^{3+} . With Cd^{2+} (Figure 2.6B), an R_p rate of $0.12 \pm 0.02 \text{ min}^{-1}$ was observed, 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}), and the PO substrate is essentially inactive with Cd^{2+} .

RNA-cleaving enzymes mostly use pro- R_p for metal binding (mostly Mg^{2+}). Examples include the hammerhead ribozyme,³¹⁶ HDV ribozyme,³¹⁷ 10-23 DNAzyme,³¹⁸ RNase P,³¹⁹ and Group II intron.³²⁰ Ce13d DNAzyme also follows the same trend and mainly relies on the pro- R_p oxygen for metal binding. The pro- S_p oxygen atoms might also contribute to metal binding, since 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+}). For cases like the hammerhead ribozyme, S_p is >100 times faster than R_p in the presence of Mg^{2+} .^{316, 321}

The role of metal in RNA hydrolysis is usually 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.³²² In the Ce13d DNAzyme, all the trivalent lanthanide ions (with PO substrate), and Cd^{2+} , Cu^{2+} , and Pb^{2+} (with PS substrate) appear to have a similar activity (e.g., ranging from 0.02 to 0.18 min^{-1} with 10 μM metal ions). To activate 2'-OH, the metal ion serves as a general base, and thus, the pK_a values of metal bound water are considered as an important factor. In cases like the 17E DNAzyme, there is a positive correlation between the metal pK_a and activity,^{119, 324} while the Ce13d DNAzyme is quite different. The pK_a for Pb^{2+} is 7.2, while for Cd^{2+} is 10.1.^{119, 323} This ~3 decade pK_a difference results in only a ~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.

The switching of metal preference by the PS modification clearly points at the significance of metal binding to phosphate. Compared to most divalent metal ions, which bind to nucleotide acids with mM affinity,³¹² lanthanide has μM affinity.³²⁵ This is probably a primary reason that lanthanides are generally more active for Ce13. It should be noted that the +3 charge alone is insufficient, since Ce13d cannot be activated by Sc^{3+} .²⁹⁹ 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. Upon introduction of the PS modification, binding to

thiophilic metal ions is enhanced. Altogether, the metals are likely to play a role in neutralizing the phosphate charge.

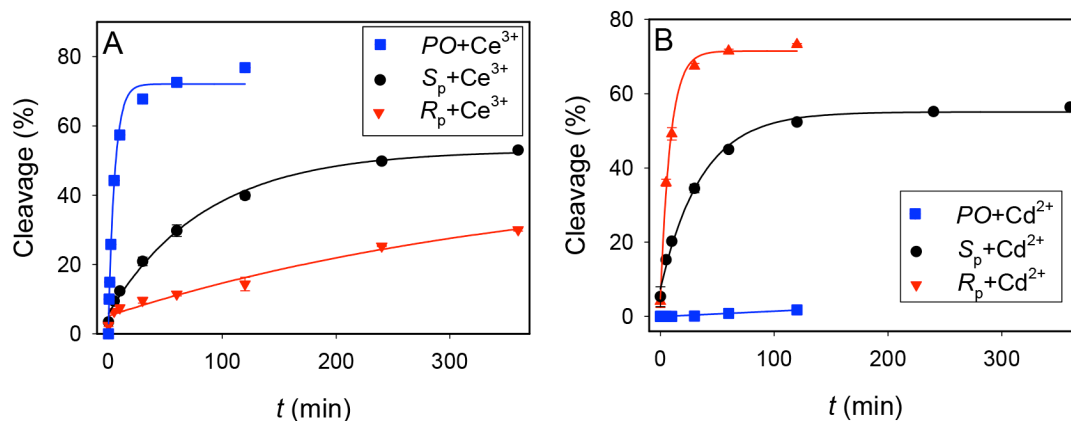


Figure 2.6 Kinetics of Ce13d DNase with three types of substrates (PO, R_p and S_p) in the presence of 10 μM Ce³⁺ (A) or Cd²⁺ (B).³¹¹

2.2.2.4 Effect of pH on the catalytic activity of Ce13d

In this section, the activity of Ce13d was studied as a function of pH. The pH profile of the enzyme can give us useful information about its catalysis mechanism. Ce13d was first to be studied with the PO substrate, in the presence of Ce³⁺. In Figure 2.7A, the log of the cleavage rate as a function of pH is plotted. The rate is typically faster at higher pH from pH 4.8 to 7. At even higher pH, the kinetics became complicated, and could not fit to a single rate. Thus, our focus is mostly on the slightly acidic region. After pH 6, the rate increase slowed down. In the pH 4.8 to 5.8 region, a linear relationship is obtained with a slope of 1.02 ± 0.12 (close to 1.0, Figure 2.7B), suggesting a single deprotonation step in the reaction process. This is a typical pH response that RNA-cleaving DNases usually adopt.³²⁴ As explained in the previous section, this deprotonation is unlikely to be related to the metal ion directly.

Using the PS substrate, Ce13d pH-dependent activity was performed in the presence of Cd^{2+} , and a similar observation was made (Figure 2.7C). The slope in the pH region from 4.8 to 5.8 is 1.04 ± 0.15 (Figure 2.7D), which also indicates a single deprotonation step for the system. At each pH, the rate of this system is slightly slower than the normal PO/ Ce^{3+} system, while the trend of the rate change is comparable. The results provided here suggest that the mechanism of these two systems might be the same, and in spite of the PO/PS switching, the coordination environment of the metal is likely to be largely maintained. The only difference is that the metal ion is changed from Ce^{3+} to Cd^{2+} due to the PS modification.

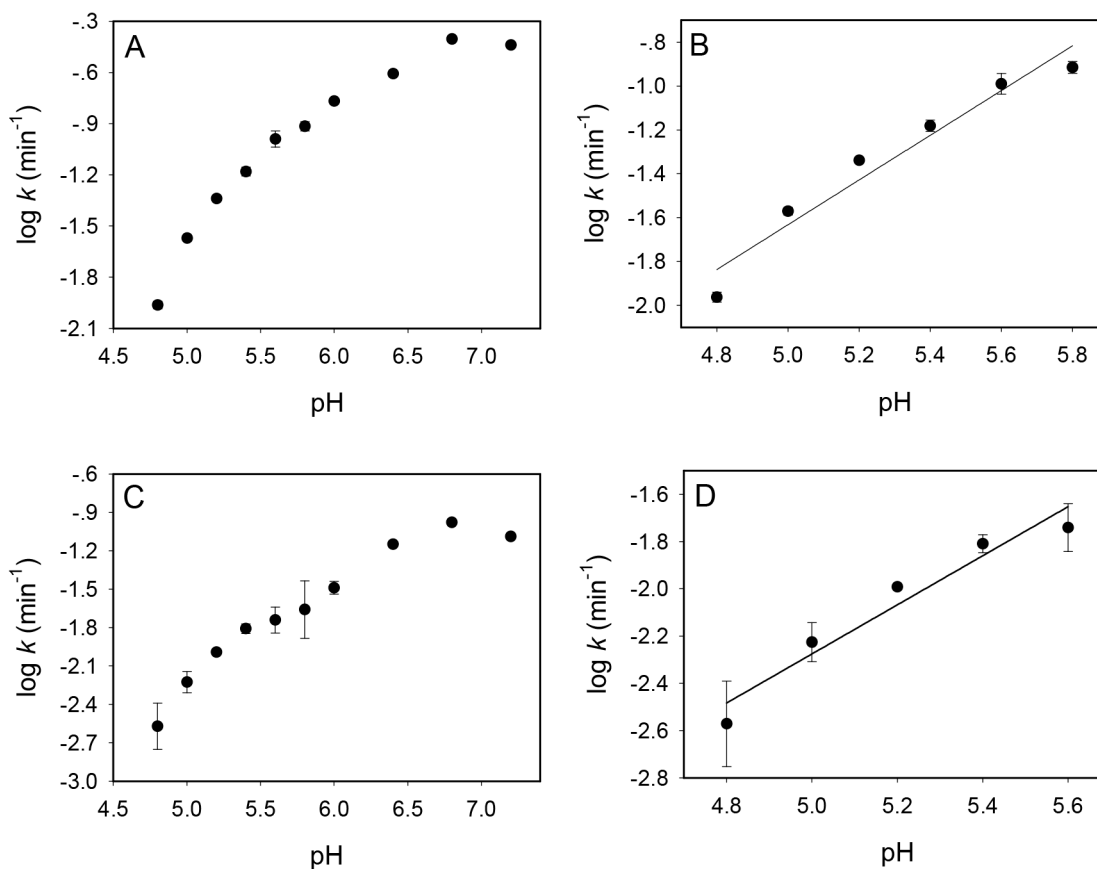


Figure 2.7 pH-dependent activity of Ce13d with (A) Ce^{3+} for the PO substrate or (C) Cd^{2+} for the PS substrate. Fitting of the data in the pH region from 4.8 to 5.8 for (B) Ce^{3+} for the PO substrate or (D) Cd^{2+} for the PS substrate.³¹¹

2.2.2.5 Enzyme mutational studies

Our preliminary studies on Ce13d indicated that the enzyme has a bulged hairpin structure (Figure 2.3). The hairpin mostly plays a structural role, and the bulged loop contains the catalytically important nucleotides.²⁹⁹ Here, to identify the important nucleotides and their roles in catalysis, we performed a systematic mutation study. Mutation of conserved nucleotides often abolishes catalysis, thus giving us clues about the mechanism of DNAzyme action. Moreover, this type of mutation may result in identifying novel DNAzyme variants with enhanced activity.

The DNAzyme loop region was initially analyzed. The numbering of the nucleotides in the loop is shown in Figure 2.3A. Ce13d loop contains 16 nucleotides, from A₃ to G₁₈. We systematically mutated all these 16 nucleotides. Each nucleotide was changed to the other three types of nucleotides (e.g., A to C, G, and T), giving a total of 48 mutants. As a result, we observed that the activity patterns for these mutants are very similar between the PO substrate with Ce³⁺ (Figure 2.8A) and the PS with Cd²⁺ (Figure 2.8B), suggesting that the nucleotides in the loop play a similar role in both cases. Among all the 16 nucleotides, A₃ is the only one relatively well tolerated to all the applied mutations, suggesting that this position does not serve an important functional purpose. In contrast, eight purines (G₄, G₅, A₉, A₁₀, G₁₁, G₁₂, G₁₅ and G₁₆) are highly conserved (marked red in Figure 1A). Mutating any of them to any other nucleotides abolishes the enzyme activity, while mutations in other nucleotides such as C₇, T₁₃, and G₁₄ do not completely disturb the enzyme activity, implying that 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 instance, C₇T, T₁₃C, and T₁₇C. Altogether, the current enzyme appears to be an optimal sequence, and most of the mutations decreased the enzyme activity.

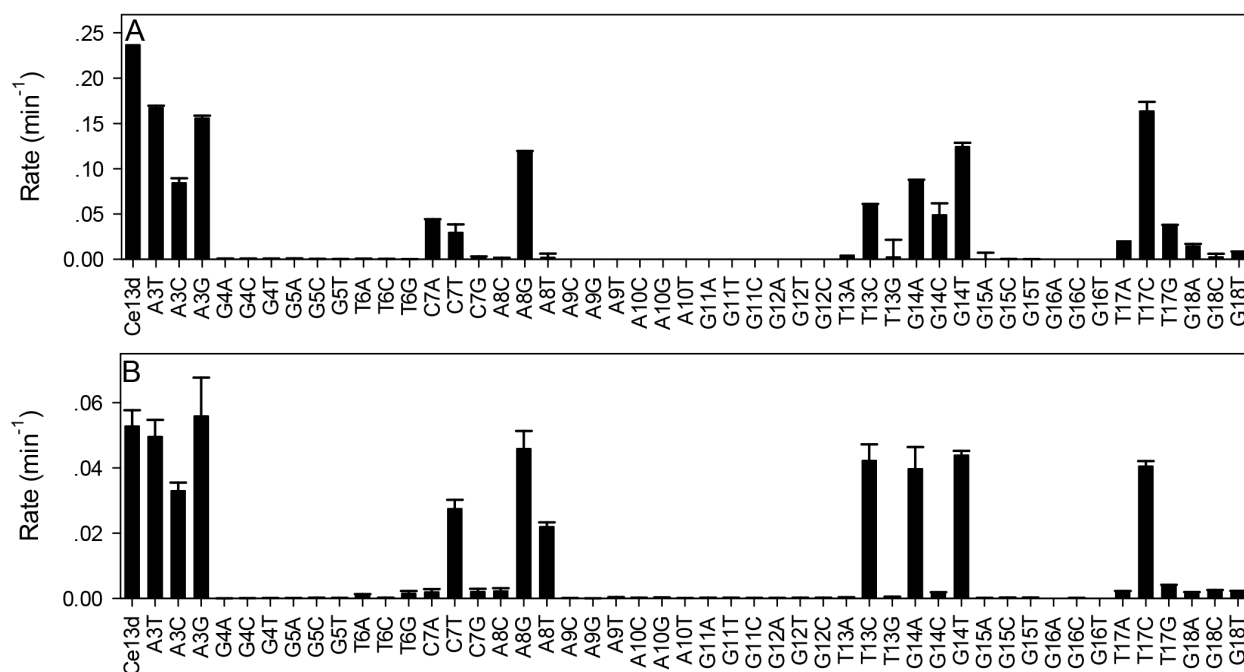


Figure 2.8 The activity of Ce13d mutants with (A) the PO substrate in the presence of 10 μM Ce^{3+} , and (B) the PS substrate in the presence of 10 μM Cd^{2+} .³¹¹

2.2.2.6 Further analysis of the conserved purine nucleotide in the catalytic core (Conserved purine to HX mutations)

As previously mentioned, eight purines are highly conserved for Ce13d activity, where there are three pairs of guanine dimers among them (G_4G_5 , $\text{G}_{11}\text{G}_{12}$, and $\text{G}_{15}\text{G}_{16}$). Two unpaired guanines are also present near the cleavage junction in the substrate. It is possible that these four pairs form a G-quadruplex structure. Such a structure is observed in a number of aptamers but not yet reported in RNA-cleaving DNazymes.³²⁶ To test the assumption, we further mutated each guanine to HX. The structures of A, G, and HX are shown in Figure 2.9A. HX can be considered to be an intermediate molecule between A and G. Compared to G, HX misses the 2-amino group, and compared to A, the 6-amino group in A is mutated to an oxo group.

However, mutating G₄, G₅, G₁₁, and G₁₅ purines to HX form only slightly decreased the activity (less than 5-fold), and also G₁₆, or G₁₂ to HX had almost no effect (Figure 2.9B). The result indicates the 2-amino groups in these guanines are not involved in important structural or functional roles. This can rule out the possibility of G-quadruplex formation. The 2-amino group is critical to maintaining the hydrogen-bonding network in a G-quadruplex, while HX cannot support it. The above discussions are mostly focused on metal binding, and we cannot rule out base mutation induced changes in hydrogen bonding patterns in the DNzyme and the related tertiary structure changes.¹¹⁶ The resolution of biochemical assays cannot probe such effects, and more accurate spectroscopic methods are needed to fully understand it.

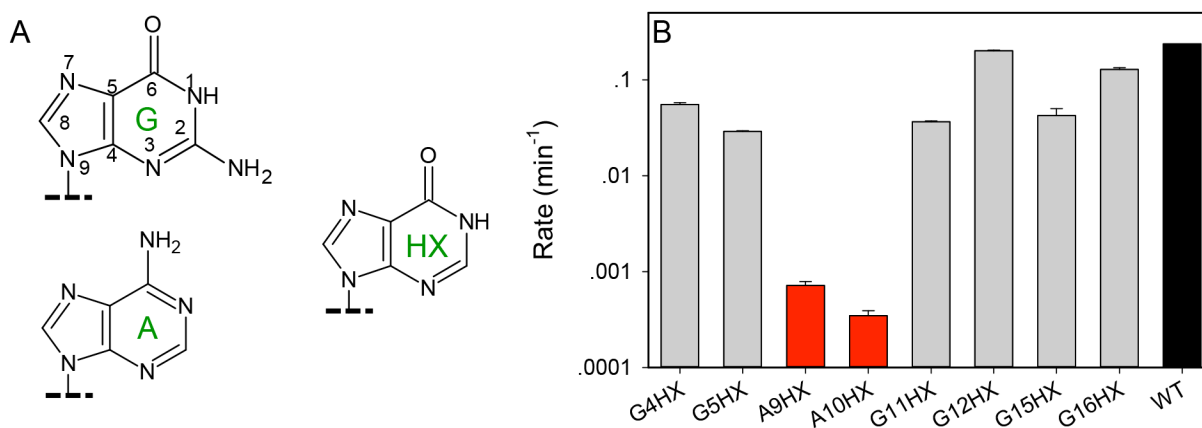


Figure 2.9 (A) Adenine, guanine, and hypoxanthine structures (HX). The amino group present in G is necessary for quadruplex formation. (B) Activity of HX-substituted Ce13d mutants plotted on the log scale. All these nucleotides are highly conserved based on the previous round of mutational studies. The rate for cleavage of the PO substrate in the presence of 10 μ M Ce³⁺ is shown. WT denotes for the wild-type DNzyme. The two adenine mutations are highlighted in red, where their activity dropped by more than 100-fold compared to the WT.³¹¹

To have a better understanding about the role of all the eight conserved purines, we also mutated the two conserved adenines to HX. Interestingly, in sharp contrast to the above guanine mutations, both mutations abolished the activity. The 6-amino group in adenine is not a good

metal ligand, since its free electron pair is shared with the conjugated ring. In comparison, 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 hydrogen bond acceptor. Therefore, these two adenines might be critical in hydrogen bonding and may play a structural role.

2.3 Summary

Here, a Ce^{3+} -dependent *in vitro* selection was performed to obtain new lanthanide-dependent DNAzymes. After conducting six rounds of selection, Ce13 was isolated as the main sequence. The Liu lab previously selected Ce13d in a Ce^{4+} selection (modified version of Ce13). Ce13d works with all the lanthanides with the normal PO substrate, but with all soft metals with the PS substrate. Since Ce13d is an interesting DNAzyme, a careful biochemical study was conducted on it with the PO substrate in the presence of Ce^{3+} , and compared with the PS substrate in the presence of Cd^{2+} . The PS-modified substrate indicates a relatively weak thio effect for both the R_p and S_p forms, while metal binding most likely takes place via the pro- R_p oxygen in the original substrate. We suggested that the metal is mainly responsible for neutralizing the phosphate charge, and not for 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 Ce^{3+} and Cd^{2+} inhibit their corresponding DNAzyme complex at high concentrations. However, Ce^{3+} 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 involve in metal binding. Lastly, a special feature of Ce13 DNAzyme is the

presence of a guanine-rich metal binding pocket, and we disproved the possibility of a guanine-quadruplex structure based on the guanine-to-HX mutations.

2.4 Materials and method

2.4.1 Chemicals

The DNA library for *in vitro* selection, primers, and fluorophore/quencher modified DNAs were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The DNA sequences used in the *in vitro* selection experiment are listed in Table 2.2. All the Ce13d mutants used in the biochemical studies were from Eurofins (Huntsville, AL). The sequences and modifications of the DNA samples are shown in Table 2.3. The metal salts, include cerium chloride heptahydrate (99.999% purity) and Cadmium chloride hydrate (99.995% purity), were from Sigma-Aldrich. Their solutions were prepared by directly dissolving their salts in water. The buffers were from Mandel Scientific (Guelph, Ontario, Canada). Tris(Hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)ethanesulfonic acid (MES) free acid monohydrate, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) sodium salt, HEPES free acid, EDTA disodium salt dehydrate, sodium chloride, sodium bromide, sodium iodide, and ammonium acetate were purchased from Mandel Scientific Inc (Guelph, Ontario, Canada). Acrylamide/bisacrylamide 40% solution (29:1), urea, and 10x TBE solution were purchased from Bio Basic Inc. SsoFast EvaGreen supermix was purchased from Bio-Rad for real-time PCR analysis. T4-DNA ligase, deoxynucleotide (dNTP) solution mix, Taq DNA polymerase with ThermoPol buffer, and low molecular weight DNA ladder were purchased from New England Biolabs. Milli-Q water was used for making all the solutions and buffers and gel solutions. Denver Instrument UltraBasic pH meter was used to measure the pH of the buffers.

Table 2.2 DNA sequences and primers used for *in vitro* selection.

Name	Sequences (from 5' to 3')
Lib-FAM	5'-GGCGAAACATCTT N50 TAGTGACGGTAAGCTTGGCAC- FAM
Lib-rA	5'-AATACGAGTCACTAT rA AGGAAGAT
Splint	5'-AAGATGTTTCGCCATCTTCCTATAGTCCACCACCA
P1 primer	5'-GTGCCAAGCTTACCG
P2 primer	5'-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACA
P3 primer	5'- FAM -AAATGATCCACTAATACGACTCACTAT rA AGG
P4 primer	5'-AACAAACAACAAC- iSp18 -GTGCCAAGCTTACCG

2.4.2 *In vitro* selection

Initially, the DNA library was prepared by ligating two pieces of DNA (Lib-FAM and Lib-rA) using a splint DNA. Three DNA strands, including Lib-FAM DNA (200 pmol) and Lib-rA DNA (300 pmol) and splint DNA (300 pmol) were mixed in buffer A (50 mM pH 7.5 Tris-HCl, pH 7.5, 10 mM MgCl₂). They were annealed at 95 °C for 1 min, and then slowly cooled down at room temperature. For the ligation, the T4 ligation protocol provided by New England Biolabs was followed. Prior to the selection, the ligated DNA product was purified with 10% denaturing polyacrylamide gel (dPAGE) at 650 V for 1 h. The purified DNA was extracted from the gel with buffer B (1 mM EDTA, 10 mM Tris-HCl, pH 7.0). The library was precipitated in ethanol overnight, and re-suspended in 60 µL of the selection buffer (50 mM MES, pH 6.0, 25 mM NaCl).

The full-length DNA library was used for the first round of selection. The random DNA pool was incubated with fresh Ce³⁺ solution. After incubation, the reaction was quenched with 8 M urea and was purified in 10% dPAGE. The piece of gel containing the selected DNA was cut and extracted from the gel and further purified with a Sep-Pak C18 column (Waters). The

purified DNA was then dried in an Eppendorf Vacufuge at 30 °C overnight. The fully dried DNA was re-suspended in 70 µL of 5 mM HEPES buffer (pH 7.5). For the following rounds of selection, the library was generated using two rounds of PCR.

2.4.3 PCR

For each round of *in vitro* selection, three PCR reactions were carried out. A real-time PCR (rt-PCR) was carried out to quantify the amount of cleaved DNA that was extracted from the gel. A total of 20 µL reaction mixture, comprising 1 µL of purified DNA template, 400 nM primer (P1 and P2), and 10 µL of SsoFast EvaGreen Supermix (Bio-Rad) was used for the reaction. The thermocycling steps provided by the vendors were followed. The steps were as follows; 95 °C for 30s, 95 °C for 5s, and 55 °C for 5s.

PCR1 was used to generate the full-length library. The PCR reaction mixture (50 µL) contained 1 µL DNA template, 200 nM of each of P1 and P2, 200 µM dNTP mixture, 1× Taq buffer, and 1.25 units of Taq DNA polymerase. The reaction was carried out for 15-20 cycles. The DNA was amplified using the following cycling steps: 94 °C for 5 min; 94°C for 30 s, 55 °C for 30 s, and 72 °C 30 s. The PCR1 product was then purified using DNA extraction kit (IBI Scientific), and used as template for PCR2. The purified product was 10× diluted with water and further amplified using P3 and P4 as the primers. The PCR2 DNA product was amplified with 12 cycles. A 200 µL PCR reaction mixture contains 4 µL diluted template from PCR1, 250 nM each of P3 and P4, 200 µM dNTP mixture, 1× Taq buffer, and 5 units of Taq DNA polymerase. The thermocycling steps are the same as PCR1. The final product was further purified in 10% dPAGE. The single-stranded FAM-labeled DNA was extracted from the gel, precipitated in ethanol overnight, and subsequently used as the library pool for the next round of selection.

2.4.4 Cloning and sequencing

The Ce^{3+} -dependent selection was stopped at round 6, and then the fresh PCR1 product was prepared for cloning. Cloning was done using the TA- TOPO Cloning Kit (Invitrogen) and Subcloning Efficiency DH5 α competent cells (Invitrogen). The protocol provided by the vendor was followed. DirectPrep 96 MiniPrep Kit (QIAGEN) was used for the plasmid DNA extraction and DNA purification. The sample was submitted to TCAG DNA Sequencing Facility (Toronto, ON) for analysis. Subsequently, the DNA sequences were aligned using ClustalW2.

2.4.5 Biochemical studies

pH-dependent studies. The DNAzyme complex was formed by annealing 5 μM FAM-labeled substrate (PO or PS) and 7.5 μM of the enzyme in water with 175 mM NaCl by warming up the sample to 80 °C for 1 min following slow cooling down to room temperature. Then, the sample was diluted 5 times into 50 mM of the various buffers. A final concentration of 10 μM metal ions was incubated with 35 μL of 1 μM DNAzyme complex in acetate (pH 4.2-5.6), MES (pH 5.8-6.8) or MOPS (pH 7.0-7.2) buffer. At designated time points, an aliquot was transferred to the gel loading buffer (11 mM EDTA, 8 M urea with bromophenol blue) to quench the reaction. The samples were analyzed by 15% dPAGE (120 V for 90 min). Gel images were documented with a Bio-Rad ChemiDoc MP imaging system.

Other kinetic studies. Kinetics studies in the presence of various metal concentrations or of the mutated DNAzymes were carried out in a similar way. The default buffer condition is buffer A (25 mM NaCl, 50 mM MES, pH 6.0). Other kinetic assays were performed in a similar way by changing the metal ion species or concentration. Most biochemical assays were run in triplicate and the standard deviations are plotted as error bars.

Enzyme concentration effect. For studying the effect of DNAzyme concentration (with the PO substrate), the 1 μM DNAzyme complex was formed as described above, and the 0.1 μM sample was prepared by diluting the 1 μM sample by 10-fold. The 10 μM sample was prepared using the same 1 μM substrate strand, but with a final concentration of 10 μM enzyme strand. These DNAzyme complexes were finally dissolved in buffer A and the reaction was stopped at 10 min after adding Ce^{3+} .

Table 2.3 Ce13d mutants and modifications used in this study. FAM = carboxyfluorescein; * denotes for phosphorothioate modification; *(Rp) and *(Sp) are the two separated chiral centers; rA = ribo- adenosine; [I] = hypoxanthine. The enzyme loops are color coded. The red nucleotides are subject to extensive mutation studies, the blue nucleotides form the hairpin and the two unpaired thymines next to the hairpin are in green. The black nucleotides are for substrate binding. The mutated nucleotides in the enzyme are in boldface.

Name	Sequences and modifications (from 5' to 3')
Ce13d	TTTCGCCCAT AGGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
PO-sub	GTC ACG AGT CAC TAT rAGG AAG ATG GCG AAA-FAM
PS-sub	GTC ACG AGT CAC TAT rA*GG AAG ATG GCG AAA-FAM
R _p -sub	GAGTCACTATrA*(Rp)GG AAGATGGCGAAA-FAM
S _p -sub	GAGTCACTATrA*(Sp)GG AAGATGGCGAAA-FAM
Ce13d-A3T	TTTCGCCCAT TGGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A3C	TTTCGCCCAT CGGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A3G	TTTCGCCCAT GGGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G4A	TTTCGCCCAT AAGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G4C	TTTCGCCCAT ACGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G4T	TTTCGCCCAT ATGTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G5A	TTTCGCCCAT AGATCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G5C	TTTCGCCCAT AGCTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G5T	TTTCGCCCAT AGTTCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T6A	TTTCGCCCAT AGGACAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T6C	TTTCGCCCAT AGGCCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T6G	TTTCGCCCAT AGGGCAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-C7A	TTTCGCCCAT AGGTAAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-C7T	TTTCGCCCAT AGGTTAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-C7G	TTTCGCCCAT AGGTGAAAGGTGGGTG CGAGTTTTTACTCGTTATAGTGACTCGT

Ce13d-A8C	TTTCGCCATAGGTCCAAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A8G	TTTCGCCATAGGTCTGAAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A8T	TTTCGCCATAGGTCTAAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A9C	TTTCGCCATAGGTCAACAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A9G	TTTCGCCATAGGTCAAGAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A9T	TTTCGCCATAGGTCAATAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A10C	TTTCGCCATAGGTCAAACGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A10G	TTTCGCCATAGGTCAAAGGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A10T	TTTCGCCATAGGTCAATGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G11A	TTTCGCCATAGGTCAAAAAGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G11T	TTTCGCCATAGGTCAAATGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G11C	TTTCGCCATAGGTCAAACGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G12A	TTTCGCCATAGGTCAAAGATGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G12T	TTTCGCCATAGGTCAAAGTTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G12C	TTTCGCCATAGGTCAAAGCTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T13A	TTTCGCCATAGGTCAAAGGAGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T13C	TTTCGCCATAGGTCAAAGGCGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T13G	TTTCGCCATAGGTCAAAGGGGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G14A	TTTCGCCATAGGTCAAAGGTAGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G14C	TTTCGCCATAGGTCAAAGGTCTGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G14T	TTTCGCCATAGGTCAAAGGTTGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G15A	TTTCGCCATAGGTCAAAGGTGAGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G15C	TTTCGCCATAGGTCAAAGGTGCGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G15T	TTTCGCCATAGGTCAAAGGTGTGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G16A	TTTCGCCATAGGTCAAAGGTGGATGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G16C	TTTCGCCATAGGTCAAAGGTGGCTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G16T	TTTCGCCATAGGTCAAAGGTGGTTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T17A	TTTCGCCATAGGTCAAAGGTGGGAGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T17C	TTTCGCCATAGGTCAAAGGTGGGCGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-T17G	TTTCGCCATAGGTCAAAGGTGGGGGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G18A	TTTCGCCATAGGTCAAAGGTGGGTACGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G18C	TTTCGCCATAGGTCAAAGGTGGGTCCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G18T	TTTCGCCATAGGTCAAAGGTGGGTTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G4HX	TTTCGCCATA[I]GTCAAAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G5HX	TTTCGCCATAG[I]TCAAAGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A9HX	TTTCGCCATAGGTCA[I]AGGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-A10HX	TTTCGCCATAGGTCAA[I]GGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT

Ce13d-G11HX	TTTCGCCATAGGTCAAAGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G12HX	TTTCGCCATAGGTCAAAGTGGGTGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G15HX	TTTCGCCATAGGTCAAAGGTGTGGTGGCGAGTTTTTACTCGTTATAGTGACTCGT
Ce13d-G16HX	TTTCGCCATAGGTCAAAGGTGGTGGTGGCGAGTTTTTACTCGTTATAGTGACTCGT

Chapter 3. *In vitro* selection of Yb³⁺ and Lu³⁺-dependent DNazymes and characterization of the Lu12 DNzyme

3.1 Introduction

DNazymes are DNA-based catalysts.^{22, 327-330} They typically require metal ion cofactors for their efficient activity.^{20, 22, 327-330} Thus, different metals were used to select selective and sensitive DNazymes.^{20, 291-298} In the previous chapter, the Ce13d DNzyme was discussed that separately selected in the Ce⁴⁺ and Ce³⁺-dependent selections. Ce13d has an excellent selectivity for the entire group of trivalent lanthanides and is useful for detecting the lanthanide ions as a group.²⁹⁹ However, there is a second level of selectivity to distinguish each lanthanide, which is much more challenging than detecting lanthanides as a group. With 15 very similar elements in the group, it is very difficult to determine the identity of each lanthanide. All the trivalent lanthanides have the same charge and similar sizes, displaying extremely similar chemical properties. They only differ by the number of inner 4f electrons, which do not readily participate in chemical bonding, and are shielded by the filled 4d, 5s, and 5p orbitals.

Towards the goal of detecting individual lanthanide ions, herein, I carried out new DNzyme selections. Two new lanthanides (Yb³⁺ and Lu³⁺) were used as the intended targets. My previous selection was carried out using the N50 library (e.g. with 50 random nucleotides), and the main outcome was the Ce13d DNzyme. Since Ce13d is quite long, I reason that it might be possible to eliminate it by using a shorter library. As such, in this chapter, *in vitro* selection of RNA-cleaving DNazymes was carried out using a library containing a region of 35 random nucleotides. A new DNzyme (Lu12) was isolated that displays better lanthanide discrimination than Ce13d.³⁰¹ Next, I performed a detailed biochemical assay on Lu12 and found

a number of quite interesting properties distinguishing this enzyme from the rest of the known DNAzymes (e.g., pH-rate slope = 2, pro- S_p instead of pro- R_p oxygen metal binding at the cleavage site, and acceptance of a diverse range of cleavage junctions). Collectively, Lu12 was suggested as a unique DNAzyme, with a different cleavage mechanism.

3.2 Results and discussion

3.2.1 *In vitro* selection with Yb^{3+} and Lu^{3+}

As mentioned above, I have decided to use the N35 library to discourage the occurrence of Ce13d. Next, a decision must be made as to which lanthanide ion to use. A total of 14 trivalent lanthanides are available for the new *in vitro* selection (Pm^{3+} is radioactive and excluded from discussion). Previously, I used Ce^{3+} for the selection experiment, which resulted in re-selecting Ce13d. The cleavage of RNA by free lanthanide ions was previously reported.²⁹⁹⁻³⁰¹ For example, a high concentration of free lanthanide (e.g. 5 mM) was shown to cleave RNA efficiently, and larger lanthanides were suggested to have much higher cleavage activity.³³¹ Lu^{3+} was reported to be the most efficient lanthanide for RNA cleavage.³³¹ Therefore, to isolate highly active DNAzymes, I chose the two largest lanthanides, ytterbium (Yb^{3+}) and lutetium (Lu^{3+}).

To isolate new lanthanide-specific DNAzymes, two independent selections with Yb^{3+} and Lu^{3+} were carried out. The *in vitro* selection was initiated with the DNA library. The N35 DNA library contained a sequence population of $\sim 10^{14}$. The randomized region is flanked with two short base paired. There is a single ribo-adenosine (rA) in the sequence, serving as a cleavage site, since RNA is more susceptible to cleavage than DNA.³¹⁰ The exact sequences of the library are shown in Figure 3.1B, where the randomized region is marked with blue and the cleavage site is colored red.

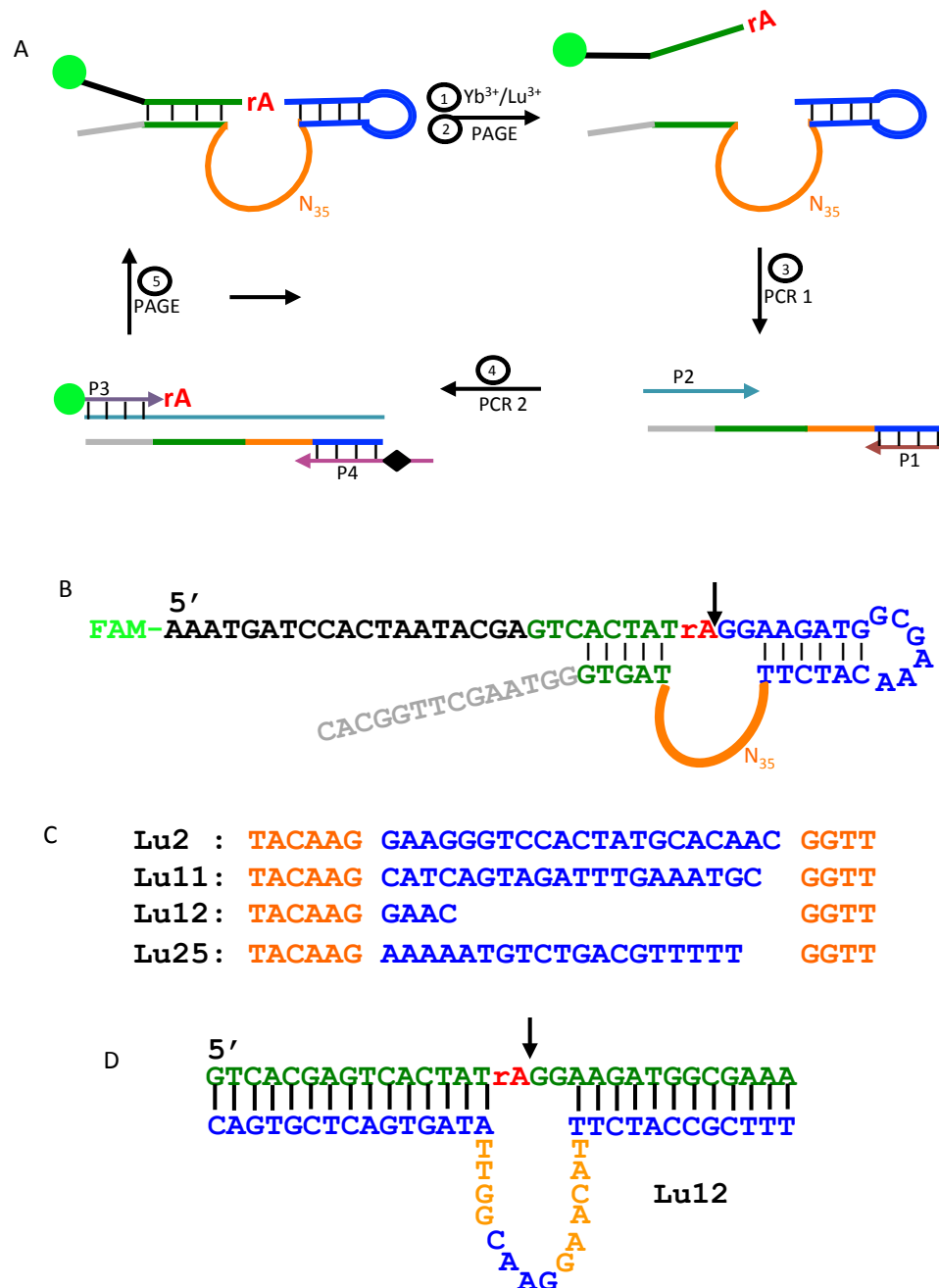


Figure 3.1 (A) $\text{Yb}^{3+}/\text{Lu}^{3+}$ -dependent *in vitro* selection procedure. The initial N35 library incubated with the metal ion to induce cleavage. Cleaved sequences are harvested after gel electrophoresis, and the full-length single-stranded library is regenerated using two rounds of PCR. The positive strand is isolated after another PAGE step and is used for the next round. (B) The N35 library sequence used for the *in vitro* selection experiment. (C) Sequence alignment from the start of the N35 region. The highly conserved nucleotides are marked in orange. (D) The trans-cleaving structures of Lu12. Important nucleotides for catalysis are marked with a different color.

In the first round, the N35 DNA library was incubated with the lanthanide ion to induce the cleavage. The cleaved sequences were separate from the rest of sequences via running on the polymerase gel. Two rounds of polymerase chain reaction (PCR) were conducted to amplify the DNA sequences and generate full-length library for the next round of selection (Figure 3.1A). Six rounds of selections were carried out with each metal ion and the activity of DNA libraries reached $\sim 50\%$. The two libraries were then cloned and sequenced. The details of the selection conditions and progress are summarized in Figure 3.2 and Tables 3.1 and 3.2.

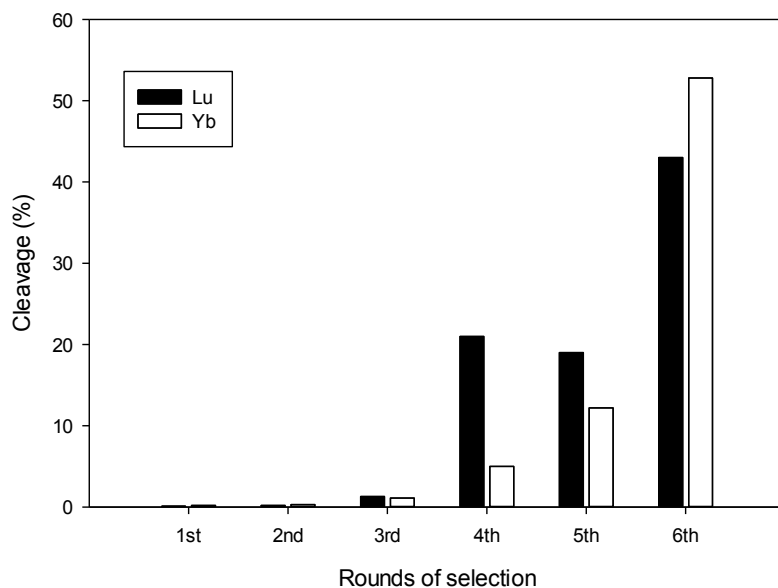


Figure 3.2 Summary of the Yb^{3+} and Lu^{3+} selections.

Table 3.1 Lu^{3+} selection progress and condition.

round #	$[\text{Lu}^{3+}]$ (μM)	incubation time (min)	cleavage (%)
1	50	60	0.1
2	50	60	0.2
3	50	80	1.3
4	50	80	21
5	20	80	19
6	20	80	43

Table 3.2 Yb³⁺ selection progress and condition.

round #	[Yb ³⁺] (μM)	incubation time (min)	cleavage (%)
1	50	60	0.1
2	50	60	0.3
3	50	80	1.1
4	50	80	5
5	20	80	12.2
6	20	80	52.8

3.2.2 Secondary structure analysis

The sequencing analysis indicated most of the DNAs selected through the Yb³⁺ selection (~ 95%) belong to Ce13d sequence family, and the rest did not form properly. While aligning the Lu³⁺ sequences, we found most of the cloned sequences belong to a single sequences family. The representatives are shown in Figure 3.1C. As indicated, the first six nucleotides are highly conserved (TACAAAG, in orange) in these sequences, followed by a highly variable region that again ended with another highly conserved nucleotides (GGTT).

Out of the sequences selected in the Lu³⁺ selection, Lu12 has the shortest sequences and was selected for the folding analysis. The Lu12 sequence was folded using Mfold software.³³² The secondary structure is shown in Figure 3.3. The trans-cleaving version of the structure is also shown in Figure 3.1D. The Lu12 DNAzyme complex contains a substrate and an enzyme strand. As shown with orange, the two conserved regions generate the two sides of the enzyme.³⁰¹ The cleavage junction (rA·G) is indicated by the arrowhead. The enzyme strand has a simple loop structure with 14 nucleotides. For those DNAzymes with longer insertions, the inserted sequences form either a hairpin or a structureless loop, which suggests the extra region may not participate in the catalytic reaction and Lu12 contains the important nucleotides. The structure of Lu12 is quite different from that of Ce13d, and it might have a different activity

trend across the lanthanide series. The secondary structure of Ce13d is also presented in Figure 3.4, for the comparison. Next, the detailed biochemical characterization of the Lu12 DNAzyme was performed.

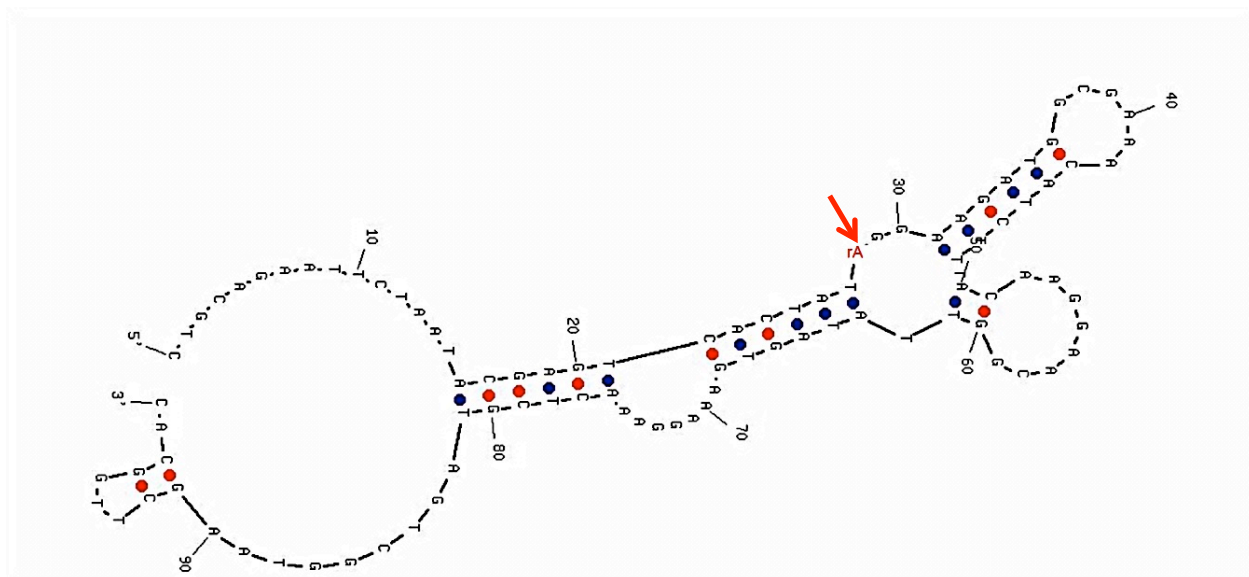


Figure 3.3 The *cis*-cleaving structure of Lu12. The rA cleavage site is marked with red arrowhead.

3.2.2 Biochemical characterization of the Lu12 DNAzyme

The initial activity study was performed by a colleague, which indicated Lu12 has similar activity for the light lanthanides and its activity decreased significantly with the last few heavy lanthanides.³⁰¹ Previously, it was reported that Lu^{3+} was among the most efficient lanthanides for cleaving a dinucleotide RNA (at high concentration of 5 mM lanthanides, and no DNAzyme added), while small lanthanides such as Nd^{3+} were almost inactive.³³¹ In this case, however, it is interesting that even though Lu12 was isolated through Lu^{3+} selection, Lu^{3+} gave the least cleavage. Lu12 DNAzyme seemed to follow an interesting pattern for its activity. To gain insight into the catalysis mechanism of Lu12, a detailed biochemical analysis was performed. In the

following biochemical assays, a light lanthanide (Nd^{3+}) was used, since it was found as one of most efficient lanthanides for cleaving the Lu12 enzyme.³⁰¹

In addition to Lu12, three other DNAzymes (Ce13d, 17E, and GR5) were also included in this study for comparison. As previously discussed, Ce13d is a general lanthanide-dependent enzyme.²⁹⁹ 17E is the most thoroughly studied DNAzyme that is active with many divalent metal ions (the best activity with Pb^{2+}),^{324, 327} while GR5 works only with Pb^{2+} .^{20, 333}

The secondary structures of these DNAzymes were presented in Figures 3.4. These four DNAzymes share the same substrate sequence. In the presence of the target metal ion, the substrate is cleaved into two pieces. The 3'-end of the substrate is labeled with a FAM fluorophore to screen the cleavage reaction on gel electrophoresis.

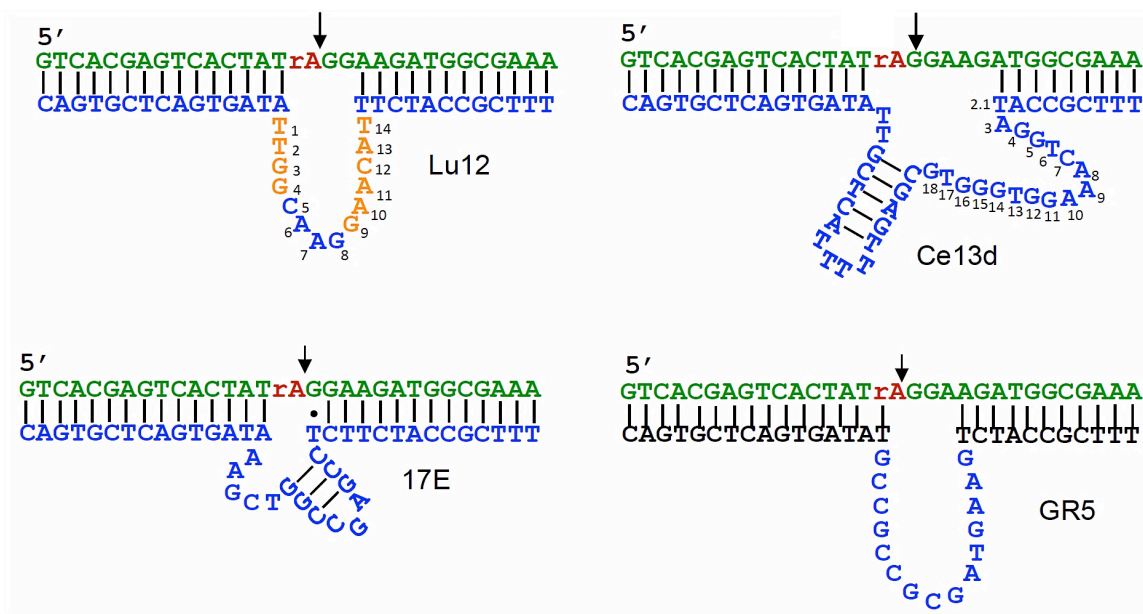


Figure 3.4 The trans-cleaving structure of the DNAzymes studied in the assay. The substrate stand is indicated in green and the cleavage site is marked with a black arrow.

3.2.2.1 Junction cleavage analysis

First, the effect of the cleavage dinucleotide junction in the substrate strand was studied. The conventional junction for the RAN-cleaving DNAszymes is rA·G. Changing the junction composition might reveal new insights regarding metal binding and catalytic mechanism. For 17E DNAszyme, cleavage of all the 16 possible junctions were studied, where the activity can differ over 5 orders of magnitude.^{120, 327, 334-335} We recently also compared a few substrate junctions for GR5 and 17E, and they both showed the same trend.³³⁶ We can conclude that enzymes with a similar mechanism (e.g., GR5 and 17E) probably follow a similar activity pattern for different substrate junctions. Here, I only picked up a few representative examples for the comparison (e.g., the ones known to be easy to cleave and more difficult to cleave). The rU·T junction was included in this study since our previous studies indicated that cleavage of pyrimidine-pyrimidine junctions is very challenging.³³⁴ Two modified nucleotides (2-aminopurine (2AP) and hypoxanthine (HX)) were also included to gain further mechanistic insights.

The rate constants for the Lu12 DNAszyme with the six different substrates are summarized in the Figure 3.5A. As indicated, the normal rA·G and rA·HX junctions have the highest activity, and as expected, the activity is the slowest with the rU·T junction. However, the difference is just slightly over 10-fold. Overall, the Lu12 DNAszyme is quite insensitive to the dinucleotide junction changes. Compared to Lu12, the other three enzymes did not show measurable rates for the rU·T junction (even after 24 h incubation). They demonstrated over 5 orders of magnitude difference in rate for the different junctions. Even by excluding the two slowest junctions (rA·T and rU·T), the other four junctions still span 3 orders of magnitude.

All the four DNAzymes cleave the rA-G junction the fastest. When only comparing this junction, the 17E and GR5 DNAzyme is ~10-fold faster than Lu12 and Ce13d (note the different y-axis scale). Despite this difference, Lu12 cleaves the other 5 types of junctions faster than all the other DNAzymes. This study clearly indicates that Lu12 is a special enzyme that might bind metal cofactor using a different mechanism.

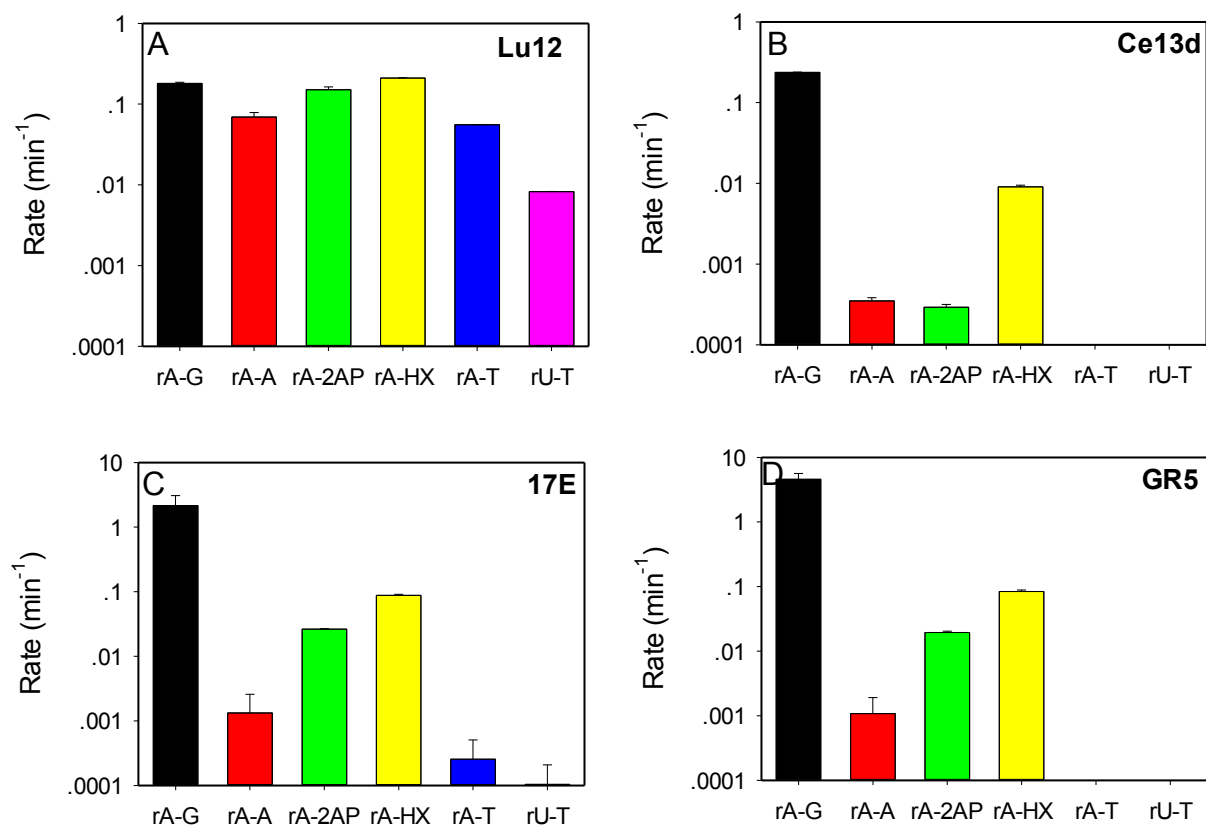


Figure 3.5 Rate values for the four DNAzymes cleaving six different substrate junctions. (A) The Lu12 assay was carried out at pH 6 with 2 μM Nd^{3+} . (B) The Ce13d assay was carried out at pH 6 with 10 μM Ce^{3+} . (C) The 17E assay was carried out at pH 6 with 10 μM Pb^{2+} . (D) The GR5 assay was carried out at pH 6 with 10 μM Pb^{2+} .

It has been speculated that pyrimidine-pyrimidine junctions are more difficult to cleave due to the base-stacking interactions at the dinucleotide junction that are suggested to play important roles in the hydrolysis of small DNAzymes.^{327, 334, 335} Purine bases seem to be involved in stronger stacking interactions than pyrimidine bases and are therefore more favourable for the cleavage junction position. Therefore, the fact that Lu12 is more tolerant for substrate junctions may indicate that base stacking is less important for it; thus, a different mechanism of metal binding. Based on this initial observation, we reason that Lu12 warrants a careful study.

3.2.2.2 pH-dependent activity studies

The previous cleavage junction study suggested a unique mechanism for the Lu12 DNAzyme. The pH profile of a DNAzyme can further help us to understand its hydrolysis mechanism. The rate of RNA cleavage is usually faster at higher pH, since high pH favours the deprotonation of the 2'-OH group to make it a better nucleophile. This deprotonation can be assisted by a metal ion or a nucleobase, depending on the specific enzyme.^{133, 322}

In Figure 3.6A, the log of Lu12 cleavage rate versus pH is graphed from pH 4.4 to 7.0 in the presence of 2 μM Nd^{3+} . Initially, the increase was rapid and followed a linear trend. After pH 5.2, the rate increase became slower, which might be related to the hydrolysis of Nd^{3+} at higher pH. Using a linear equation, the slope of the rate versus pH from 4.4 to 5.2 was calculated to be 2.1 (Figure 3.6B). Since most of the previously reported DNAzymes and ribozymes have only a single deprotonation step indicated by a slope of ~ 1 or less, this result is quite interesting.^{133, 245, 322, 324, 327, 337} For comparison, we also included the data for Ce13d (open dots, Figure 3.6B, slope = 1.06), where the difference in slope can be clearly observed. The results provided here suggest

two proton transfer steps are involved in the catalytic step of Lu12. One proton transfer can be linked to the 2'-OH group at the cleavage site, while it is unclear at this moment about the origin of the other proton transfer step.

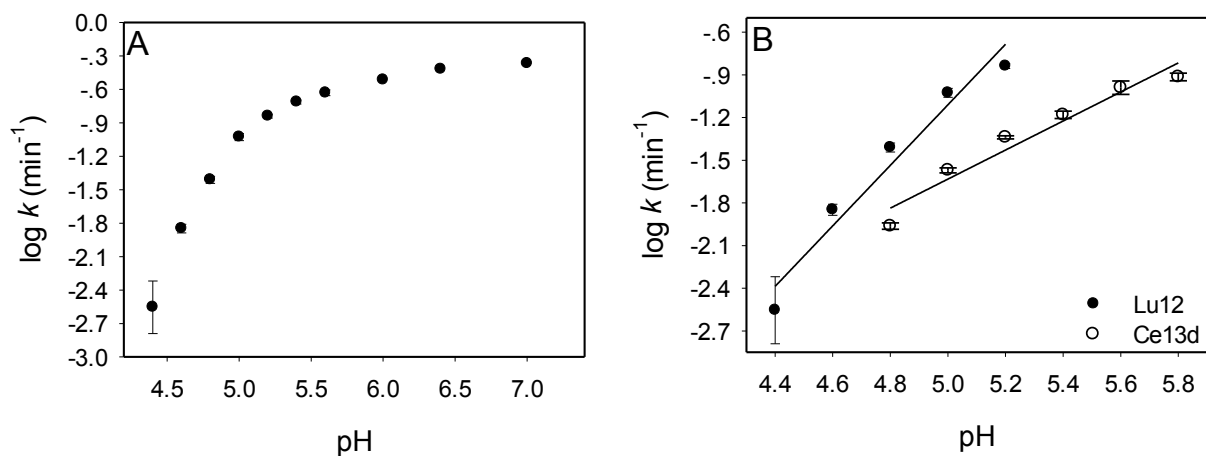


Figure 3.6 The cleavage of the Lu12 DNAzyme over a wide pH range with 2 μM Nd^{3+} (A) and the linear part in the low pH region (B). The slope of Ce13d is also shown for comparison.

3.2.2.3 Metal concentration effects

The previous pH-dependent study suggested two proton transfer steps for the hydrolysis of Lu12. Here, the effect of metal binding was studied to clarify if Lu12 binds multiple metal ions as well. Most known metal-dependent ribozymes or DNAzymes bind only a single metal ion for catalysis, as revealed by the metal concentration-rate profile. Recently, we reported new enzymes that bind two or three lanthanide ions cooperatively for catalysis.³⁰⁰ We first performed a study over a wide Nd^{3+} concentration and found a bell-shaped profile, where the highest activity was observed with 1-2 μM Nd^{3+} . Any further increase of metal concentration induced inhibition of activity. When the increasing region was fit, the Lu12 DNAzyme shows a single

metal binding without cooperativity trend observed. Conclusively, we reason that the two proton transfer steps are not linked to two different metal ions.

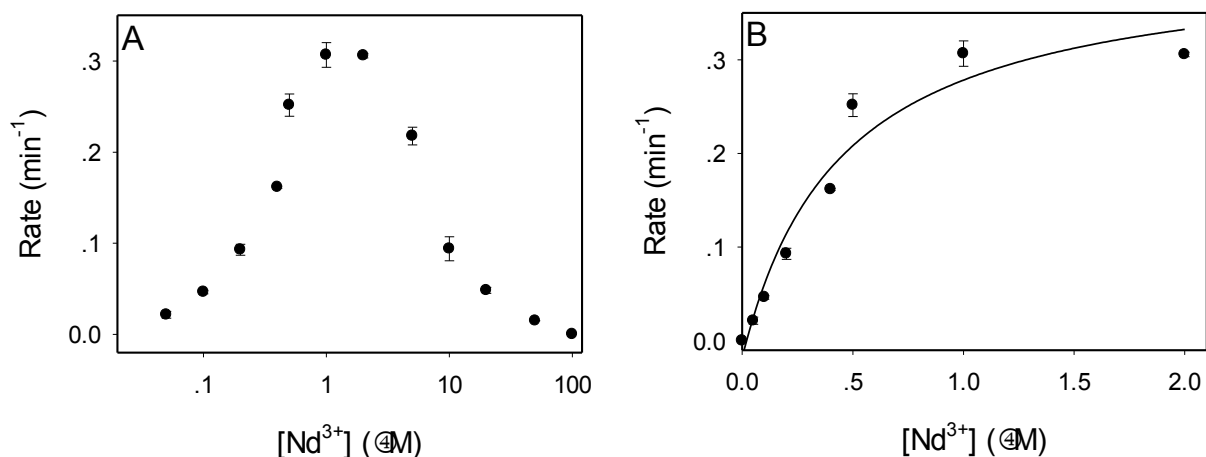


Figure 3.7 Nd^{3+} concentration dependent activity of the Lu12 DNAzyme over a wide metal concentration (A), and the fitting to a single metal binding curve in the low Nd^{3+} concentration region (B).

3.2.2.4 Phosphorothioate-modified substrate

Replacing a non-bridging oxygen atom by sulfur (so called phosphorothioate modification) at the cleavage junction is a powerful means of probing metal binding sites (Figure 3.8A).^{308, 316, 318, 319, 338} Lanthanide ions are hard Lewis acids that prefer oxygen-based ligands such as phosphate, and therefore, this modification can reveal the functionality of the site.

For the Lu12 enzyme, the normal phosphate (PO) substrate yielded ~90% cleavage, while the PS substrate is cleaved only ~60% after 3 h (Figure 3.8B). The PS substrate has a two-phase kinetics; the initial fast phase is similar to the PO substrate, which is followed by a slower phase. The PS modification had a larger effect on the Ce13d DNAzyme, and only ~20% cleavage was

observed (Figure 3.8C). This is another indication that these two enzymes have different mechanisms.

The above assays were performed with the non-separated substrate containing both diastereomers (R_p and S_p). To analyze each diastereomer separately, the HPLC separation was also performed with a colleague (Figure 3.8D).³¹⁵ Surprisingly, we found that the R_p substrate has a higher activity, reaching a rate of 0.17 min^{-1} , which is nearly the same as the normal PO substrate. Therefore, replacing the pro- R_p oxygen by sulfur has no effect on the enzyme activity. On the other hand, when the other fraction from HPLC separation was used, two rates were observed. The faster rate accounts for only a small fraction of cleavage ($\sim 10\%$) with a rate of 0.15 min^{-1} , which is similar to the R_p rate and is attributed to the R_p sample remaining in that fraction. This type of in-complete separation is often seen from such HPLC-based purifications.³¹⁶ The slower rate is 0.0015 min^{-1} , which is ~ 100 -fold slower than the R_p rate; therefore, Lu12 still has a strong thio effect, but the metal binding ligand is S_p instead of R_p . This arrangement of metal binding is more difficult to rationalize, since S_p is pointed away from the 2'-OH, which is directly responsible for catalysis.

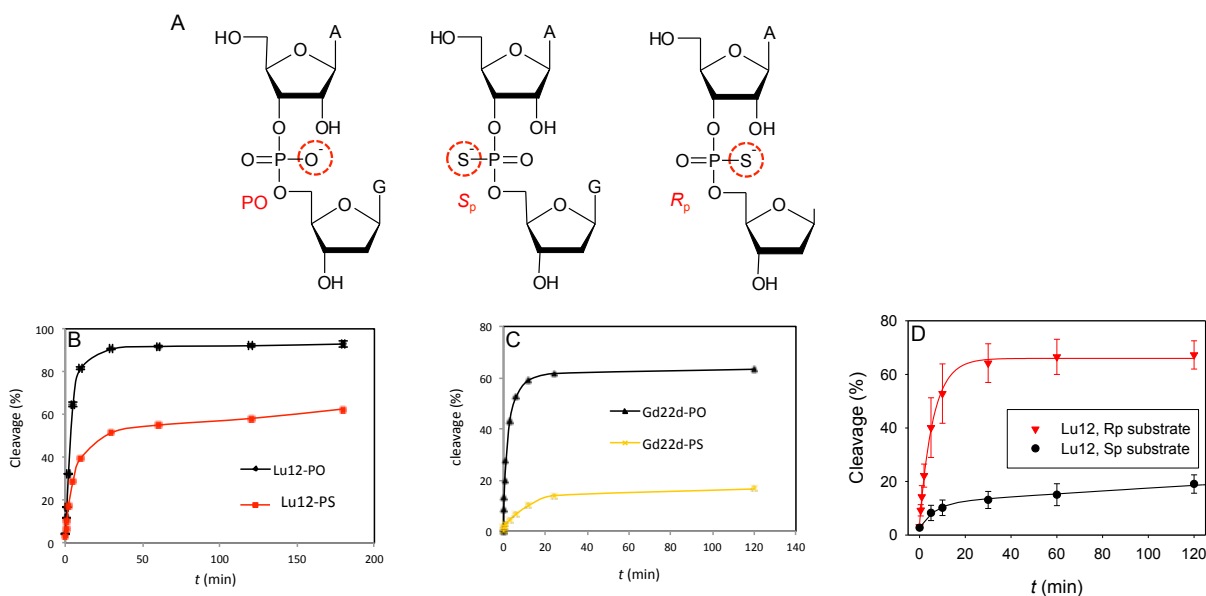


Figure 3.8 The structure of the cleavage junction with the normal PO linkage and the two diastereomers of the PS modified junctions. Cleavage kinetics of the (B) Lu12 DNAzyme and (C) the Ce13d DNAzyme of the PO and PS substrates. Note that the PS substrate was not separated and contained both diastereomers in this assay. (D) The activity of HPLC separated isomers with the Lu12 DNAzyme.

3.2.2.6 Enzyme mutational analysis

The above work mainly focused on the phosphate of the DNAzyme. Next, we aim to identify the important bases. Previous sequence alignment indicated that the nucleotides in orange are highly conserved (Figure 3.1D). However, it remains unclear about potential mutations. To achieve this goal, systematic mutation studies were carried out. The rates of the mutants are shown in Figure 3.9 (for the numbering see Figure 3.4A). A smaller bar indicates a more detrimental effect of the mutant. This study indicates that most nucleotides in the catalytic core of the Lu12 are important for its activity. The least important one appears to be T₁₄, since it can be mutated to all the other three nucleotides. We suspect that forming a base pair with the substrate might be its role since it maintains the most activity when mutated to a C, which forms

a Watson-Crick base pair with the substrate (the original enzyme would thus be a G-T wobble pair). The other tolerated change is A11, which can be changed to a guanine with even greater activity. On the other hand, most other nucleotides cannot be mutated without significantly inhibiting the enzyme activity. Overall, Lu12 is a well-defined small DNAzyme.

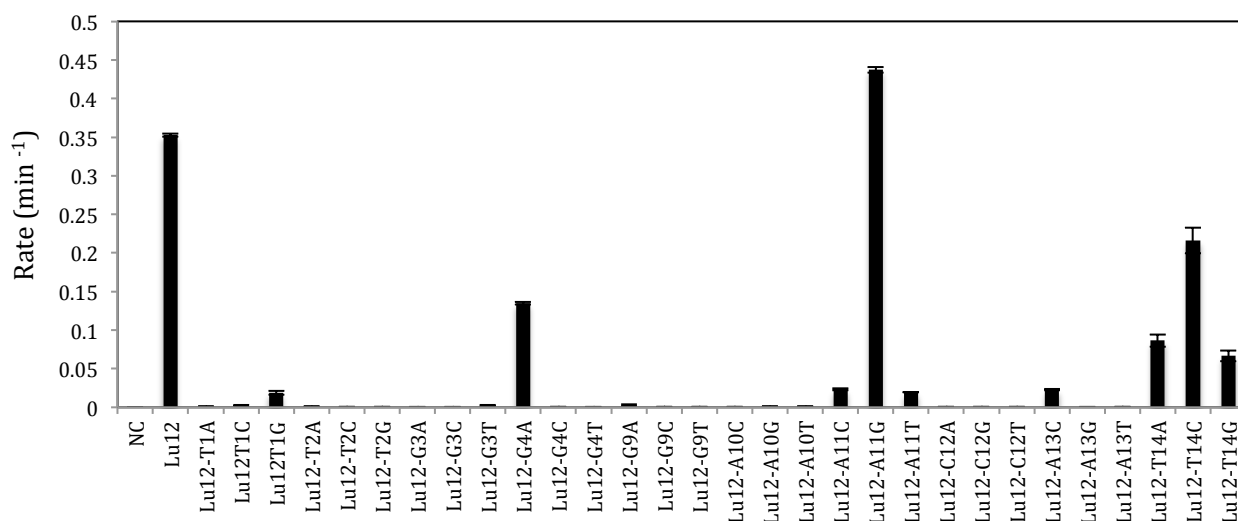


Figure 3.9 Cleavage activity of Lu12 mutants with the PO substrate in the presence of 2 μ M Nd^{3+} .

3.2.2.7 The Lu12 catalytic mechanism

In a typical catalytic mechanism of ribozymes and DNAzymes, the 2'-OH group is activated through deprotonation by a general base, and the 5'-O is activated by a general acid.³²² Metal ions are available to coordinate the electron-rich groups of nucleic acids (e.g., imino nitrogens, phosphate oxygens, and keto oxygens) in the catalysis. In some cases, a single metal ion can coordinate two or more groups from separate secondary structural motifs, which results in bridging domains and stabilizing a specific tertiary structure.³³⁹ For example, in cases like the

HDV ribozyme, the catalytic Mg^{2+} ion interacts through inner-sphere coordination with the 2'-OH group of U(-1) and activates it for nucleophilic attack on the scissile phosphate.³⁴⁰ The same metal ion interacts with the pro- R_p oxygen at the scissile phosphate,³⁴¹ and also interacts through outer-sphere coordination with two water molecules with a G25:U20 reverse wobble. In this cleavage reaction, C75 is implicated as the proton donor.

DNAzymes for the cleavage of RNA usually go through a single deprotonation step,^{133, 245, 322, 324, 327, 337} while, unlike other normal DNAzymes, Lu12 is involved in a double deprotonation step, yet still using a single Ln^{3+} metal ion. One of the steps is probably linked to the 2'-OH deprotonation, but the other one still requires more research. The pH profile of the enzyme also supports the deprotonation step, and the metal ion can assist the event by lowering the pK_a of the 2'-OH group and activating it for nucleophilic attack. The Lu12 enzyme uses the pro- S_p oxygen at the scissile phosphate for binding the metal ion. Since the pro- S_p oxygen is not in close proximity to the 2'-OH, thus, probably prior to the transition state the Lu12 enzyme should also adopt a conformational change to bring the functional groups involved in the catalysis together and let them interact with the single Ln^{3+} metal ion.

Lu12 is quite insensitive to the dinucleotide junction changes, which is inconsistent with other known DNAzymes (e.g., 17E and GR5). Clearly, Lu12 has a different pattern for the metal binding and catalysis, and mainly relies on the nucleotides in the core region for the activity. These nucleobases are sensitive to substitution mutation, and are therefore critical for the catalysis. They might have essential structural or functional roles, which are still unclear. Two exceptions are G₄ and A₁₁, which can tolerate purine-to-purine substitution. Changing the A₁₁ nucleotide to C and T completely disturbed the activity, while substituting it with a G even increased the cleavage rate. This can be related to the different pK_a values introduced by this

change, at this position. A more detailed analysis of the core nucleotides can further clarify the catalytic mechanism of the Lu12 DNAzyme. For example, nucleotides with deleted or modified functional groups or PS-modified enzyme mutants can be employed to more deeply study their roles in the catalysis.

3.3 Summary

To obtain new DNAzymes that can discriminate each lanthanide ion, two independent *in vitro* selections were carried out using Yb^{3+} and Lu^{3+} metal ions. A new DNAzyme named Lu12 was isolated, which has a descending activity in the presence of the last few heavy lanthanides. A detailed biochemical study was performed on Lu12 that revealed a number of interesting properties. Unlike other selected DNAzymes (e.g., 17E, GR5, and Ce13d), Lu12 is quite insensitive to the dinucleotide junction changes. It has a pH-rate slope of 2. Lu12 adopts a unique mechanism that might contribute to the better tolerance of the cleavage junction composition. Collectively, a double deprotonation step mechanism was proposed for the Lu12 DNAzyme. We then combined Lu12 and Ce13d to design ratiometric sensors for lanthanide detection, allowing us to distinct the last few heavy lanthanide ions.³⁰¹

3.4 Materials and methods

3.4.1 Chemicals

The list of chemicals used for the *in vitro* selection, PCR, and the activity assay are the same as previously described in chapter 2, section 2.4.1. The lanthanides that were used in the

experiment, include ytterbium chloride hexahydrate, lutetium chloride hexahydrate and neodymium chloride hexahydrate, were purchased from Sigma-Aldrich with the purity of 99.999%, $\geq 99.99\%$ and 99.9%, respectively. The *trans*-cleaving enzyme and the mutants were purchased from Eurofins (Huntsville, AL). The sequences of various mutants used in this chapter are listed in Table 3.3.

3.4.2 *In vitro* selection

The methods of *in vitro* selection and PCR are the same as previously described in chapter 2. The conditions and progress of Yb^{3+} and Lu^{3+} -dependent selection are summarized in Tables 3.1 and 3.2.

3.4.3 Cloning and Sequencing

The two *in vitro* selections were stopped after 6 rounds. The fresh PCR1 product from each *in vitro* selection was then cloned. The cloning and sequencing analysis are described in chapter 2.

3.4.4 Activity assay

To make the enzyme-sub complex, a final concentration of 0.7 μM of the FAM-labeled substrate strand and 1.1 μM of each enzyme were annealed in a buffer containing 25 mM NaCl and 50 mM MES, pH 6. Next, a specific concentration of a metal ion was added to 5 μL of 1 μM the enzyme-sub complex in the former buffer to initiate the hydrolysis reaction. After 1 h incubation time (or a specific amount of time), the samples were quenched by 8 M urea. Cleaved fragments were then separated using 15% denaturing polyacrylamide gel and quantified by a

Bio-Rad ChemiDoc MP imaging system. The MES and MOPS buffers (50 mM with 25 mM NaCl) were used for the pH-dependent activity assay.

Table 3.3 List of the mutants used in this chapter. The important nucleotides are colored green.

Name	Sequences and modifications (from 5' to 3')
Lu12	TTTCGCCATCTT TAC AAG GAA CGG TT ATAGTGAC TCGTGAC
PO-sub	GTC ACG AGT CAC TAT rAGG AAG ATG GCG AAA-FAM
PS-sub	GTC ACG AGT CAC TAT rA*GG AAG ATG GCG AAA-FAM
R _p -sub	GAGTCACTATrA*(Rp)GG AAGATGGCGAAA-FAM
S _p -sub	GAGTCACTATrA*(Sp)GG AAGATGGCGAAA-FAM
Lu12-T1A	TTTCGCCATCTT TAC AAG GAA CGG TA ATAGTGAC TCGTGAC
Lu12-T1C	TTTCGCCATCTT TAC AAG GAA CGG TC ATAGTGAC TCGTGAC
Lu12-T1G	TTTCGCCATCTT TAC AAG GAA CGG TG ATAGTGAC TCGTGAC
Lu12-T2A	TTTCGCCATCTT TAC AAG GAA CGG AT ATAGTGAC TCGTGAC
Lu12-T2C	TTTCGCCATCTT TAC AAG GAA CGG AT ATAGTGAC TCGTGAC
Lu12-T2G	TTTCGCCATCTT TAC AAG GAA CGG AT ATAGTGAC TCGTGAC
Lu12-G3A	TTTCGCCATCTT TAC AAG GAA CGA TT ATAGTGAC TCGTGAC
Lu12-G3C	TTTCGCCATCTT TAC AAG GAA CGC TT ATAGTGAC TCGTGAC
Lu12-G3T	TTTCGCCATCTT TAC AAG GAA CGT TT ATAGTGAC TCGTGAC
Lu12-G4A	TTTCGCCATCTT TAC AAG GAA CAG TT ATAGTGAC TCGTGAC
Lu12-G4C	TTTCGCCATCTT TAC AAG GAA CCG TT ATAGTGAC TCGTGAC
Lu12-G4T	TTTCGCCATCTT TAC AAG GAA CTG TT ATAGTGAC TCGTGAC
Lu12-G9A	TTTCGCCATCTT TAC AAA GAA CGG TT ATAGTGAC TCGTGAC
Lu12-G9C	TTTCGCCATCTT TAC AAC GAA CGG TT ATAGTGAC TCGTGAC
Lu12-G9T	TTTCGCCATCTT TAC AAT GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A10C	TTTCGCCATCTT TAC ACG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A10G	TTTCGCCATCTT TAC AGG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A10T	TTTCGCCATCTT TAC ATG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A11C	TTTCGCCATCTT TAC CAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A11G	TTTCGCCATCTT TAC GAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A11T	TTTCGCCATCTT TAC TAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-C12A	TTTCGCCATCTT TAA AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-C12G	TTTCGCCATCTT TAG AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-C12T	TTTCGCCATCTT TAT AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A13C	TTTCGCCATCTT TCC AAG GAA CGG TT ATAGTGAC TCGTGAC

Lu12-A13G	TTTCGCCATCTT TGC AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-A13T	TTTCGCCATCTT TTC AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-T14A	TTTCGCCATCTT AAC AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-T14C	TTTCGCCATCTT CAC AAG GAA CGG TT ATAGTGAC TCGTGAC
Lu12-T14G	TTTCGCCATCTT GAC AAG GAA CGG TT ATAGTGAC TCGTGAC

Chapter 4. *In vitro* selection with group 3A metal ions: Ga³⁺, In³⁺, Al³⁺ and Tl³⁺

4.1 Introduction

In vitro selection of metal-specific DNAzymes is a powerful technique to obtain biosensors for detecting different metal ions.³⁴²⁻³⁴⁷ In this method, DNAzymes are selected based on their particular activities toward specific metal ions.³⁴²⁻³⁴⁷ Different metal ions can be used for the DNAzyme selection. We assume trivalent ions are more efficient for the activity of DNAzymes, and the main goal of this thesis is to explore the use of trivalent metal ions for DNAzyme catalysis. Trivalent metal ions have a higher charge density, and they may be better at assisting DNAzyme catalysis. In the previous chapters, examples of efficient DNAzymes that can cleave the substrate in the presence of trivalent lanthanide ions were obtained, for example, the Ce13d and Lu12 DNAzymes. As mentioned, lanthanides are ideal co-factors for the activities of DNAzymes and ribozymes.^{245, 328, 342, 343, 345, 348-352} They have high charge density and are hard Lewis acids, making them interact strongly with DNA phosphate backbone.^{353, 354} In addition, their first pK_a of bound water molecules is close to neutral, appropriate for acting as the general base or acid catalyst.³⁴²

Here, in this chapter, the group 3A trivalent metal ions (including Ga³⁺, In³⁺, Al³⁺ and Tl³⁺) are used as the intended metal ions for performing *in vitro* selection to obtain new DNAzymes. Compared to the lanthanides, these metal ions have a much lower pK_a of bound water, which may not be optimal for the RNA hydrolysis that takes place around pH 7.²²¹ At pH 7, the bound water is deprotonated and it can even lead to extensive hydrolysis of the metal ions. If extensively hydrolyzed, these metals may not bind tightly to the phosphate groups of

DNAzymes,^{355, 356} or their bindings in some cases, as suggested for Al^{3+} and Ga^{3+} , can result in destabilizing the DNA structure.²⁶⁴⁻²⁶⁷

Although there are potential limitations for these metal ions interacting with the DNA molecules, *in vitro* selection might still provide a solution to obtain desired DNAzymes. Finding DNAzymes that specifically activate by any of these elements would be very precious for both analytical applications and for fundamental metal binding studies.

The elements in this group are mostly toxic. Particularly thallium, the most hazardous element in the group, is extremely toxic even in trace amounts. Although there are different methods for detecting these elements in the environment, such as inductively coupled plasma mass spectroscopy (ICP-MS), differential pulse anodic stripping voltammetry, and atomic absorption spectrophotometry,²⁵¹⁻²⁵³ these methods are sophisticated, expensive, and time-consuming. Also, some methods may not differentiate between thallium and similar metal ions. We lack good analytical methods that can accurately and easily detect thallium. DNA-based biosensors like DNAzymes are great alternatives. DNAzymes can successfully detect different metal ions, and can be turned into practical metal ion-biosensors.¹⁹⁸⁻²⁰³ However, despite the importance of group 3A metal ions (more particularly thallium), no specific DNAzymes or ribozyme have been reported for these elements thus far. The objective of this work is to select a selective and sensitive RNA-cleaving DNAzyme for Ga^{3+} , In^{3+} , Al^{3+} , and Tl^{3+} metal ions. Between these four elements, thallium, due to its high toxicity, has an excessive importance. Thus, in addition to the selection with the normal library, we have repeated the Tl^{3+} -dependent selection with a phosphorothioate-modified (PS-modified) library. Our previous works on the PS-modified DNAzymes indicated that it is possible to obtain DNAzymes by performing selections using a PS-containing library.^{204, 311} The processes of *in vitro* selections with group 3A

metal ions and the results obtained with the normal and the PS-modified libraries will be discussed.

4.2 Results

4.2.1 *In vitro* selection using N50 DNA Library

To isolate group 3A metal-dependent DNazymes, the *in vitro* selection technique was employed. The steps of the *in vitro* selection are the same as previously described for Ce^{3+} and Lu^{3+} selections. The process begins with an oligonucleotide library consisting of randomly generated sequences. For group 3A metal ions, the N50 DNA library was selected, which is estimated to contain $\sim 10^{14}$ random DNA sequences. There is a single RNA linkage (rA) in the DNA strand, serving as the putative cleavage site, since the RNA molecule is 1,000,000 times more susceptible to hydrolysis than DNA.³⁵⁷ In each round, DNA sequences with the ability to hydrolyze the RNA phosphodiester bond in the presence of the target metal ion, were isolated. The DNA library is incubated with the intended target metal ion to induce the cleavage. Cleaved sequences are separated from the rest of the library by running on the poly acrylamide gel. They are then amplified and enriched by PCR to produce the library for the next round of selection. The selection is stopped when the activity of the DNA library reached $\sim 30\text{-}40\%$, and the selected DNAs are sequenced and analyzed. The processes of the *in vitro* selection are shown in Figure 4.1.

For group 3A, four independent selections were done, which are described in the following sections, 4.2.1.1 and 4.2.1.2. Due to the significance of Tl^{3+} metal ion, a more careful study was done with this metal ion.

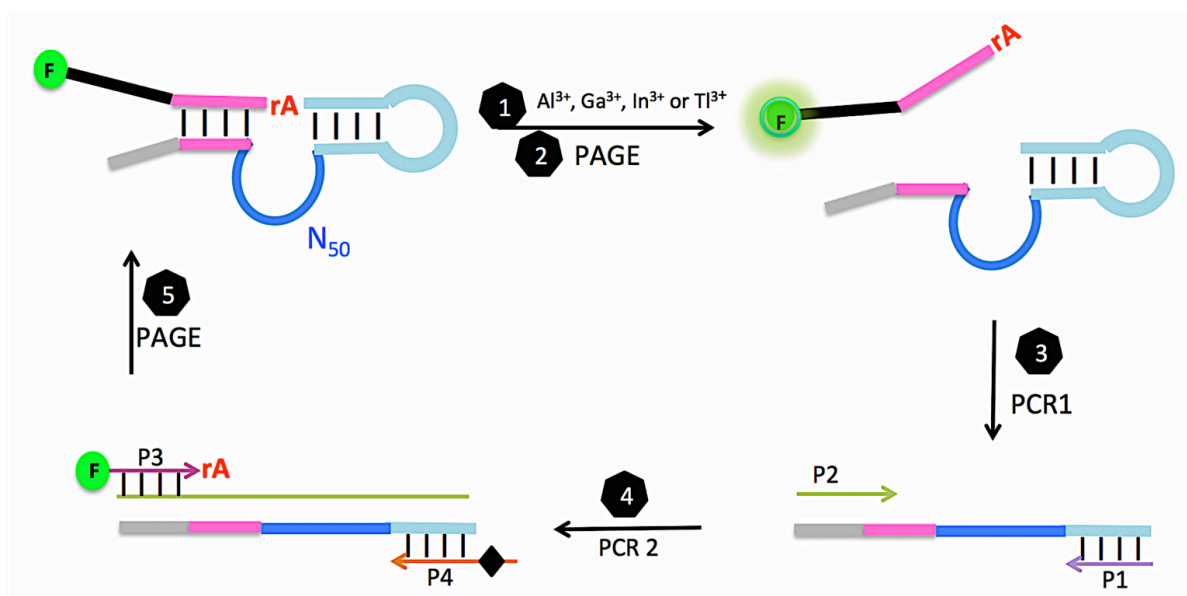


Figure 4.1 Schematic representing the *in vitro* selection for Al^{3+} , Ga^{3+} , In^{3+} , and Tl^{3+} -dependent DNAzymes, using N50 library. To generate a full-length library for the next round of selection, four different primers are used, which are shown as P1, P2, P3 and P4. The cleavage site is a single riboadenosine, indicated as red rA. The substrate cleavage results in increasing the florescent signal.

4.2.1.1 Ga^{3+} , In^{3+} , and Al^{3+} -dependent selections

Three independent selections were carried out using Ga^{3+} , In^{3+} , and Al^{3+} metal ions as cofactors, respectively. The condition for each round of the selection and the percentages of cleavage after each round of selection are described in Table 4.1, 4.2, and 4.3. Conducting five rounds of selection with each metal, the activity of the DNA library barely reached >10 % (Figure 4.2). The lack of high activity of the library suggests that these metal ions may not be efficient cofactors for RNA cleavage under our selection conditions.

With relatively small ionic sizes and high charges, these metal ions readily form hydrolysis complexes, which may precipitate and weaken their electrostatic interaction with DNA. Therefore, they may not bind to the DNA backbone so tightly,³⁵⁵ and probably much

higher concentrations of these metal ions are required to activate the DNA. Another parameter that can help accelerate their catalysis is by applying a higher pH. A higher pH usually promotes RNA cleavage, due to the deprotonation of the 2'-OH group as a better nucleophile. However, a high pH will further enhance metal ion hydrolysis. The effects of these parameters were separately tested on the selection.

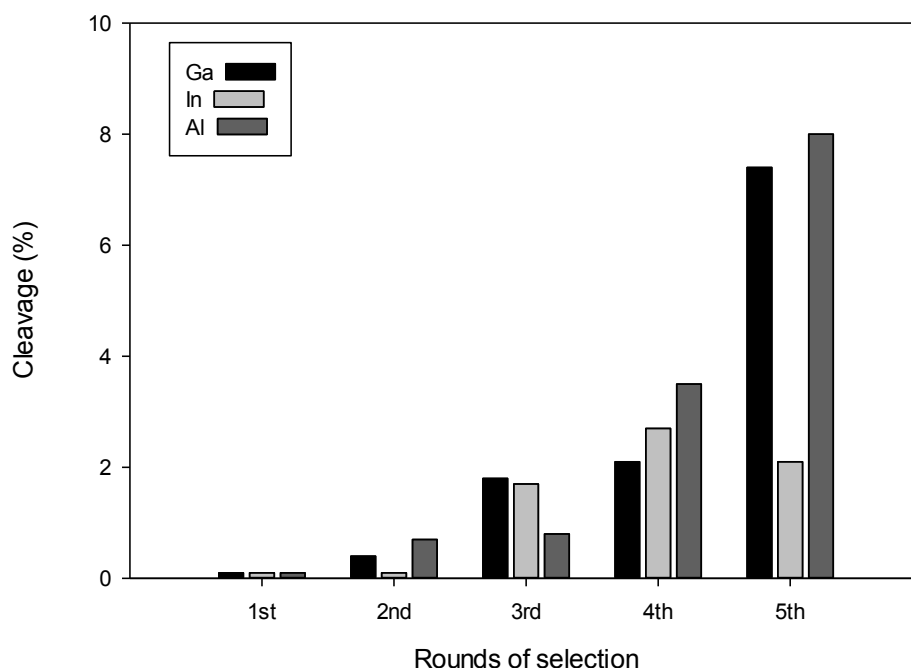


Figure 4.2 Summary of three independent *in vitro* selections with Ga^{3+} , In^{3+} , and Al^{3+} metal ions. A total of five rounds of selection were done with each metal.

Table 4.1 Ga^{3+} -dependent selection conditions and progress

round #	$[\text{Ga}^{3+}]$ (μM)	incubation time (min)	cleavage (%)
1	100	60	0.1
2	100	30	0.4
3	100	30	1.8
4	50	30	2.1
5	100	30	7.4

Table 4.2 In^{3+} -dependent selection conditions and progress.

round #	$[\text{In}^{3+}]$ (μM)	incubation time (min)	cleavage (%)
1	100	60	0.1
2	100	30	0.1
3	100	30	1.7
4	50	30	2.7
5	100	30	2.1

Table 4.3 Al^{3+} -dependent selection conditions and progress.

round #	$[\text{Al}^{3+}]$ (μM)	incubation time (min)	cleavage (%)
1	100	60	0.1
2	100	30	0.7
3	100	30	0.8
4	50	30	3.5
5	100	30	8

Out of the three selections, Al^{3+} -dependent selection seemed to be more reliable, as in each round of selection showed a gradual improvement. It also indicated the highest activity after five rounds of selection ($\sim 8\%$). Thus, it was selected for the trial selection. Starting from round one, the effects of the higher metal ion concentration and the higher pH were tested in rounds two and three of Al^{3+} selection, respectively. In order to eliminate the influence of other factors, in each round of selection, double/triple amounts of the DNA library were prepared and equally divided between the samples. However, neither of these factors appeared to significantly help in promoting the amount of cleavages in the Al^{3+} selection (Table 4.4). There are other factors that might improve the selection. For instance, maybe a more complex DNzyme structure is required for the group 3A metal ion, which is formed by a larger DNA sequence. Here, we could not test all the possibilities, and future studies could consider these factors.

Selections with Ga^{3+} , In^{3+} , and Al^{3+} metal ions were stopped at this point, but further analyses were done on the selected DNA sequences.

Table 4.4 Conditions and progress of Al^{3+} trial selection.

round #	$[\text{Al}^{3+}]$ (mM)	pH	incubation time (min)	cleavage (%)
1	0.1	6	60	0.1
2	1	6	30	0.3
	10	6	30	0.3
	50	6	30	0.2
	1	6	30	0.8
3	1	7.6	30	0.5

4.2.1.2 Tl^{3+} -dependent *in vitro* selection

Thallium is the most hazardous element in the group, and thus, it is the main focus of this chapter. In the periodic table, it is situated between lead (Pb^{2+}) and mercury (Hg^{2+}). Pb^{2+} is an efficient co-factor metal ion in the RNA-hydrolysis, and Hg^{2+} is also shown to directly activate DNazymes and ribozymes.¹⁹⁸⁻²⁰³ Therefore, it is reasonable to assume that Tl^{3+} can also activate the DNzyme catalysis.

Here, efforts were made to select a Tl^{3+} -dependent DNzyme with the ability to catalyze the hydrolysis of a single phosphodiester bond. To accomplish this, the *in vitro* selection was performed with the Tl^{3+} metal ion, and with the N50 DNA library. To ensure the technique was working properly, Ce^{3+} -dependent selection was also included as the control, and was done side by side with the Tl^{3+} selection. Both low and high concentrations of Tl^{3+} were tested in the Tl^{3+} -dependent selection. As shown in Figure 4.4, in the first five rounds, no significant activities of the DNA library were observed with Tl^{3+} , but afterwards, cleavages started appearing in the gel (~5 % in round six). This is when the Ce^{3+} selection was saturated with the active sequences. Details about Ce^{3+} selection were described in chapter 2.

In Tl^{3+} -dependent selection, a higher concentration of metal ion seemed to be more effective. When conducting a ten round selection with 500 μM Tl^{3+} salts (high Tl^{3+}

concentration), the cleavage activity reached ~ 20 percent. The Ti^{3+} selection was stopped at this point, where the selected sequences were cloned and analyzed.

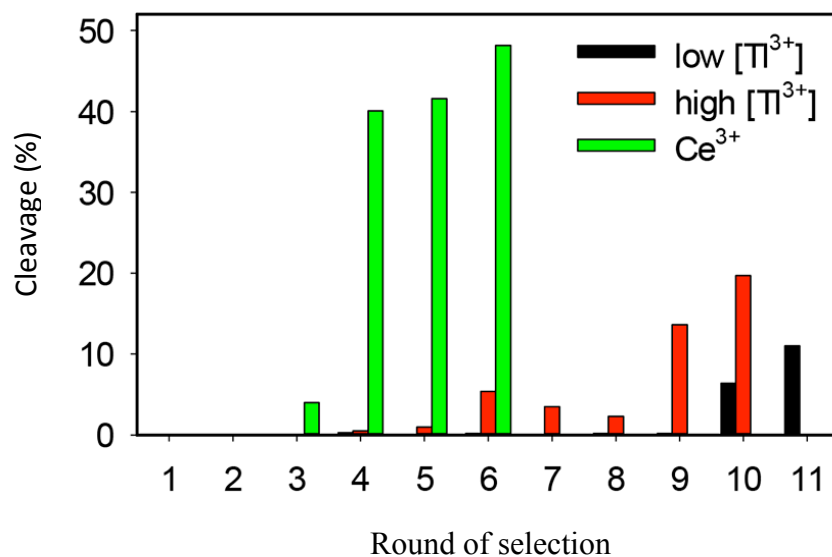


Figure 4.4 Summary of Ti^{3+} -dependent selection using N50 library.

Table 4.5 Ti^{3+} dependent selection conditions and progress.

round #	$[\text{Ti}^{3+}]$ (μM)	incubation time (min)	cleavage (%)
1	1000	60	0.1
2	1000	60	0.1
3	1000	60	0.1
4	1000	60	0.5
5	1000	60	1
6	1000	60	5.4
7	1000	60	3.6
8	20	60	2.3
9	500	60	13.6
10	20	60	6.6
10	500	60	19.7
11	20	60	11

4.2.2 Sequence alignment and secondary structure analysis

The Al^{3+} , Ga^{3+} , and In^{3+} selections were stopped after round 5, and the Tl^{3+} selection (with high metal concentration) was stopped after ten rounds. Next, the DNA sequences obtained from the four independent *in vitro* selections were sequenced and aligned. Table 4.6 shows the alignment of Tl^{3+} , Ga^{3+} , In^{3+} , and Al^{3+} selection sequences. As indicated, these sequences all belonged to four families. The nucleotides in the random region of the DNA library are highlighted with four different colors for these four sequence families.

Table 4.6 Alignment of Tl^{3+} , Ga^{3+} , In^{3+} and Al^{3+} selection sequences. The marked A is the cleavage site. Nucleotides in the random region of the DNA library are highlighted.

Clone #	Sequence (from 5'-end)
Al11	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al26	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
In25	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
In16	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
In01	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Ga25	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al04	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al13	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al14	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Ga06	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al06	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al22	--TGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 55
Al23	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al32	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Ga16	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al21	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al16	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Al18	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Tl21	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Tl6	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Tl2	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
In02	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Ga29	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCATA-GC 56
In10	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Al24	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
In22	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Ga10	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGT-GC 56
Ga32	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
In12	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
In27	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Al03	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Al25	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Ga20	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Ga27	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
A02	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCG--TAGCAGA-GC 56
Al12	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCG--TAGCAGA-GC 56
Ga03	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGC-AAACATCA--TAGCAGA-GC 55
Ga15	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCG--TAGCAGA-GC 57
In11	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCGGA-GC 56
Ga19	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCGGA-GC 56
Al07	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCGGA-GC 57
Al08	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCGGA-GC 57
Tl13	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCGGA-GC 56
Tl20	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCGGA-GC 56
Tl4	-CTGCAG-ATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 55
In28	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATC---TAGCAGA-GC 56
Al28	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATC---TAGCAGA-GC 56
Ga28	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATC---TAGCAGA-GC 56
In17	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCAAAACATC---TACCAA-GC 55
Tl29	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCG--TAGCAGA-GC 56
Tl9	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Tl26	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Tl7	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 56
Ga02	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Ga05	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAGA-GC 57
Tl22	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCA---GC 54
Ga12	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCA--TAGCAG---C 55
Tl27	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATC---TAGCAG---C 53
In07	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATC---TTGAGGA-AA 55

Al15	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATC---	TTGAGGA-AA	56
Tl8	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TGAAGGGCGA	57
Tl23	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TG-AGGGS	56
Tl5	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TG-AGGGCGA	56
Tl19	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TG-AGGGCGA	56
Tl28	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TGAAGGGCGA	57
Tl14	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TG-AGGGCGA	56
Tl25	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TG-AGGGCGA	56
Tl30	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TG-AGGGCGA	56
Tl15	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TGAAGGGCGA	57
Tl24	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCT--	TGAAGGGCGA	57
Tl10	-CTGCAGAATTCTATTACGAGTCACTATAGGAAGATGGCGAAACATCT--	TGAAGGGCGA	57
Tl1	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	54
Al30	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	54
Al17	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Al20	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Tl3	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
In20	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
In15	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
In03	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
In19	-----AGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	37
Al19	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Ga21	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCTT-AG	55
Ga22	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCTT-AG	55
Tl16	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
In26	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga26	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga17	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga04	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga09	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Al29	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCGT-AG	55
In24	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	TGAGCCAT-AG	55
In23	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga31	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga23	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Ga07	TCTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	56
Al09	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Al01	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Ga24	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Ga01	--TGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	54
Al10	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Ga18	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
Tl18	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACATCTCT	GAGCCAT-AG	58
Tl31	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCAT-AG	55
In09	-CTGCAGAATTCTAATACGAGTCACTATAGGAAGATGGCGAAACAT---	GAGCCGA-AA	55

Al11	GTCTG---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al26	GTCTG---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGT-----	105
In25	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
In16	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
In01	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Ga25	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al04	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al13	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al14	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Ga06	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al06	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al22	GTCTT---GATGT-AG-TAAATCTCTTTTATAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	109
Al23	GTCTA---GATGTAAG-TAAATCTTTTATCAGCGAG-GCGAAA	TAGTGACGGTAAGCTTG	112
Al32	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Ga16	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112

Al18	GTCTA---GATGTAAG-TAGATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Tl21	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Tl6	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Tl2	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
In02	GTCTA---GATGTAGG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga29	GTCTA---TATGTAG--TAAATCTTTTCTCACCAG-ACGAAA	TAGTGACGGTAAGCTTG	110
In10	GTTTA---GATGTAAG-TATATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Al24	GTTTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
In22	GACTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga10	GACTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAGA	TAGTGACGGTAAGCTTG	111
Ga32	GACTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
In12	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
In27	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Al03	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Al25	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga20	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga27	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
A02	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Al12	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga03	GTCTA---GATGTAAG-TAAATCTTCTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	110
Ga15	GTCTA---CATGTAAG-TAAATCTTCTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
In11	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga19	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Al07	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Al08	GTCTA---GATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Tl13	GTCTAG-AGATGTAAG-TAGATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	113
Tl20	GTCTAG-AGATGTAAG-TAGATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	113
Tl4	GTCTAG-AGATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
In28	GTCTAG-AGATGTAGC-AAAATCTTTTCTTAGCAAG-ACGAAA	TAGTGACGGTAAGCTTG	113
Al28	GTCTAG-AGATGTAG--AAAATCTTCTCTTAGCAAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Ga28	GTCTAG-AGATGTAGC-AAAATCTTCTCTTAGCAAG-ACGAAA	TAGTGACGGTAAGCTTG	113
In17	GTCTAA-AGATGTACC-AAAATCTTCTCTTACCAAA-ACAAAA	TATGACGGTAAGCTTG	112
Tl29	GTCTAG-AGATGAAG--TAAATCTTCTCTTAGCAAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Tl9	GTCTAG-AGATGTAG--CAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Tl26	GTCTAG-AGATGTAG--CAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Tl7	GCCTAG-AGATGTAA--GTAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Ga02	GTCTAG-AGATGTAA--GTAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	113
Ga05	GTCTAG-AGATGTAA--GTAATCTTTTCTCAGCGGG-ACGAAA	TAGTGACGGTAAGCTTG	113
Tl22	GTCTAG-AGATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	111
Ga12	GTCTAG-AGATGTAAG-TAAATCTTTTCTCAGCGAG-ACGAAA	TAGTGACGGTAAGCTTG	112
Tl27	GTCTAAGAGATGTAAGTAAAATCTTCTCTTAGCAAG-ACGAAA	TAGTGACGGTAAGCTTG	112
In07	ATAGAT--GATAAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	110
In18	ATAGAT--GATAAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	110
In04	ATAGAT--GATAAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	111
In30	ATAGAT--TATAAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	110
Ga30	ATAGAT--TATAAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	110
Ga13	ATAGAT--GATGAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	110
In08	ATAGAT--GATGAGCA--AAGAATTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	111
Al15	ATAGAT--GAGAAGCA--AAGAGTTCTGTGTCAGCGAC-ACGAAA	TAGTGACGGTAAGCTTG	111
Tl8	GGCTC---AATGAGAAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	113
Tl23	GGCTC---AATGAGAAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl5	GGCTC---AATGAGAAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl19	GGCTC---AATGAGAAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl28	GGCTC---ARTGAGAAGTACRACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	113
Tl14	GGCTC---AATGAGGAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl25	GGCTC---AATGAGAAGTGCAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl30	GGCTC---AATGAGAAGTGCAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl15	GGCTC---AATGATAAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	113
Tl24	GGCTC---AATGATAAGTACAACGTGCTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	113
Tl10	GGCTC---AATGAGAAGTACRACRT-CTTGAATTGC-GGAAAA	TAGTGACGGTAAGCTTG	112
Tl1	GTCAAAGGTGGGTGCG---AGTCGTATCATATCGACGAAATAA	TAGTGACGGTAAGCTTG	111
Al30	GTCAAAGGTGGGTGCG---AGTCGTATCATATCGACGACATAA	TAGTGACGGTAAGCTTG	111
Al17	GTCAAAGGTGGGTGCG---GTCGTATCATATCGAC-CAGCA	TAGTGACGGTAAGCTTG	111

In20	GTCAAAGGTGGGTGTG----GTCGTATCATATCGAC-CAGCAATAGTGACGGTAAGCTTG	111
In15	GTCAAAGGTGGGTGTG----GCCGTATCATATCGAC-CAGCAATAGTGACGGTAAGCTTG	110
In03	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
In19	GTCAAAGGTTGGTGAG---GGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	93
Al19	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
Ga21	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
Ga22	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
Tl16	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
In26	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga26	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga17	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga04	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga09	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	112
Al29	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
In24	GTCAAAGGTGGGTGAG---AGTCGTATCATAACGAC-TAGCAATAGTGACGGTAAGCTTG	111
In23	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga31	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga23	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	112
Ga07	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	112
Al09	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	111
Al01	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	111
Ga24	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	111
Ga01	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	110
Al10	GTCAAAGGTGGGTGAG---AGTCGTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	111
Ga18	GTCAAAGGTGGGTGAG---AGTCCTTATCATATCGAC-TAGCAATAGTGACGGTAAGCTTG	111
Tl18	GTCAAAGGTGGGTGCG---TGTCGTAT---ATCGAC-TAAGTATAGTGACGGTAAGCTTG	111
Tl31	GTCAAAGGTGGGTGCG---TGTCGTATCTTATCGAC-TAAGTATAGTGACGGTAAGCTTG	111
In09	GT-AAAGGTGGGTGAG---AGTCGTATCATATCAAC-TAAAAATAGTGACGGTAAGCTTG	110

As indicated, the sequences of each family are either the same or only differ in a few nucleotides. From each group, different sequences were selected for folding analysis. Using the Mfold³⁵⁸ software, their secondary structures were predicted. However, most of the selected sequences could not adopt the proper DNAzyme structure and were folded into structures atypical for RNA-cleaving DNAzymes. Examples of the improperly folded structures are shown in Figure 4.5. Therefore, these sequences might undergo non-specific cleavage. Among all the selected sequences, only a few sequences could fold into the proper DNAzyme structure, where there are several sequences very similar to the Ce13d DNAzyme. The Ce13 sequence is a common sequence and we usually obtain this sequence when using the N50 library. The Ce13d-like sequences and the corresponding secondary structures are shown in Figure 4.6.

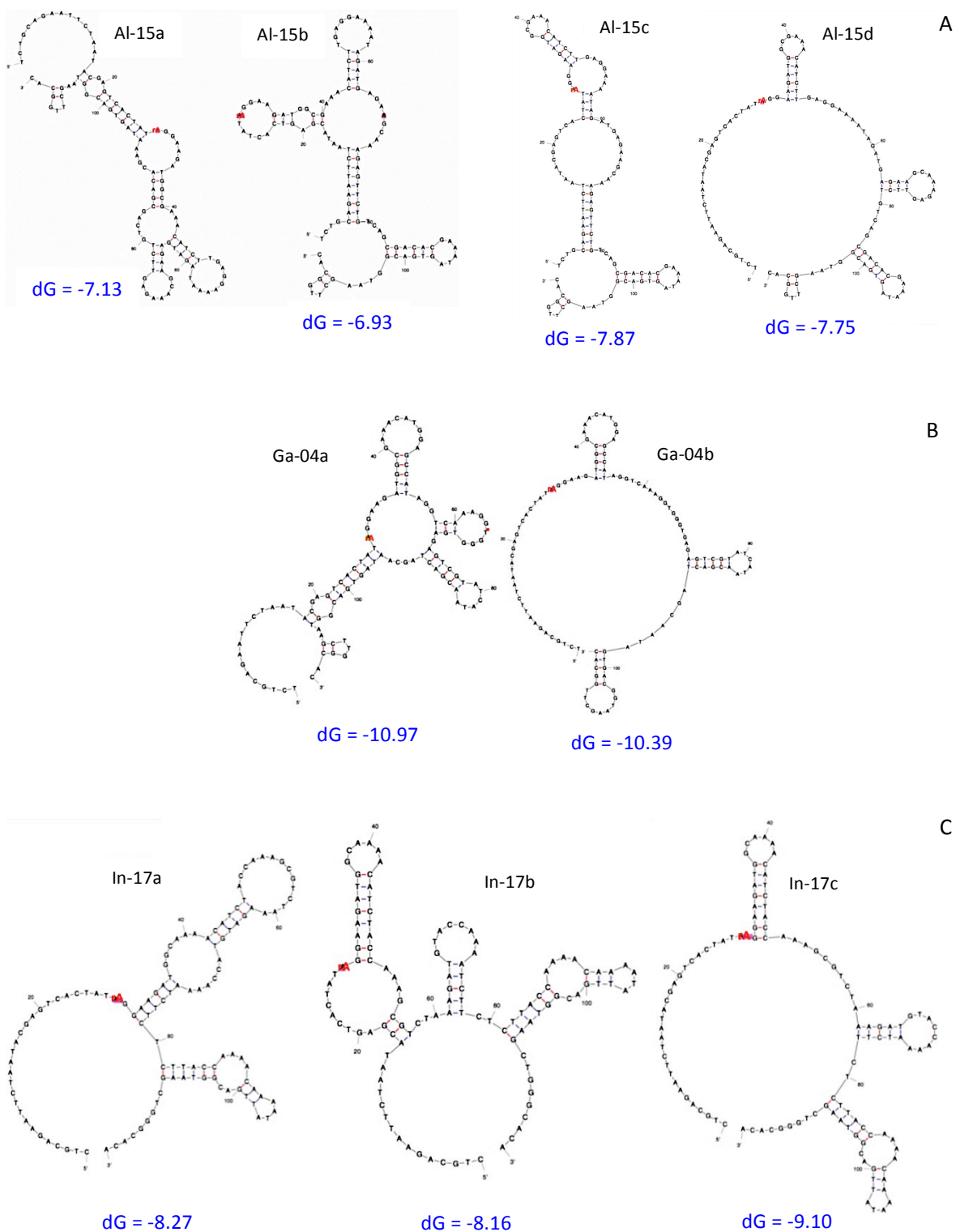


Figure 4.5 Examples of the improperly folded structures. Possible secondary structures predicated for the Al-15 (a), Ga-04 (b) and In-17 (c) sequences, using Mfold software.³⁵⁸ The cleavage site (rA) is shown in red.

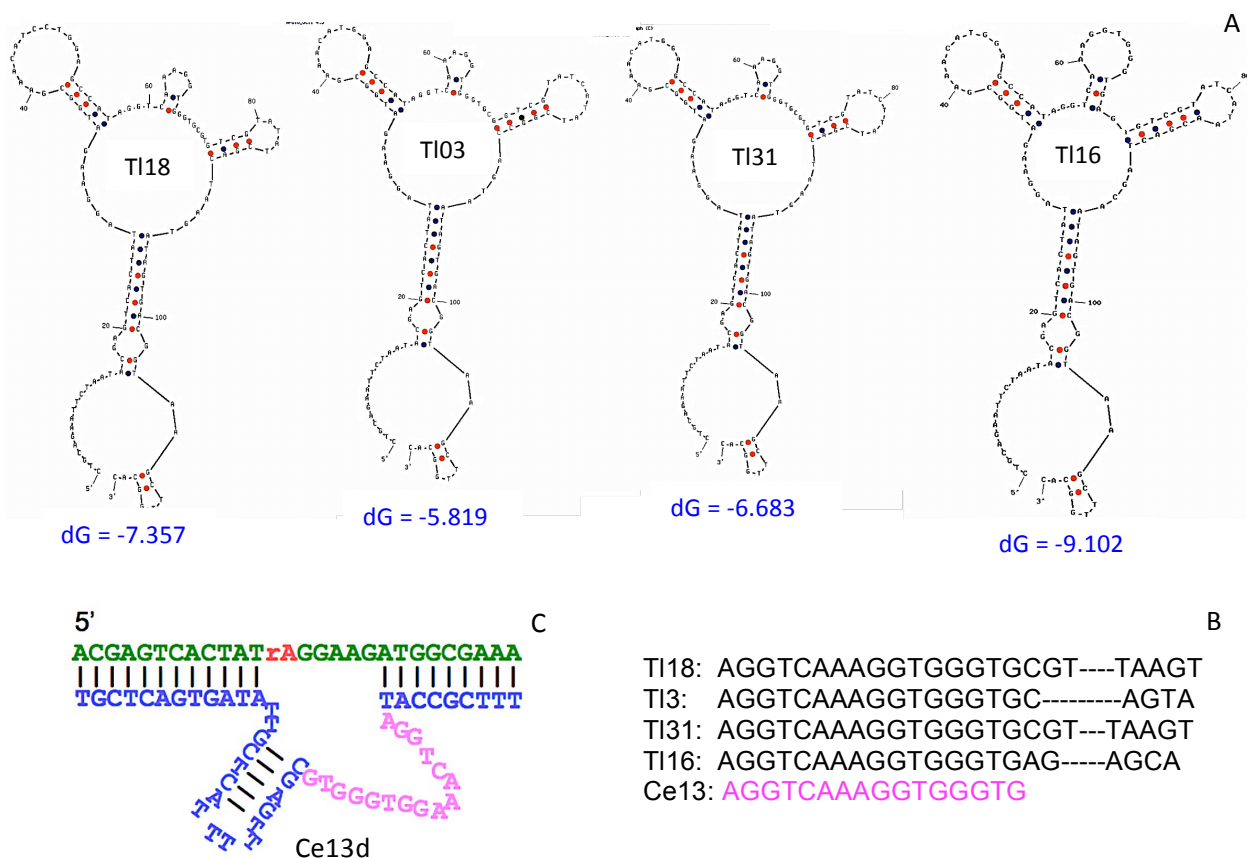


Figure 4.6 (A) Secondary structures of the Ce13-like sequences predicated by Mfold software,³⁵⁸ (B) sequences in the catalytic core of the enzymes, (C) the secondary structure of the Ce13d enzyme. The nucleotides in the catalytic core of enzyme are highlighted.

In addition to those Ce-13like sequences, the sequence analysis showed three new sequences, TI-13, TI-22, and TI-27, which were not selected in any of our former *in vitro* selections. These three sequences were able to adopt the typical DNAzyme structure, as predicted by Mfold software.³⁵⁸ The *cis*-cleaving structures of these three enzymes are indicated in Figure 4.7, and are called TI-13a, TI-22a, and TI-27a. They have very similar structures and only differ in the number of nucleotides in the catalytic core. There are some extra nucleotides in the catalytic core of TI-22a and TI-13a compared to the smallest enzyme, TI-27a. TI-13a enzyme has the largest catalytic core with nine nucleotides. In the catalytic core of the enzymes, there is a

long loop, while not all the nucleotides in this loop are assumed to be required for the enzyme activity. Thus, part of the loop was truncated and substituted with TTTT nucleotides (truncated regions are indicated with red boxes in the Figure 4.7). The binding arms of the enzymes were also optimized, and sequences producing the unnecessary region in the arms were deleted. The following studies were performed with the optimized and truncated versions of enzymes.

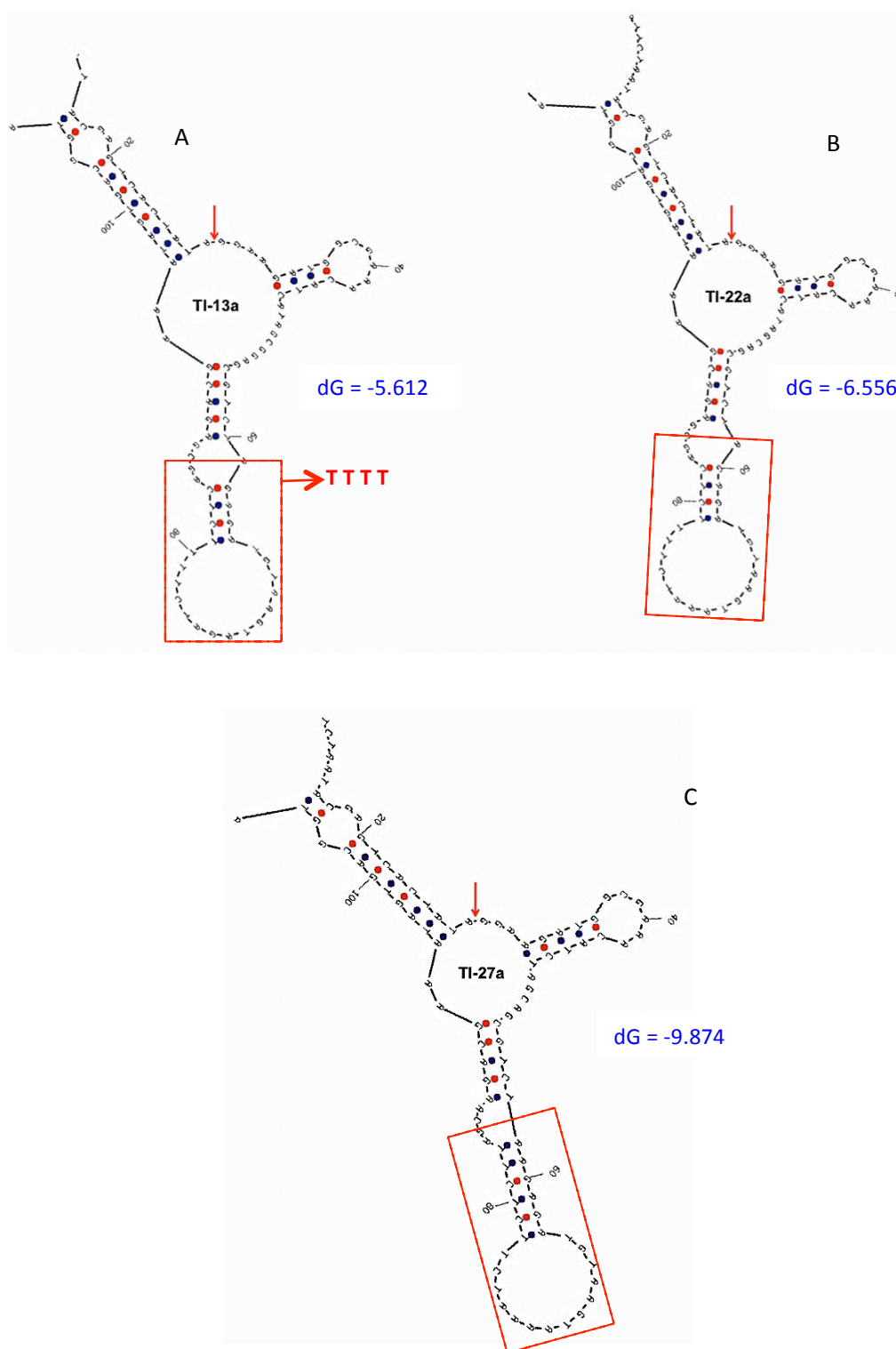


Figure 4.7 The *cis*-cleaving structures of the TI-13a, TI-22a, and TI-27a enzymes (A, B, and C, respectively), predicted by Mfold software.³⁵⁸ The truncated regions are marked with red boxes. The site of cleavage is shown with a black arrow.

4.2.3 Activities of the Tl-13a, Tl-22a, and Tl-27a DNAzyme complexes

To monitor the activity of an individual enzyme, each sequence was functionalized with a quencher, and hybridized with a FAM-labeled substrate strand to form the DNAzyme complex. The substrate strand contained a single ribo-adenosine (rA) nucleotide, serving as the cleavage site. The ability of the enzymes to cleave this rA site was assessed. The three DNAzyme structures are shown in Figure 4.8A. The sequences producing the enzyme strand are indicated in the Figure 4.8B.

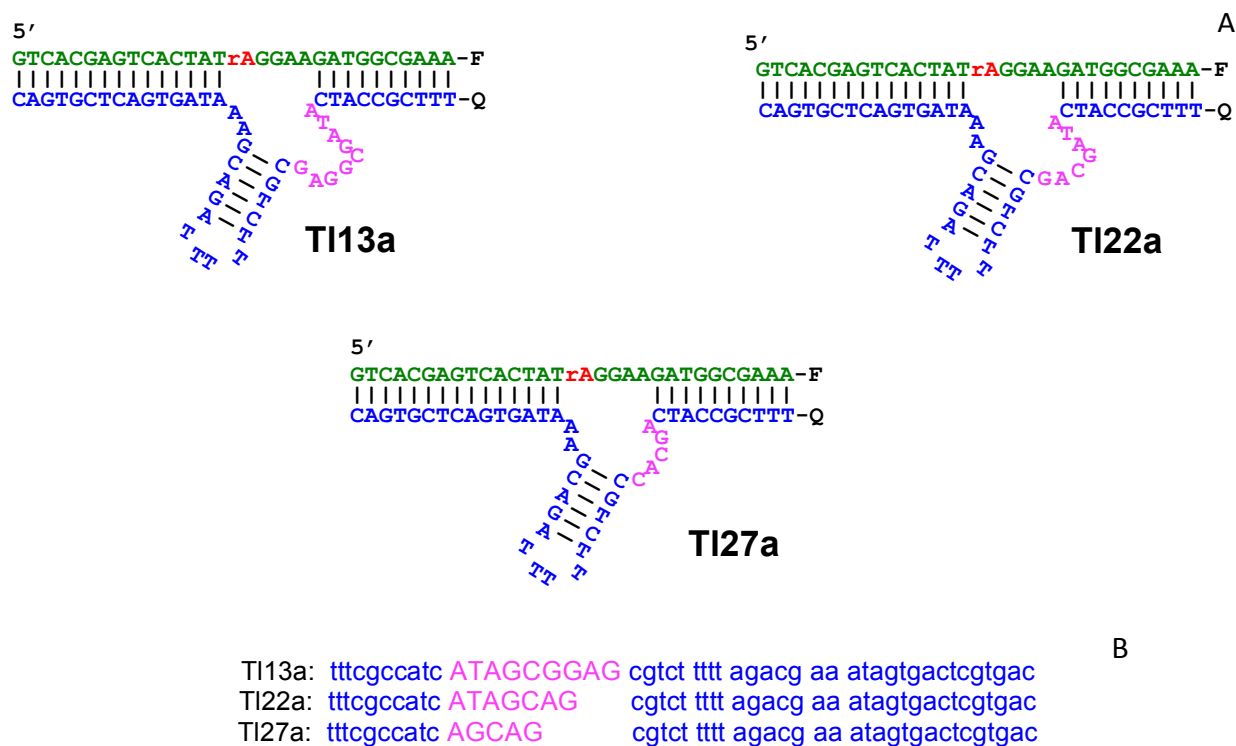


Figure 4.8 (A) *trans*-cleaving structures of Tl-13a, Tl-22a, and Tl-27a DNAzymes. The FAM-substrate hybridized with Quencher-labeled enzyme. (B) The sequences of enzyme strands. The conserved nucleotides are shown in blue, the varied nucleotides in the catalytic core are highlighted in pink.

4.2.3.1 Activity with group 3A metal ions

First, the activities of selected enzyme in the presence of each of group 3A metal ions were monitored. Tl-13a, Tl-22a and Tl-27a DNAzyme complexes were respectively incubated with three various concentrations (10uM, 100uM, and 1000uM) of Ga^{3+} , In^{3+} , Al^{3+} , and Tl^{3+} metal ions. While, as shown in Figure 4.9A, B, and C, no activity was detected for any of the selected DNAzymes in the presence of group 3A metal ions.

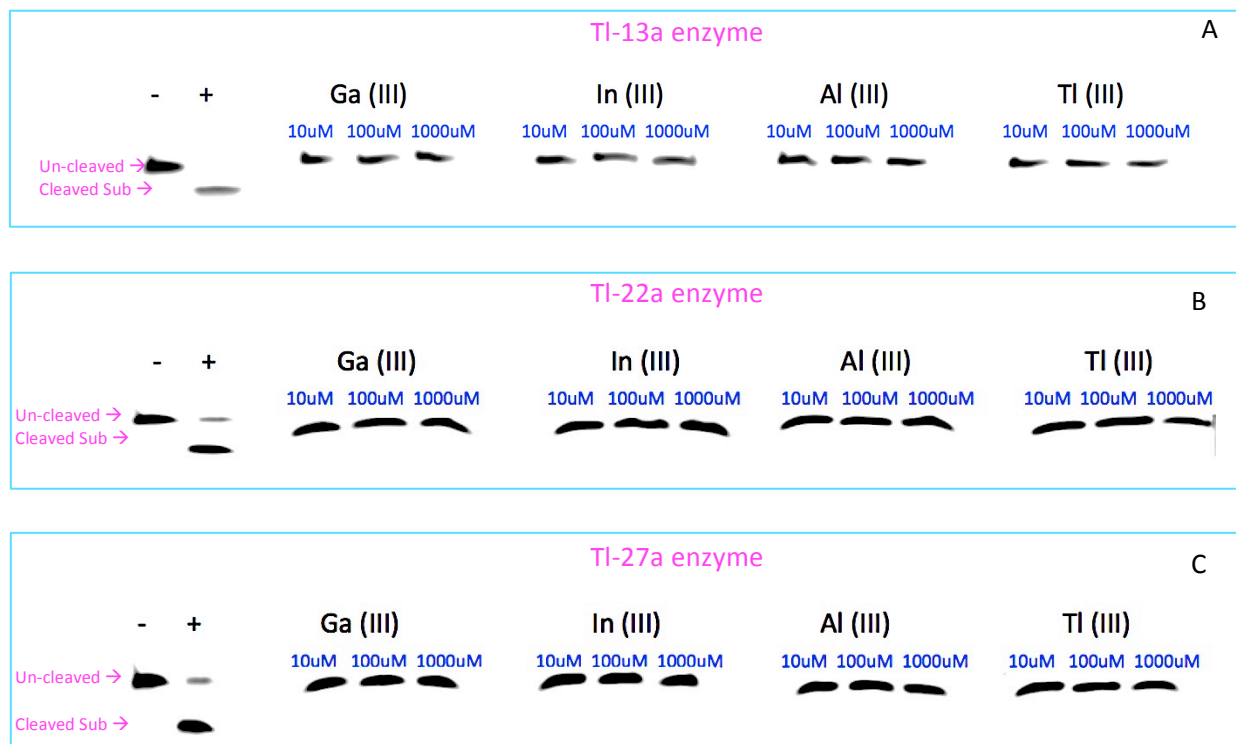


Figure 4.9 The DNAzymes' activity with different concentrations of group 3A metal ions. The activities of the Tl-13a, Tl22a, and Tl27a with four group 3A metal ions are indicated in figure a, b and c, respectively.

Thallium was previously shown to act as a transition metal ion,³⁵⁹ and is thought to have a better connection with soft ligands such as thiol. Hence, activities of the enzymes were reassessed with phosphothioate-modified RNA substrate (PS-modified sub), and tested with two

various concentrations of Tl^{3+} (10 and 100 μM). In the PS-sub, one of the non-bridging oxygen at the cleavage site is substituted with sulfur, and thus Tl^{3+} was expected to bind to this substrate more tightly. For the comparison, activities of enzymes with the normal (PO) substrate are also shown. The enzyme-substrate with no metal ion was included as the control for the assay. Results are indicated in Figure 4.10. Compared to the negative control (no metal added), the enzymes did not indicate significant Tl^{3+} -dependent activities with either PO or PS in 1hr incubation time.

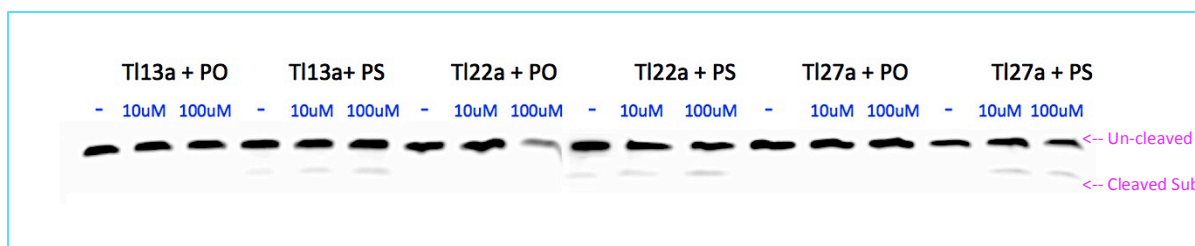


Figure 4.10 Enzymes activities with the PO and the PS substrate, in the presence of various Tl^{3+} concentrations. The (-) shows the activity of the complex in the absence of Tl^{3+} metal ion.

4.2.3.2 Activity with other metal ions

The three enzymes demonstrated no activities with group 3A metal ions. It is possible that during the incubation time, these metal ions were extensively hydrolyzed and could not interact with the complex. Thus, enzymes were also tested with various metal ions, including Mg^{2+} and Pb^{2+} . DNAzyme complexes were incubated with the individual metal ion for 1 hr. Again, no activity was observed for any of the selected DNAzymes. The results are shown in Figure 4.11A,B for 10 μM and 500 μM of different metal ions, respectively.

As described in chapter one (Introduction), Mg^{2+} is known as an effective co-factor for the activity of different ribozymes and DNAzymes. The Pb^{2+} divalent metal ion is yet another

effective co-factor, and is known to be effective for RNA-hydrolysis. While the enzymes did not show any activities with Mg^{2+} metal ion, only trace amounts of the activities were observed at a high concentration of Pb^{2+} for all the three enzymes.

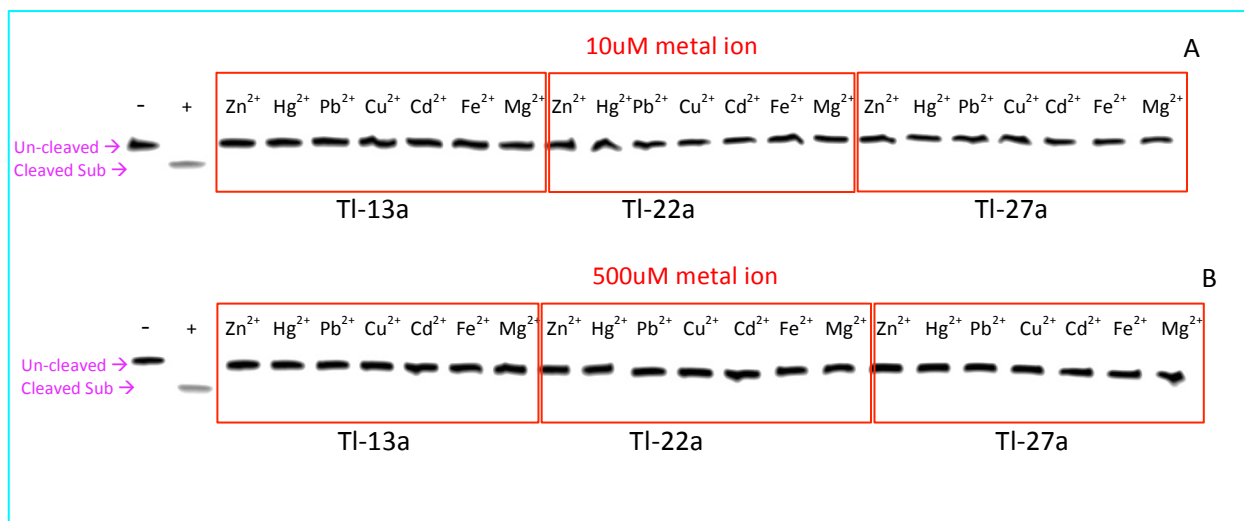


Figure 4.11 Enzymes activities in the presence of different metal ions, with two concentrations, 10uM (A) and 500uM (B).

4.3 Discussion

There are several possibilities that can explain why the selection of the group 3A metal ions did not work. The pK_a is an important parameter in the DNA hydrolysis event. Unlike lanthanides, group 3A metal ions have very low first pK_a of bound water and are very strong acids. In the aqueous solutions, these metal ions are hydrolyzed extensively, and hence, they cannot have effective roles in the hydrolysis. But, as also previously mentioned, lanthanides appear to have an optimal pK_a of bound water, and are thus ideal co-factors for the hydrolysis mechanism. In addition, group 3A metal ions do not have tendencies for binding to the phosphate groups of DNA molecules.³⁵⁵ While the primary requirement for a catalyst is to bind to the phosphate groups to be involved in the mechanism and facilitate the hydrolysis.

Accordingly, group 3A metal ions are not able to perform this role, efficiently. Instead, they may bind to non-specific sites on the DNA molecule and do not let the DNA form the efficient structures. Further studies are required to confirm these hypotheses. To test the hypothesis, a phosphorothioate modified DNA library (PS-modified RNA substrate) was used and the effect of this modification on improving the group 3A-dependent *in vitro* selection was studied.

4.3.1 Using phosphorothioate modified library for Tl^{3+} selection

As mentioned, we assumed that the failure in the *in vitro* selection with group 3A elements is because these metals do not bind phosphate tightly, or they may interact with non-specific sites in the DNA and destabilize the DNA structure. Thus, we thought of introducing a PS modification in our library. In the modified DNA library (PS), one of the non-bridging oxygen atoms in the phosphate backbone is substituted by sulfur. Using the PS-modified library, our lab successfully obtained a new DNAzyme that works specifically with Cd^{2+} .²⁰⁴ We have previously performed group 3A-dependent selection with an N50 DNA library. This time, the N35-PS DNA library was used to select more specific sequences and avoid isolating the same sequences. Out of the group 3A metals, we are mostly interested in Tl^{3+} due to its strong toxicity. Hence, the *in vitro* selection was carried out with the N35 PS-modified library in the presence of the Tl^{3+} metal ion (Figure 4.12). The modified library is thought to be a better choice for Tl^{3+} -dependent DNAzyme selection, since this metal has a higher affinity towards the sulfur compared to the oxygen. Compared to the normal DNA, the PS-DNA is suggested to have better stability against nuclease degradation.³⁶⁰ However, the PS-substrate shows some non-specific cleavage with Tl^{3+} , that is, a simple mixing of the PS-modified library with Tl^{3+} induced ~16% cleavage.³⁶¹ We initially thought that a population of it might be non-specifically cleaved (e.g., each PS modification brings in two diastereomers and one of them might be cleaved but the other

one was retained). Therefore, the library was initially treated with Hg^{2+} (with also strong thiophilicity) for a period of time. The non-cleaved population was harvested and used for the Tl^{3+} -dependent selection.

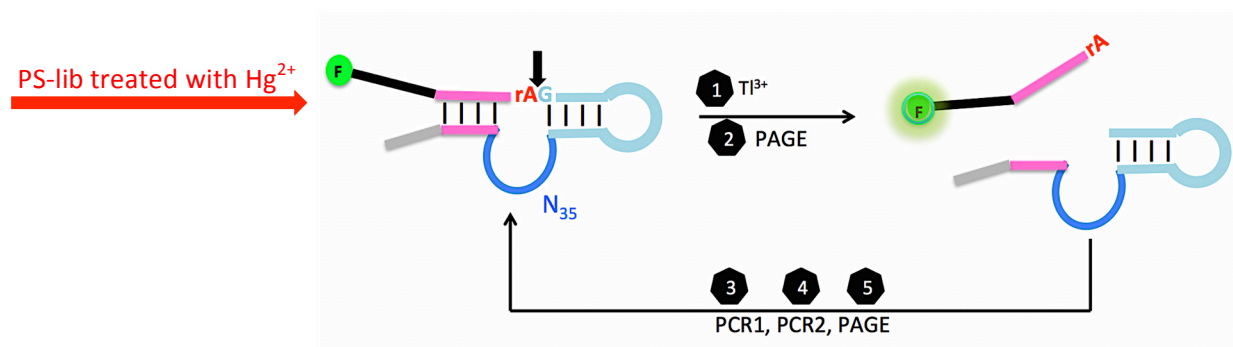


Figure 4.12 The *in vitro* selection process using the PS-modified RNA library. In order to eliminate the non-specifically cleaving sequences, the library was treated with Hg^{2+} prior to the selection.

After performing ten rounds of *in vitro* selection, in round eleven, the specific activities of selected sequences were tested toward the Tl^{3+} metal ion. The results, however, indicated the amount of cleavages in the presence and absence of metal ion were almost the same (Figure 4.13A, B). It seems the PS-modified DNA library is cleaved spontaneously, and the observed activity is not specific to the Tl^{3+} metal ion.

Although after more than ten rounds of selection with the Tl^{3+} metal ion, the library was enriched with active DNA sequences, but the selected active sequences were not sequenced or quantified. Our goal was to select the DNazymes with specific activities in the presence of group 3A (particularly Tl^{3+}), while the activity observed was not corresponding to the Tl^{3+} metal ion.

Later, by reading the literature, we learned that PS-modified RNA linkages can be cleaved in the presence of strongly thiophilic metals such as Hg^{2+} , and the uncleaved fraction undergoes desulfurization to become the normal PO linkage.³⁶² Therefore, our Hg^{2+} treated PS library was actually converted to the PO library, and the selection was again performed on the PO system. A later study by a colleague indicated that, after the Tl^{3+} treatment, the PS substrate is either cleaved or desulfurized back to the PO substrate,³⁶³ in a way similar to Hg^{2+} .³⁶¹ This is the reason that the selection using the modified library did not work. Apparently, after incubating the library with Tl^{3+} , we were actually doing the PO selection again (Figure 4.13C). This recent study indicated that while Tl^{3+} desulfurizes the PS substrate to the normal substrate, it activates the substrate to be cleaved by the Tm7 enzyme, which is an Er^{3+} -dependent RNA-cleaving DNzyme.³⁶³ The described system is been engineered into a catalytic beacon for the detection of Tl^{3+} metal ion.

Table 4.7 Tl^{3+} dependent selection conditions and progress, using the PS-modified library.

round #	$[\text{Tl}^{3+}]$ (μM)	incubation time (min)	cleavage (%)	
			Negative control (no metal)	Hg-treated N35 PS library
1	50	60	NA	0.1
2	50	60	NA	0.1
3	50	60	NA	0.1
4	50	60	NA	0.3
5	50	60	NA	0.1
6	100	60	NA	0.2
7	100	120	NA	0.1
8	100	120	NA	0.2
9	100	120	NA	0.2
10	100	30	NA	6.4
11	100	15	12.9	11

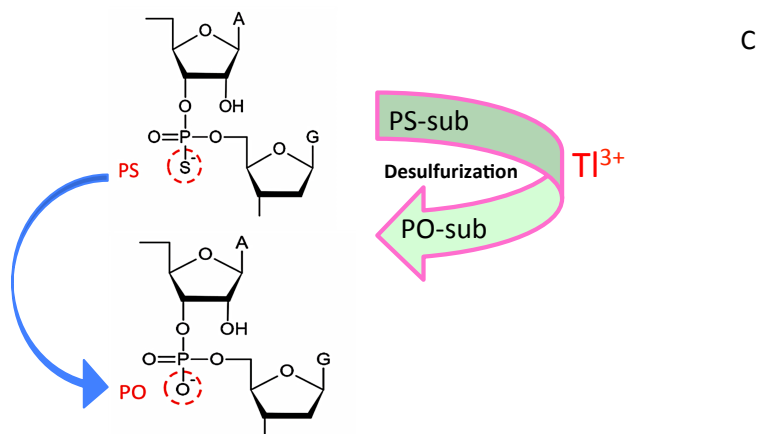
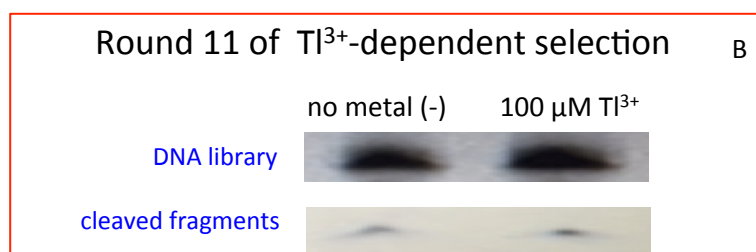
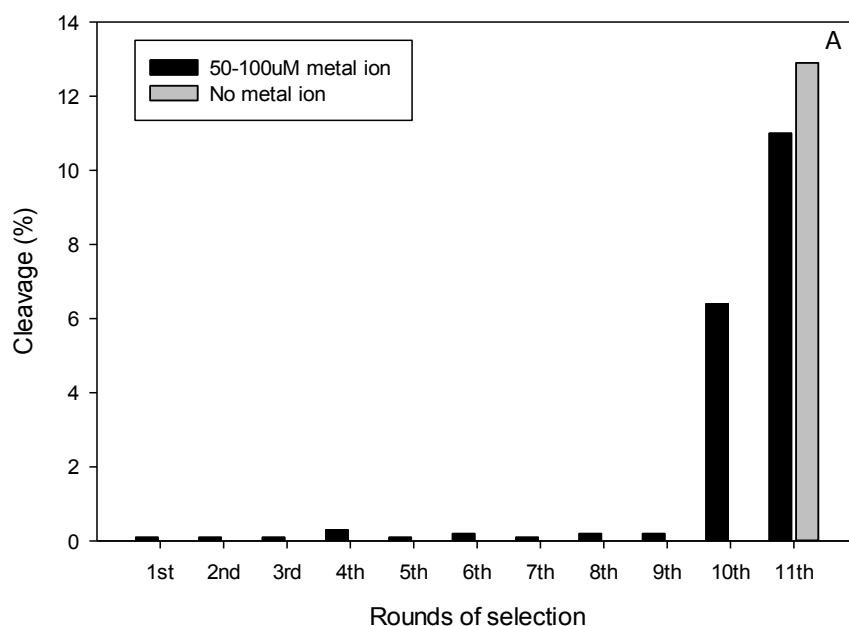


Figure 4.13 The Tl^{3+} -dependent selection using Hg-treated N35 PS library. The summary of eleven rounds of *in vitro* selection with Tl^{3+} , using the PS-library (A), last round of selection with and without Tl^{3+} metal ion (B), PS-substrate desulfurization after incubating with Tl^{3+} (C).

4.4 Summary

My efforts toward the isolation of DNAzymes specific to the group 3A metal ions using *in vitro* selection are reported in this chapter. Four independent selections were carried out with Ga^{3+} , In^{3+} , Al^{3+} , and Tl^{3+} , respectively, using the N50 DNA library. The resulting sequences were aligned into four sequence families. The secondary structure analysis indicated that most of the selected sequences could not fold into the typical DNAzyme structure, and only a few sequences were able to adopt the proper structure. Among those sequences, some of them were similar to the Cel3d enzyme. Only three new potential DNAzymes were identified that did not show significant activities with any of group 3A metal ions or other divalent and trivalent metal ions. The possible reasons for the failure of the *in vitro* selection with the group 3A metal ions were discussed, which are mainly the very low pK_a of these metal ions in aqueous solution, as well as the inability of these metals to tightly bind to phosphate group of the DNA molecule. The Tl^{3+} -dependent selection was repeated with a PS-modified library, but the selection still did not work since Tl^{3+} can desulfurize the substrate back to the normal PO substrate.

4.5 Materials and methods

4.5.1 Chemicals

The list of chemicals used for the *in vitro* selection, PCR, and the activity assay are the same as previously described in chapter 2, section 2.4.1. The metal salts with ultra-high purity (99.999% purity), were used for the selections. The *trans*-cleaving enzyme strands were purchased from Eurofins (Huntsville, AL). Sequences of the enzyme strands are indicated in Figure 4.8B.

4.5.2 *In vitro* selection

The processes of the selection and PCR are the same as previously described in chapter 2. The conditions for different selections done in this chapter are summarized in Tables 4.1, 4.2, 4.3, 4.4, and 4.6.

4.5.3 Cloning and Sequencing

The *in vitro* selections using the N50 library were stopped after 5 rounds for Ga^{3+} , In^{3+} , and Al^{3+} , and after 10 rounds for Tl^{3+} . The PCR1 product from each *in vitro* selection was then cloned. The cloning and sequencing analysis are described in chapter 2.

4.5.4 Activity assay

To test the activity of the individual enzyme-sub complex, a final concentration of 0.7 μM of the FAM-labeled substrate strand and 1.1 μM of each enzyme were annealed in a buffer containing 25 mM NaCl and 50 mM MES, pH 6. The reaction was initiated by adding a specific concentration of a metal ion to 5 μL of 1 μM the enzyme-sub complex in the former buffer. After 1 h incubation time, samples were quenched by 8 M urea. The products were then separated using 15% denaturing polyacrylamide gel and quantified by a Bio-Rad ChemiDoc MP imaging system.

Chapter 5. *In vitro* selection of Cr³⁺-dependent DNazymes and studying Cr³⁺ cleaving the Ce13d DNzyme

5.1 Introduction

Chromium Cr³⁺ ion is a well-known mutagen and carcinogen.³⁶⁴ It is a bio-accumulative metal ion that cannot be disposed by the body, and thus, can amplify the tissue damage.^{365, 366} Cr³⁺ usually forms complexes with negatively charged phosphate groups.³⁶⁶ It has been suggested that the interactions of Cr³⁺ with nucleic acids can induce mutational damages into human cells.³⁶⁶ In addition, recent studies indicated that Cr³⁺ inhibits some enzymes and inactivates important cellular pathways. Hence, it is necessary to always monitor the Cr³⁺ levels in the environment. Available instrumental analytical methods include inductively coupled plasma (ICP), atomic emission spectrometry (AES),²⁷⁰ flame atomic absorption spectrometry (FAAS),²⁷¹ chromatography,²⁷² fluorometry,²⁷³⁻²⁷⁶, and electrochemical methods.^{277, 278} These methods usually need sophisticated laboratory equipment and are very time-consuming. DNazymes may provide a simple detection method for trivalent chromium, as they do not need expensive, laborious, or sophisticated laboratory equipment.

Many DNazymes have been isolated thus far using the *in vitro* selection method,^{20-31, 367} and they all display metal-dependent catalytic activities. Many of the selected DNazymes have been converted into biosensors for detecting specific metal ions.¹⁹⁸⁻²⁰³ In this chapter, chromium (Cr³⁺) is the intended target metal ion for the *in vitro* selection.

The chromium chloride salt, [CrCl₂(H₂O)₄]Cl, is a dark green complex. Freshly prepared solution of chromium chloride is green and after aging in water, it becomes violet in color. This is attributable to the substitution of chloride by water in the inner coordination sphere.

DNA interaction with chromium has been studied previously. The two most stable forms of chromium are Cr^{6+} and Cr^{3+} . Cr^{6+} reacts with intracellular materials to reduce to the Cr^{3+} form, which binds DNA. Cr^{3+} binds DNA via guanine N_7 and the nearest phosphate group.³⁶⁵ At high Cr^{3+} concentrations, DNA condensation occurs. However, no major alteration of DNA conformation was observed, and DNA biopolymer seems to remain in the B form structure.³⁶⁵ Studying single-strand DNA suggested that Cr^{3+} bound in a dose-dependent manner to the template DNA and prevents DNA replication.³⁶⁸ Recently, Cr^{3+} was shown to cause mutational damages in shuttle vector systems replicated in human cells.³⁶⁹ The interaction of Cr^{3+} with DNA may be responsible for the carcinogenic activity of chromium.

Here, to better understand the selection and the interaction between Cr^{3+} and DNA, we carried out studies on the cleavage of a previously selected DNAzyme, Ce13d, by Cr^{3+} . This preliminary study could give us information about the condition for efficient activity of Cr^{3+} . Using the above information, we conducted a Cr^{3+} -dependent *in vitro* selection to isolate a Cr^{3+} -specific DNAzyme.

5.2 Results and discussion

5.2.1 Studying Cr^{3+} cleaving the Ce13d DNAzyme

Previous *in vitro* selection using Cr^{3+} by another member of the Liu lab resulted in the Ce13d DNAzyme, which is active with lanthanide ions. Preliminary assays indicated that Ce13d is also moderately active with Cr^{3+} . So, Ce13d provides a molecule for studying the interaction between Cr^{3+} and DNA. I used Ce13d to do the primary study to gain better understanding about this metal ion before starting a new Cr^{3+} selection. It is known that in water, the Cr^{3+} metal ion forms different complexes other than the initial chromium (III) chloride hydrate,

$[\text{CrCl}_2(\text{H}_2\text{O})_4]\text{Cl}$. As a possible factor affecting the selection, the activities of different Cr^{3+} complexes (produced from the primary Cr^{3+} compound) were also studied and compared.

5.2.1.1 Comparing the activities of different Cr^{3+} solutions

Cr^{3+} ions tend to form octahedral complexes. The colors of these complexes are different, and are determined by the ligands attached to the Cr^{3+} . For example, the commercially available chromium chloride hydrate, $[\text{CrCl}_2(\text{H}_2\text{O})_4]\text{Cl}$, is a dark green complex. After dissolving it in water, the green solution gradually turns to violet. This is due to the substitution of chloride by water in the inner coordination sphere. The related compounds have different colors, such as pale green, $[\text{CrCl}(\text{H}_2\text{O})_5]\text{Cl}_2$ and violet, $[\text{Cr}(\text{H}_2\text{O})_6]\text{Cl}_3$. This kind of reaction is also observed with other water-soluble Cr^{3+} salts (e.g., chrome alum).

In Figure 5.1A, different Cr^{3+} solutions are shown, which are made by dissolving the chromium chloride salts, $[\text{CrCl}_2(\text{H}_2\text{O})_4]\text{Cl}$, in water over a period of three months. Cr^{3+} complexes may have different functionalities in terms of activating a DNAzyme to catalyze RNA hydrolysis. The activities of the Cr^{3+} complexes were evaluated using the Ce13d DNAzyme. The activity detected by each Cr^{3+} complex is indicated in Figure 5.1B, below the corresponding solution. The negative control sample is the Ce13d enzyme-sub complex with no metal ion added. Rates are also calculated for different solutions, based on 1 h incubation time (Figure 5.1C).

As shown, these Cr^{3+} complexes induced almost the same activity. We can conclude that these closely related complexes, ($[\text{CrCl}_2(\text{H}_2\text{O})_4]\text{Cl}$, $[\text{CrCl}(\text{H}_2\text{O})_5]\text{Cl}_2$ and $[\text{Cr}(\text{H}_2\text{O})_6]\text{Cl}_3$), have very similar activity in the catalysis. Cr^{3+} has very slow ligand exchange rate, and it is likely that

the metal exerts its role in catalysis based on its charge and outer sphere interaction with the DNA.

Thus, converting the initial Cr^{3+} complex to the other related Cr^{3+} complexes seems not to be an important factor in the selection, but for the consistency it is suggested to use the fresh Cr^{3+} solution.

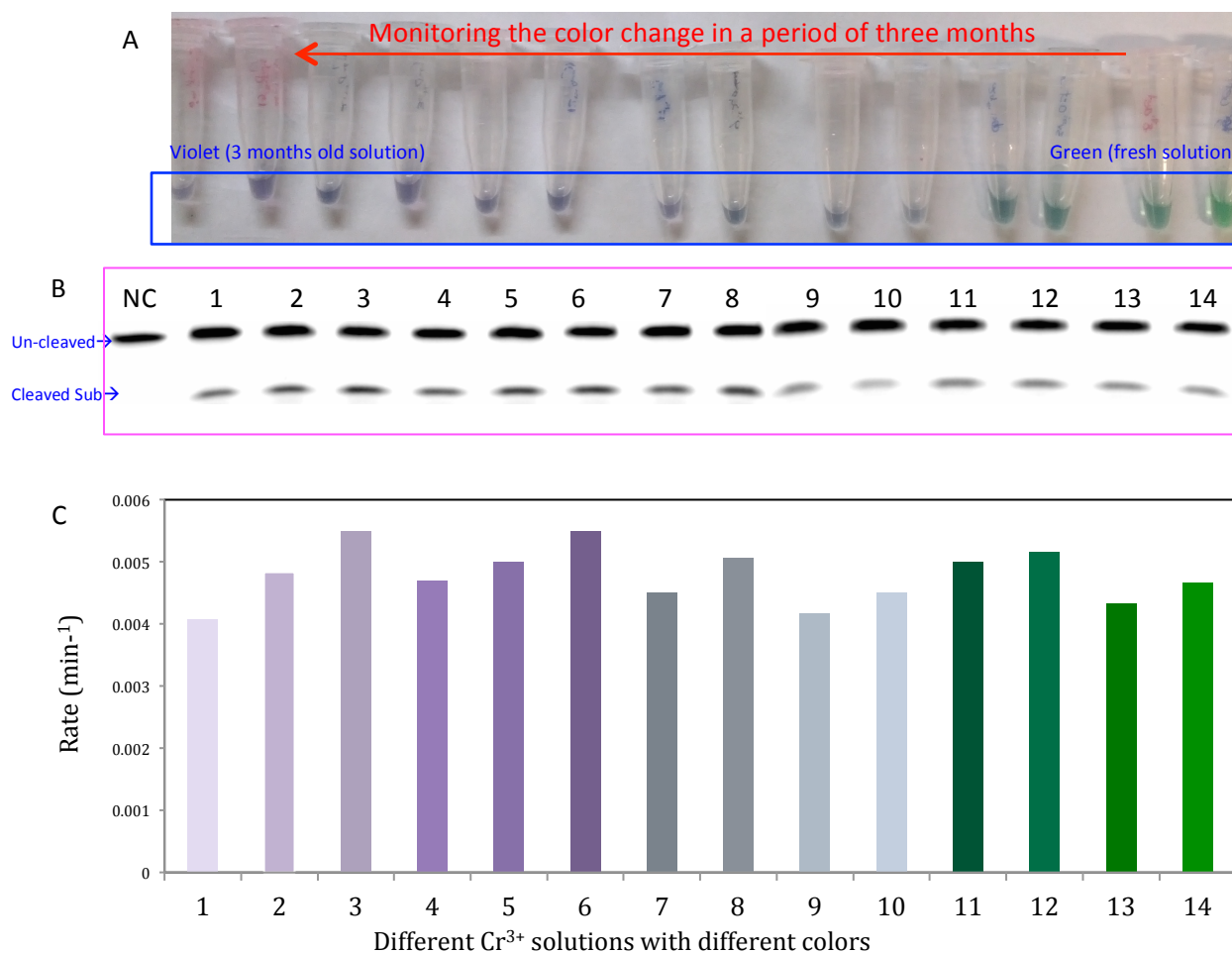


Figure 5.1 Ce13d enzyme activity with different Cr^{3+} complexes. (A) Various Cr^{3+} solutions with different colors. (B) Activity of the enzyme with 10 μM of different Cr^{3+} solutions in one-hour incubation time. (C) The calculated rates for the corresponding samples.

5.2.1.2 Metal concentration-dependent study

Optimizing metal ion concentration is a key step in the *in vitro* selection experiment, since a low metal concentration cannot induce the necessary activity, and a high concentration may disrupt the DNA secondary structure. The optimal concentration may vary for the DNAzyme selection using different metal ions. For example, trivalent lanthanides have very strong affinities with DNA phosphate and bases, and the inhibition can start at low concentrations ($\sim 10\ \mu\text{M}$).³¹¹ Here, a careful study was performed with various concentrations of Cr^{3+} , using Ce13d DNAzyme.

The Ce13d activity at various concentrations of Cr^{3+} was measured and the corresponding rates were calculated. The results are shown in Figure 5.2. The DNAzyme complex with no metal ion was the control, which did not show any noticeable activity after 1 h incubation. At low Cr^{3+} concentrations, the cleavage rate increased with an increase in Cr^{3+} concentration. The cleavage activity of Ce13d was at the maximum with $100\ \mu\text{M}\ \text{Cr}^{3+}$, where the cleavage reached $\sim 36\%$. We decided that $50\ \mu\text{M}$ was the optimal concentration (with $50\ \mu\text{M}\ \text{Cr}^{3+}$ cleavage is $\sim 33\%$), since greater than $50\ \mu\text{M}$ of Cr^{3+} appears to non-specifically bind DNA and retard their mobility in gel. For example, the DNA band completely disappeared with $1\ \text{mM}$ ($1000\ \mu\text{M}$) Cr^{3+} .

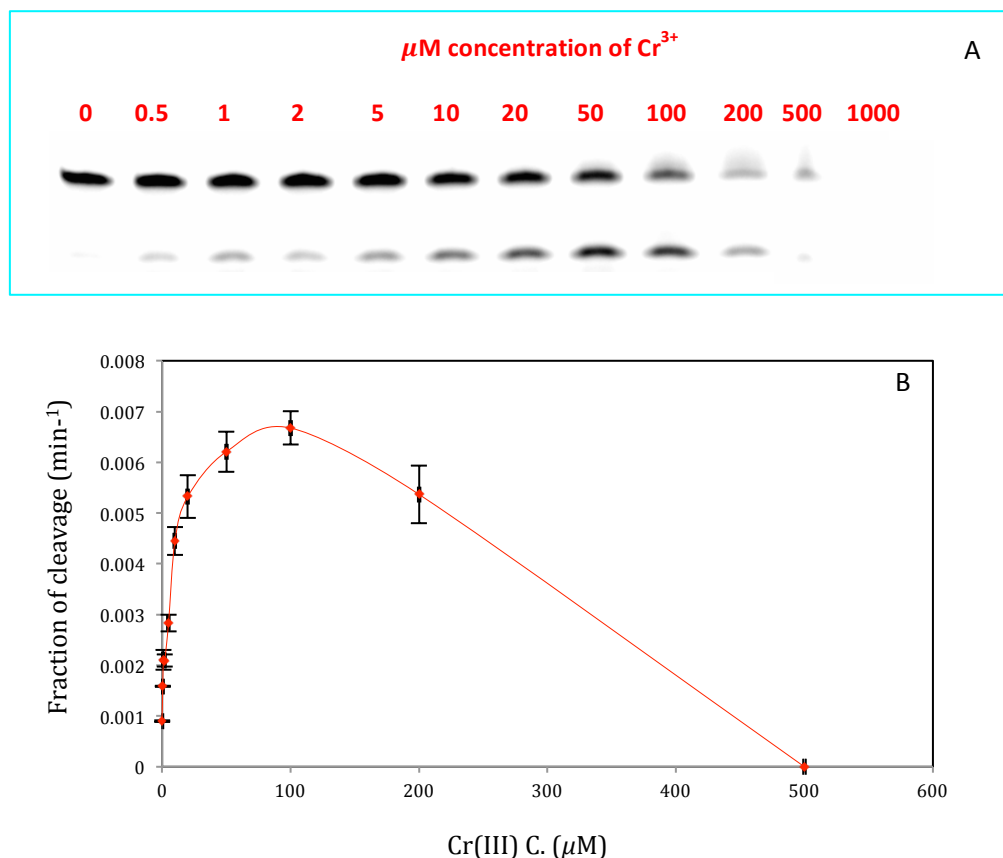


Figure 5.2 Cel3d DNase activity with various concentrations of Cr^{3+} (μM). (A) The cleavage activity of Cel3d with different Cr^{3+} concentrations, and (B) the calculated rates in 1hr incubation time are shown.

Compared to other trivalent metal ions that we tested (e.g., lanthanides and group 3A metals), Cr^{3+} seems to have a moderate affinity toward DNA molecules. At 50 μM , Cr^{3+} did not disturb the DNA structure or inhibit the hydrolysis, while lower concentrations of a trivalent lanthanide can strongly inhibit the catalysis.³¹¹ On other hand, Cr^{3+} has higher affinity compared to group 3A metal ions which do not have significant interactions with nucleic acids at neutral pH.³⁶³

5.2.1.3 pH-dependent study

Here, we studied the activity of Cr^{3+} as a function of pH, using the Ce13d enzyme. pH is an important parameter in metal dependent-DNAzymes. In general, a higher pH promotes RNA cleavage due to the deprotonation of the 2'-OH group as a better nucleophile. At a very low pH, hybridization does not occur, and thus, no DNAzymes complexes can be formed. Also, at a very high pH, the metal ions are extensively hydrolyzed and precipitation happens. In this study, the activity was measured at three different moderate pHs, 5.2, 6.2 and 7.2, and the corresponding cleavage rates are represented in Figure 5.4A, B. Usually, at higher pH, the rate is faster and this is also consistent with our results for Cr^{3+} cleaving the Ce13d enzyme. The cleavage rate measured at pH 7.2 is ~1.7-fold higher than at pH 6.2, and ~ 2.4-fold higher than the rate at 5.2. If the pH effect is used only for activation of the 2'-OH, we would expect a 10-fold increase in rate for every pH unit increase. Here, the rate increase was much less, and it is possible that the hydrolysis of Cr^{3+} at higher pH has decreased its effective concentration as a free ion. The combination of these two effects has led to the current results. Overall, Cr^{3+} can be used in the pH range between 5.2 and 7.2.

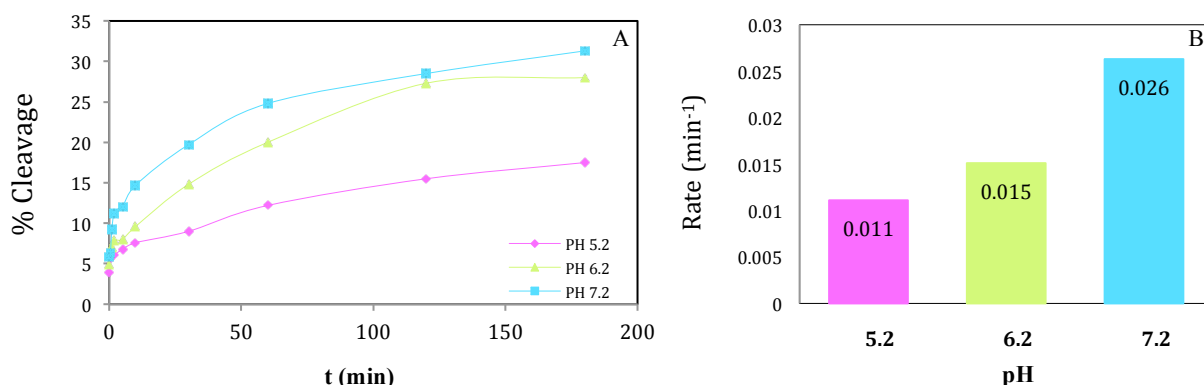


Figure 5.4 The Ce13d activity induced by Cr^{3+} , at different pHs. The percentage of cleavage at different time points (A), and corresponding rates (B) for three different pHs, 5.2, 6.2, and 7.2, are demonstrated.

5.2.1.4 Temperature-dependent assay

Each enzyme has a temperature range in which a maximal rate of reaction is achieved. This maximum is known as the temperature optimum of the enzyme. Note that the temperature optimum of each enzyme is different. Usually, enzymes are inactive at very low temperatures. Very high temperatures can also have negative effects on the enzymes structures and can inhibit their efficient activity. We usually perform *in vitro* selection at room temperature, which seems to be optimal for most of the DNAzymes. Here, to have a better understanding about the activity of Cr^{3+} in the hydrolysis, its function was studied at three different temperatures. At all these temperatures the enzyme is active, and is the matter of comparison to find the optimum activity.

The Ce13d activity induced by Cr^{3+} at three different temperatures, 4 °C, 22 °C (or room temperature) and 37 °C (or body temperature), was monitored. The cleavage rates are demonstrated in Figure 5.5. As shown, cleavage rate increases from 4 °C to 22 °C, but increasing temperature afterwards does not improve the activity rate. Therefore, room temperature is optimal for Cr^{3+} activity, and can be used for the Cr^{3+} selection.

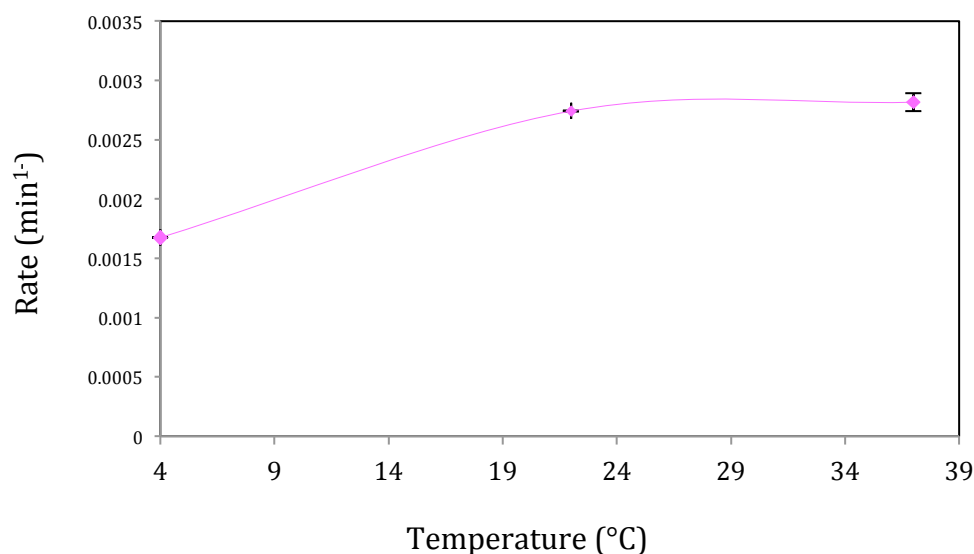


Figure 5.5 The effect of temperature on the activity of Cr^{3+} cleaving Ce13d enzyme.

5.2.1.5 Salt concentration-dependent assay

Enzymes typically require some salts for their efficient activities. The presence of salt usually affects the conformation, folding, stability, and activities of the protein and DNA enzymes. However, high concentrations of any salt decreases water activity, and that may reduce enzyme activity in turn. In addition, too much salt can also disturb the enzyme's secondary structure and inhibit enzyme activity. Thus, it is essential to find the optimal salt concentration for the activity of different enzymes. The most common salt is NaCl, because of its compatible ions. Here, the activity of Ce13d enzyme with different concentrations of NaCl was tested. For the comparison, the activity was also studied with different amounts of NaNO_3 . The Cr^{3+} -dependent activity of Ce13d enzyme with various concentrations of NaCl and NaNO_3 are indicated in Figure 5.6.

As shown, the lowest 25 mM of each salt appears to be sufficient and optimum for the DNAzyme activity. Higher salt concentration inhibited the activity. DNAzyme hybridization is facilitated by salt and thus the inhibited activity cannot be attributed to the loss of DNAzyme structure. We reason that at higher salt concentrations, the electrostatic interaction between Cr^{3+} and DNA is screened, or the salt can compete with Cr^{3+} for DNA binding, leading to decreased activity.

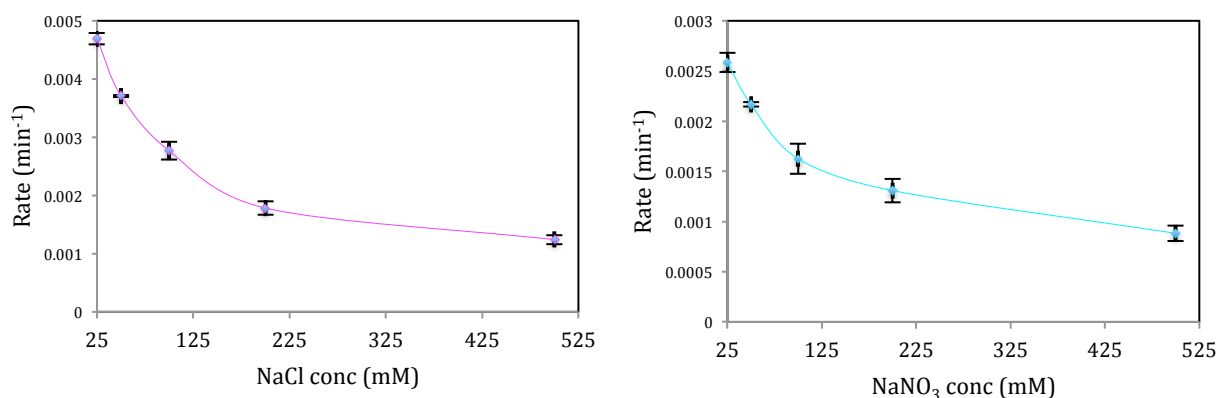


Figure 5.6 Salt-dependent activity of Ce13d induced by Cr^{3+} . The Ce13d DNAzyme activity with various concentrations of each of the NaCl and NaNO_3 salts is shown.

In the preceding sections, the best condition for the activity of Cr^{3+} was found. This information is used to help selecting a specific DNAzyme for Cr^{3+} .

5.2.2 Cr^{3+} -dependent *in vitro* selection

As mentioned in the introduction, the ultimate goal of this chapter is to isolate an RNA-cleaving DNAzyme with specific activity toward Cr^{3+} , which can turn into a selective and sensitive biosensor. Efforts toward isolating Cr^{3+} -dependent DNAzyme, using a modified selection method, will be described in the following sections.

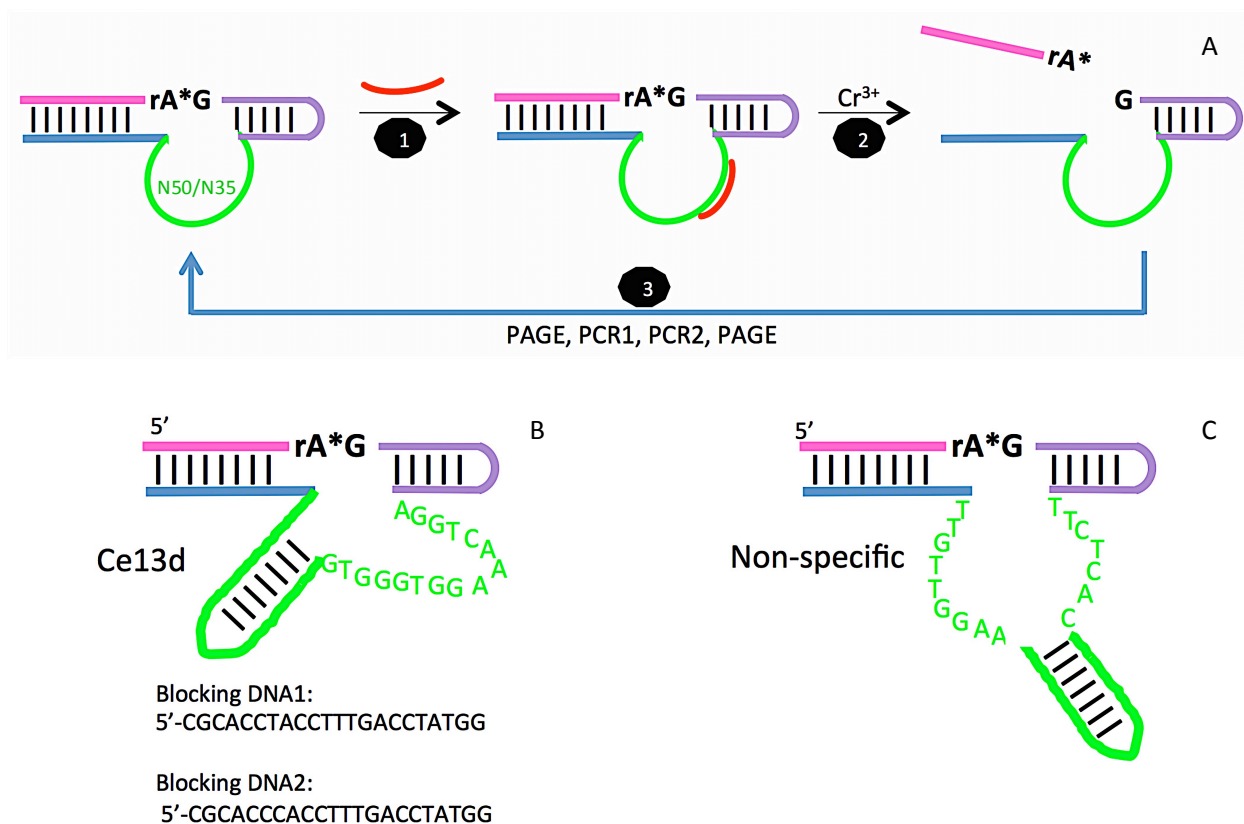


Figure 5.7 (A) Scheme represents the blocked *in vitro* selection method. In each round of selection, prior to the incubation step, Ce13d sequence is blocked in the random region of the DNA library (1). The library is incubated with the target metal ion to induce the cleavage (2). The cleaved sequences containing the random nucleotides are used to produce the library for the next round of selection, and are separated from the rest of sequences by running on the poly acrylamide gel (3). Figures (B) and (C) show the Ce13d and non-specific DNAzymes. The nucleotides in the catalytic core are shown in green. The Ce13d blocking DNAs, which are used to inactivate Ce13d sequence in the selection, are also indicated.

5.2.2.1 Ce13d blocked selection using N50 library

Recently, a Cr^{3+} -dependent selection was carried out in our lab by a colleague, which mainly resulted in isolating the known Ce13d sequence (unpublished data). Ce13d is only moderately active with Cr^{3+} , but is highly active with all trivalent lanthanides, Y^{3+} , and to a lesser extent with Pb^{2+} . Here, I aim to obtain new DNAzymes with better selectivity for Cr^{3+} .

Our group has recently developed a new blocking method. Using this method, a specific Cd^{2+} -dependent DNAzyme was obtained.³¹⁵ In that work, to eliminate the Ce13d sequence, a DNA complementary to the Ce13d catalytic core was used to inactivate that population. Since Ce13d has a long stretch of highly conserved nucleotides, its activity can be inhibited by adding an oligonucleotide complementary to this highly conserved sequence. The selection progress using this method is shown in Figure 5.7A.

Accordingly, the *in vitro* selection with Cr^{3+} was carried out using a DNA library containing 50 random nucleotides (N50), and prior to each round of selection, blocking was accomplished by hybridizing Ce13d complementary sequences to the DNA library. The two blocking DNA sequences used in the assay are represented in Figure 5.7B. The blocked library was incubated with Cr^{3+} to induce cleavages and then the cleaved fragments were separated by PAGE to produce the library for the next round. Cleavage site was a single riboadenosine (rA) nucleotide, which is known to be less stable compared to the rest of nucleotides in the library.³⁵⁷ The un-blocked library was included as the control for the assay, and the two independent un-blocked and the Ce13d blocked selections were done side by side.

Our Cr^{3+} -dependent study with the Ce13d enzyme suggested the optimal metal ion concentration ($50\ \mu\text{M}$) for the selection. Thus, initially $50\ \mu\text{M}$ of Cr^{3+} metal ion was used, which decreased to $20\ \mu\text{M}$ in round three and four to select the most active sequences (Table 5.1). After conducting four rounds, the activity of blocked library reached $\sim 42.2\%$. The selection is usually stopped when the activity reaches to 30-40 %. But, to ensure observed activity is specific to the Cr^{3+} metal ion, a round of negative selection (selection with no metal ion) was also conducted. While, as indicated in Figure 5.8, the amount of cleavages with Ce13d blocked-library in round four (with $20\ \mu\text{M}$) and five (with no metal ion) were almost the same. This implied the obtained

activity is not specific to Cr^{3+} metal ion. On the other hand, in each round, the blocked library indicated less activity compared to the un-blocked, which is due to the suppression of highly active Ce13d population. The result endorses that the method was successful in deleting the Ce31d sequence, while, a different strategy is needed to eliminate both the Ce13d and non-specific form the selection.

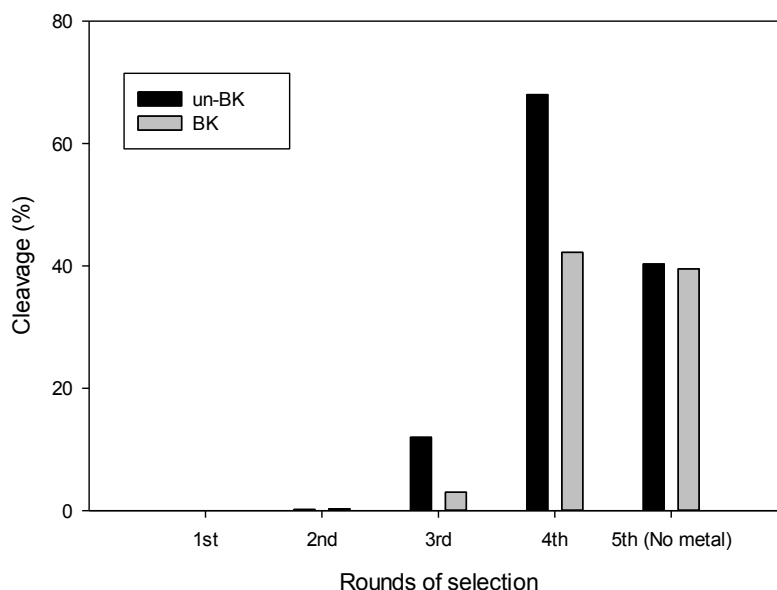


Figure 5.8 The Cr^{3+} -dependent selection, using the Ce13d blocked method.

Table 5.1 Condition and progress of Cr^{3+} selection

round #	$[\text{Cr}^{3+}]$ (μM)	incubation time (min)	cleavage (%)	
			Un-BK	BK-Gd22
1	50	60	0.1	0.1
2	50	60	0.2	0.3
3	20	60	12	3
4	20	60	68	42.2
5	No metal	60	40.3	39.5

5.2.2.2 Cr³⁺ selection using N35 library

Using a shorter library may eliminate the two DNazymes (the Ce13d and the non-specific), since they require relatively long sequences. Thus, the *in vitro* selection was repeated with a library containing 35 random nucleotides (N35). To ensure producing non-specific cleavage is not due to the contamination of the selection and extraction buffers, they were also freshly made, and new PCR supplies were used. Both the Ce13d and non-specific sequences were only selected with N50 library, and since we use the N35 library there is no need to block these sequences in the selection.

Five rounds of *in vitro* selections with Cr³⁺ were conducted and the activity of N35 library reached about 25-30 %. The specific activity of selected sequences was again tested. The library was equally divided and its activity in the presence and absence of the target metal ion was separately evaluated. The activity of library after the round six selection, with no metal ion and with 25 μ M Cr³⁺, is shown in Figure 5.9. As indicated, the amounts of hydrolysis with and without metal ion are the same, which again implies the detected activity is not specific to the presence of Cr³⁺ metal ion.

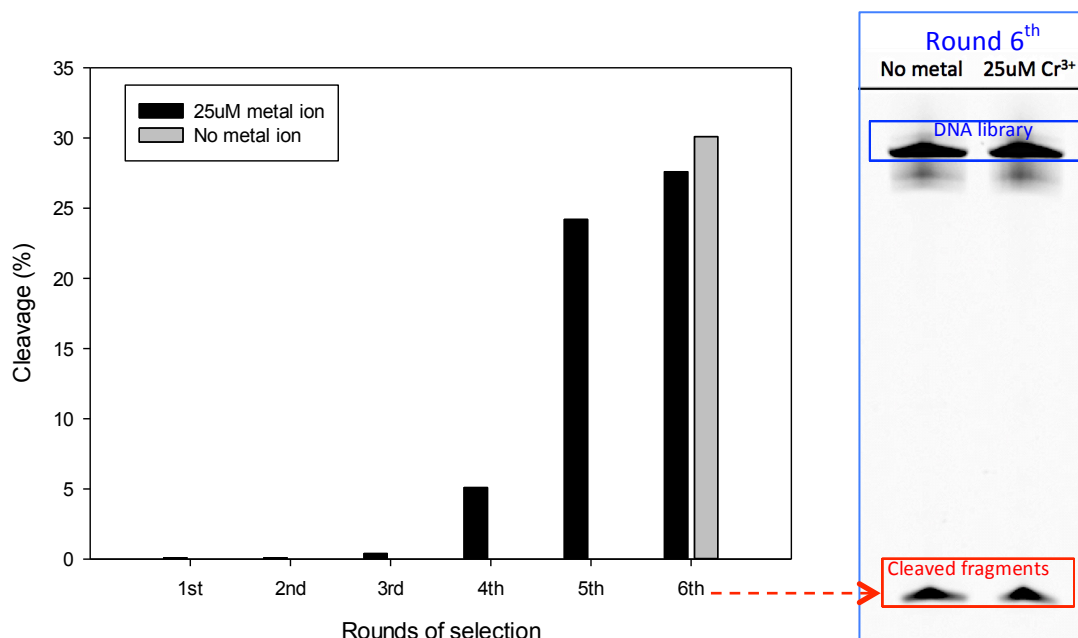


Figure 5.9 summary of the Cr^{3+} -dependent selection using N35 library. The gel shows the last round of selection with no metal ion and with $25\mu\text{M}$ metal ion.

Table 5.2 Conditions and cleavages of the Cr^{3+} *in vitro* selection, using N35 library.

round #	$[\text{Cr}^{3+}]$ (μM)	incubation time (min)	cleavage (%)	
			Negative control (no metal)	BK-non specific N35 library
1	25	60	NA	0.1
2	25	60	NA	0.1
3	25	60	NA	0.4
4	25	60	NA	5.1
5	25	60	NA	24.2
6	25	60	30.1	27.6

Apparently, changing the library to N35 could not help in eliminating the non-specific activity. Our goal was to use the *in vitro* selection to obtain a new DNAzyme with specific activity toward the Cr^{3+} metal ion. However, the selected DNA sequences showed almost the same amount of activity in the presence and absence of Cr^{3+} . It is possible that there are

sequences other than the one we previously identified, or the non-specific hydrolysis may evolve during the overnight DNA precipitation and we enriched the sequence by PCR.

The non-specific sequence previously identified with N50 library (Figure 5.7C),³⁷⁰ and it would be interesting to compare that sequence with the one(s) obtained in our selection with N35 library. Also, there is still a small chance of finding a Cr^{3+} -dependent DNAzyme among the selected sequences. Thus, we have decided to use the sequences selected in round six in the presence of Cr^{3+} , for cloning and sequencing.

5.2.3 Analyzing the selected sequences

5.2.3.1 Sequencing and DNAzyme secondary structure analysis

Deep sequencing was used to find all the possible sequences. About 40000 sequences were identified. Since it would be very hard to analyze all the sequences, only the sequence families that had the highest populations were considered. As expected, the majority (< 90%) of the selected sequences belong to the previously reported non-specific sequence family. The non-specific (also called the EtNa DNAzyme) is a Na^+ -dependent DNAzyme, and it is accelerated in organic solvents.³⁷⁰ As mentioned, this DNAzyme was previously identified in our lab through the *in vitro* selection using the N50 DNA library,³⁷⁰ and here, we have reselected it with N35 library. Interestingly, comparing the two sequences indicates that the nucleotides producing the catalytic core are completely conserved. The structure of the non-specific DNAzyme selected using the N35 library is indicated in Figure 5.10. The *trans*-cleaving structure is also shown, in which the redundant sequences are deleted and the structure is simplified. The conserved nucleotides between the two sequences are highlighted with red and the nucleotides in the catalytic core are marked with a black dashed box (Figure 5.10A).

Since our focus is on selecting a DNAzyme for the Cr^{3+} metal ion, this sequence was excluded in the following activity studies. The fact that EtNa was isolated indicates that the Cr^{3+} -dependent activity cannot compete with the background cleavage during DNA ethanol precipitation (where a high concentration of Na^+ and ethanol were used).

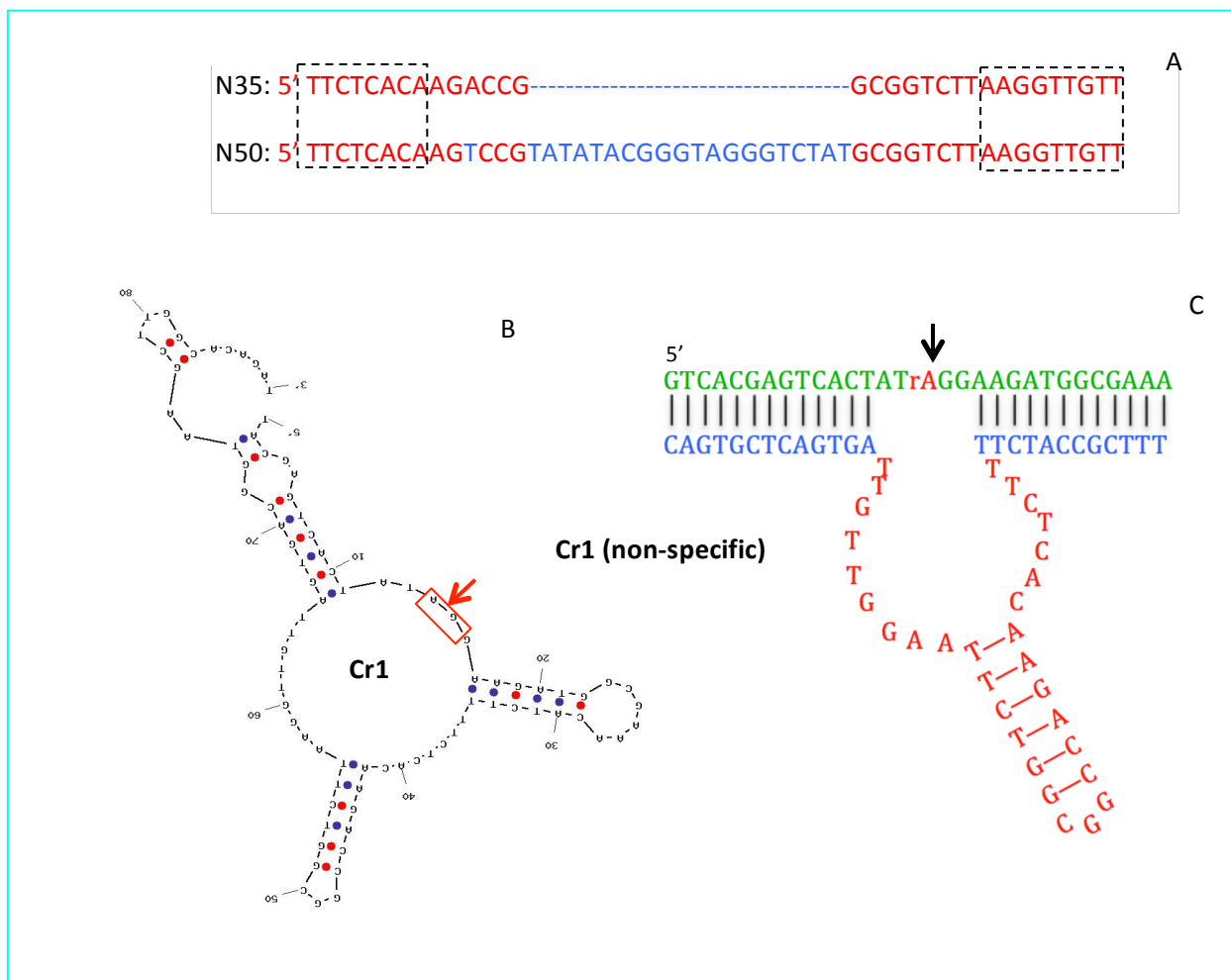


Figure 5.10 Analyzing the non-specific sequence selected in the Cr^{3+} selection. (A) The nucleotides in the catalytic core of non-specific DNAzyme selected with N50 and N35 libraries are demonstrated. Conserved nucleotides between the two sequences are marked as red. The nucleotides in the catalytic core are marked with a black dashed box. (B), (C) The *cis* and *trans*-cleaving structures of the non-specific sequence (or here called Cr1) are shown, respectively. The redundant sequences are deleted in the *trans*-cleaving structure. The cleavage site is marked with an arrow.

Other dominant sequences identified in the Cr³⁺ selection were, Cr2 (at about 2%), Cr3 (at about 2%), Cr5 (at >1%), Cr6 (at >1%) and Cr7 (at >1%), which are listed in Table 5.3. The remaining 3% included the sequences with less than 0.01% population, which was not analyzed. Cr3a sequence correspond to the previously identified 17E DNAzyme. 17E has been previously isolated in many *in vitro* selections over the past two decades.^{371, 114} The DNAzyme can activate with different metal ions, such as Mg²⁺ and Pb²⁺, but it is highly active in the presence of zinc.¹¹⁹ The 17E enzyme (or here named Cr3a) was included as a control for the following activity studies.

Table 5.3 Dominant sequences selected in the Cr³⁺-dependent selection.

Name	Sequence
Cr1	TTTCGCCATCTT TTCTCACAAGACCGGCGGTCTTAAGGTTGTT - -AGTGACTCGTGAC
Cr2	TTTCGCCATCTT GGTCTAAATCTTTTCTCAGCGAGACGAA ATAGTGACTCGTGAC
Cr2d	TTTCGCCATCTT GGTCTATCTCTCAGACGAA ATAGTGACTCGTGAC
Cr3a	TTTCGCCATCTT TTCTCAGCGAGACGAA ATAGTGACTCGTGAC
Cr5	TTTCGCCATCTT TCTTCTCTCAGCGAGACGAA ATAGTGACTCGTGAC
Cr6	TTTCGCCATCTT ATGACTAAATCTTTTCTCAGCGAGAGGAA ATAGTGACTCGTGAC
Cr6d	TTTCGCCATCTT ATGACTTCTTTTCTCAGGAA ATAGTGACTCGTGAC
Cr7	TTTCGCCATCTT GGTCTAAATCTTTTCTCAGCGAGAGGAA ATAGTGACTCGTGAC

5.2.3.2 Activities of selected DNAzymes

Using Mfold³⁵⁸ software, the secondary structures of selected sequences were predicted. The simplified *trans*-cleaving structures of these DNAzyme complexes are shown in Figure 5.11. The Cr2 and Cr6 DNAzymes have larger catalytic cores compared to other ones. Truncating the redundant sequences, in some cases, may significantly improve the activity of DNAzyme. Thus,

the truncated version of these DNazymes (named Cr2d and Cr6d) were also designed and included for the activity studies (Table 5.3).

After optimizing the sequences, each enzyme strand was hybridized with a designed substrate to produce the DNzyme complex. The activity of each individual DNzyme was evaluated based on the ability to hydrolyze a single phosphodiester bond (rA·G) present in the substrate strand. The cleavage site is marked with a black arrow (Figure 5.11).

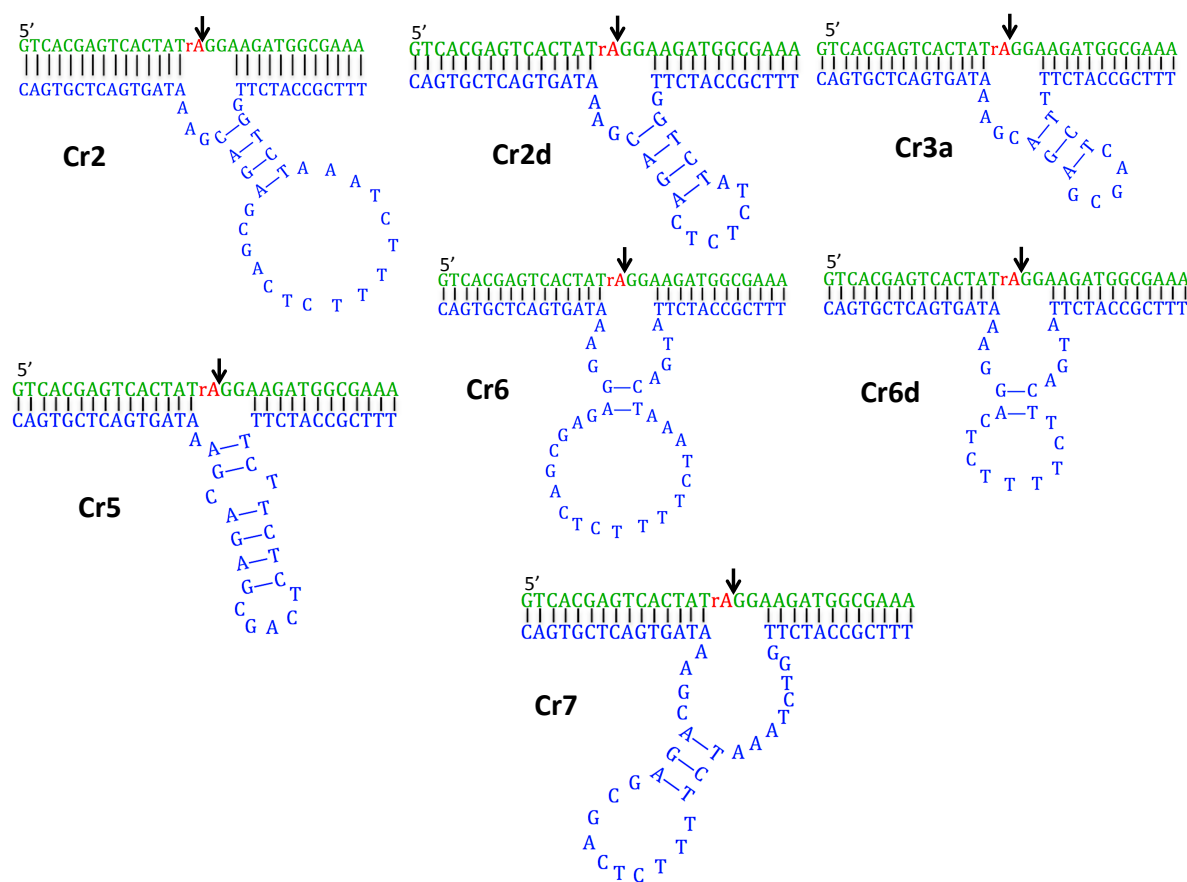


Figure 5.11 *Trans*-cleaving structures of the DNazymes selected through Cr³⁺ selection. The substrate strand is shown in green and the enzyme strand is shown in blue. The cleavage site is indicated with a black arrow.

We have hypothesized that spontaneous hydrolysis may occur during the overnight DNA precipitation, in the process of preparing the library for each round of selection. Thus, the enzyme activities were first tested in ethanol for the same period of time (overnight). For the chance of finding a specific Cr^{3+} -dependent DNase, activities were also separately tested with Cr^{3+} . However, Cr3a (or 17E) was the only DNase activate in the organic solvent, and also showed slight activity with Cr^{3+} (Figure 5.12A,B).

Activity tests were continued with some of the possible factors. The enzyme activities were tested in selection buffer (50 mM MES, pH 6.0, 25 mM NaCl, with no metal added), and separately tested with each of Mg^{2+} , Ca^{2+} heavy metal ions.

At the time of doing the Cr^{3+} selection, an independent Ba^{+} -dependent *in vitro* selection was also going on in our lab. It could be possible that Cr^{3+} selection was contaminated with Ba^{+} , and the sequences might be selected due to activation by Ba^{+} metal ion. So, enzymes were also tested with Ba^{+} .

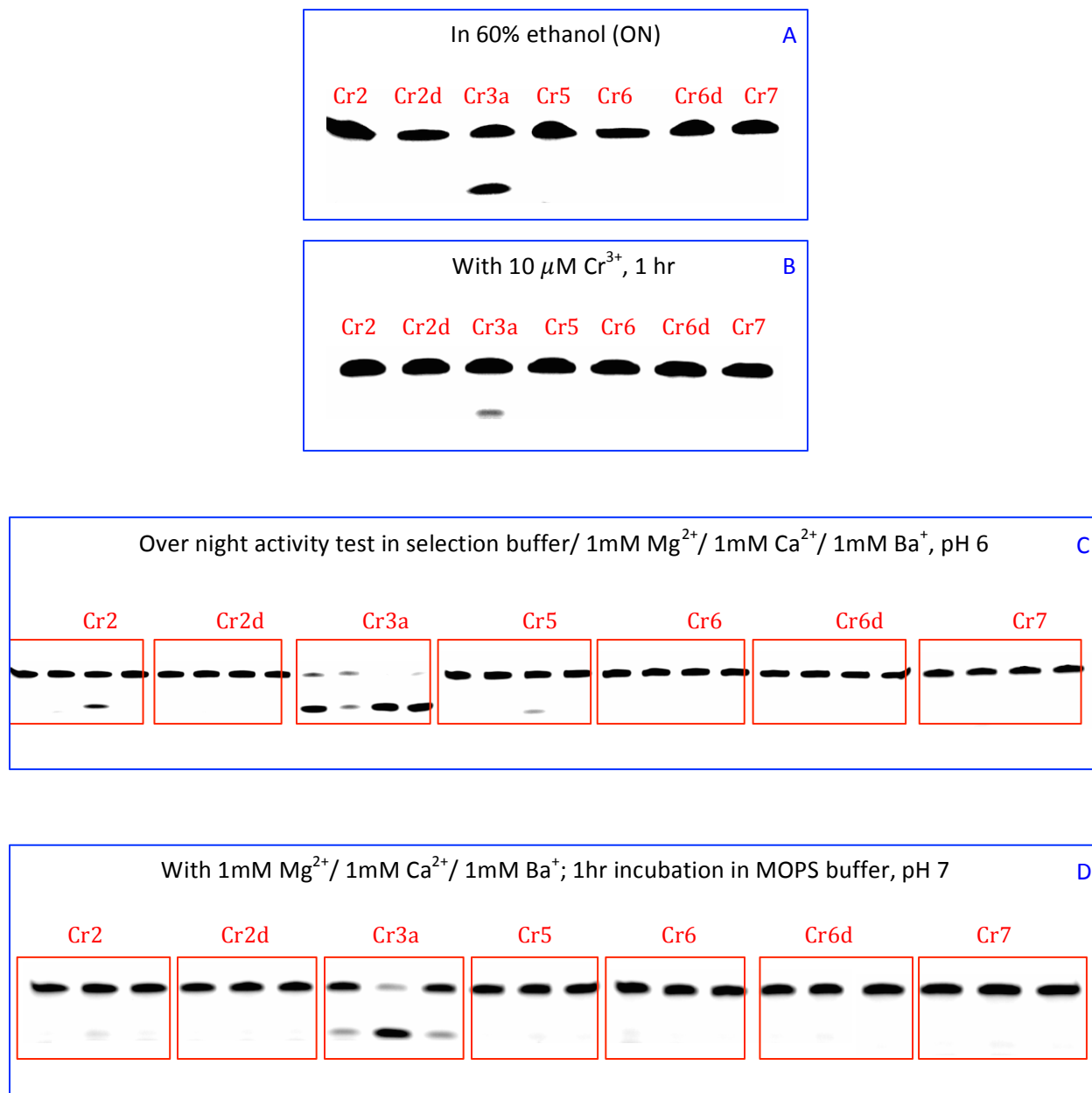


Figure 5.12 DNases activities in different conditions. Their activities tested in 60% ethanol (A), with Cr^{3+} (B), in selection buffer, 1mM Mg^{2+} , 1mM Ca^{2+} , 1mM Ba^+ for overnight (C) and with Mg^{2+} , Ca^{2+} , Ba^+ , for 1hr in MOPS buffer, pH 7 (D).

Figure 5.12, C shows the activities of the DNases with all the factors that were tested. To ensure the enzymes had sufficient time to complete the hydrolysis, the activity test was carried out overnight. However, the highly active Cr3a DNase (or 17E) was again the only

one showing some activity. Cr2 showed moderate Ca^{2+} -dependent activity, while truncating the catalytic loop abolished its activity, as is shown in Cr2d. Cr5 only showed slight Ca^{2+} -dependent activity and other DNAzymes were almost inactive. The DNAzymes were tested at a higher pH ($\sim$ pH 7, 1 hr incubation), but only trace activities were detected with some enzymes (Figure, 5.12D), except Cr3 that again had the most activity. Note that, at high pH, non-specific hydrolysis may occur, and to avoid that, activities were only monitored for 1hr at pH 7.

5.3 Summary

A key factor that governs any selection and its success is the conditions at which selection is carried out.³⁷² In this work, I performed extensive in vitro selection experiments under different conditions in the presence of Cr^{3+} . Although no new DNAzymes were obtained, this work indicates that Cr^{3+} can cleave the Ce13d DNAzyme, a previously isolated DNAzyme that is active in the presence of trivalent lanthanide ions. After knowing this, the activity of Cr^{3+} with the previously identified Ce13d enzyme was explored. This is the first study using Cr^{3+} as the metal cofactor for DNAzyme catalysis. It indicates that it is possible to use Cr^{3+} to assist RNA cleaving based on DNAzymes. The recommended condition is 50 μM Cr^{3+} (as the target) and 25 mM NaCl, at 23 °C and pH $\sim$ 7. Furthermore, two new Cr^{3+} -dependent selections were conducted to isolate a specific DNAzyme, using the N50 and N35 libraries, respectively. The main difference here from the previous attempts was to include a blocking DNA to eliminate the Ce13d type of sequence, or to use a smaller N35 library to discourage Ce13d. However, a non-specific cleaving DNAzyme was obtained as the major product, and accompanied with a small fraction of 17E. It might be that Ce13d is already an optimal sequence for Cr^{3+} , although it is not specific for Cr^{3+} .

Later, a lab member in Dr. Liu's lab could develop a different strategy and converted the Ce13d into a selective DNzyme sensor for Cr^{3+} and Cr^{VI} . In this method, by using the phosphate buffer, the lanthanide activity was fully masked and at the same time, Pb^{2+} was also inactivated, so leaving Cr^{3+} the only metal that can activate Ce13d.³⁷³ The detection of Cr^{VI} was achieved after its reduction by NaBH_4 to Cr^{3+} .

5.4 Materials and Methods

5.5.1 Chemicals

The chemicals used for the *in vitro* selection, PCR, and the activity assays are the same as previously described in chapter 2, section 2.4.1. DNAs for selections including the blocking DNAs are from Integrated DNA Technologies (Coralville, IA). The blocking DNA sequences are indicated in Figure 5.7B. The trans-cleaving enzyme strands were purchased from Eurofins (Huntsville, AL). Sequences of the enzyme strands are indicated in Table 5.3.

The metal salts at the highest available purity (99.999%) were purchased from Sigma-Aldrich. Tris(hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), EDTA, NaCl, NaNO_3 and ammonium acetate were from Mandel Scientific (Guelph, Ontario, Canada).

5.5.2 Enzymes activity assay

To produce the DNzyme complex (enzyme-substrate), FAM-labeled substrate (0.7 μM) and enzyme (1.1 μM) were annealed in a buffer containing 25 mM NaCl and 50 mM MES, pH 6. (This is the same for Ce13d DNzyme and different DNzymes selected in Cr^{3+} selection) A

final of 10 μM Cr^{3+} (or other metals) was added to initiate the cleavage reaction. The products were separated on a dPAGE gel and analyzed using a ChemiDoc MP imaging system (Bio-Rad).

For testing the activity of different Cr^{3+} complexes, a final of 10 μM from each Cr^{3+} solution was separately incubated with 1 μM Ce13d enzyme-sub complex. The reaction was quenched after 1hr, run on dPAGE gel, and analyzed.

5.5.3 Cr^{3+} activity assay with Ce13d

In the metal-dependent assay, 1 μM of Ce13d enzyme-sub complex with different concentrations of Cr^{3+} was separately incubated and samples were quenched with 8 M urea after 1hr. For the temperature-dependent assay, three tubes each containing 1 μM of Ce13d enzyme-sub complex and a final of 10 μM Cr^{3+} were separately incubated in the fridge ($\sim 4^\circ\text{C}$), at room temperature ($\sim 23^\circ\text{C}$), and in a water bath ($\sim 40^\circ\text{C}$) for 1 hr. For pH-dependent activity assay, the MES and MOPS buffers (50 mM with 25 mM NaCl) were used. Different concentrations of NaCl and NaNO_3 salts were used for salt-dependent assay.

5.5.4 Blocked *in vitro* selection

The library was prepared as described in chapter 2, and used for the first round of selection. For subsequent rounds, the library was generated by PCR. In each round, before the selection step, the library was annealed with 150 pmol of each of the blocking DNA to inactivate the corresponding sequence. The library was then incubated with Cr^{3+} to induce the cleavage. The incubation time and metal concentration for each round are summarized in Table 5.1 and 5.2, for the Cr^{3+} selections using N50 and N35 libraries. After a specific period of time, the reaction was quenched by 8 M urea and the cleaved product purified by 10% dPAGE. The selected DNAs were extracted from the gel, purified with a Sep-Pak C18 column (Waters), and

then suspended in 70 μ L HEPES buffer (5 mM, pH 7.5). Two PCR steps and four primers, as previously described, were used to amplify the selected DNA. For the negative round of selection, the library was incubated at room temperature for a specific period of time (described in Table 5.1 and 5.2) to monitor the non-specific hydrolysis.

5.5.5 Deep sequencing

The full-length library was generated from the round six library using PCR, and used for deep sequencing. The Illumina sequencing adaptors were added to the PCR products by another PCR reaction. For this step two different primers, P701 (5'-CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTGCAGAATTCTAATACGAGTCAC) and P501 (5'-AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTCCCTACACGACGCTCTTCCGATCTGTGCCAAGCTTACCG), were used as the forward and reverse primers, respectively, each containing a unique index sequence. The PCR product was purified with 2 % agarose gel and extracted using a gel extraction kit from IBI Scientific. Subsequently, the extracted DNA was eluted in 25 μ L Milli-Q water and quantified using a NanoDrop Spectrophotometer.

Chapter 6. Final conclusions and future recommendation

The research here is the summary of my thesis work on the isolation of new RNA-cleaving DNAzymes using various trivalent metal ions as cofactors. In chapters two and three, emphasis was placed on isolation and characterization of lanthanide-dependent DNAzymes. Trivalent lanthanides and some of their complexes have been shown to efficiently cleave RNA, but a high lanthanide concentration of a few mM is needed. Using Ce^{3+} and Lu^{3+} , the Ce13d and Lu12 DNAzymes were isolated, respectively. Ce13d also was previously selected using Ce^{4+} . Surprisingly, Ce13d has almost no activity with Ce^{4+} , but is highly active with all trivalent lanthanides (Ln^{3+}) and Y^{3+} . Ce13d works with all Ln^{3+} ions with a similar activity, 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+} .

In general, Ce13d provides a general probe for the group of lanthanides with excellent sensitivity. Our work on the PS-modified Ce13d DNAzyme indicated that new DNAzymes might be obtained by performing selections using PS-containing libraries. Later, our group has selected a different DNAzyme for Cd^{2+} by using the PS-modified library for the selection.³¹⁵

In the next step, to select new DNAzymes that might distinguish individual lanthanide, a different strategy (e.g. using shorter DNA library) was employed and the Lu12 DNAzyme was obtained. Lu12 is a unique DNAzyme with very different biochemical characteristics, such as, accepting a diverse range of dinucleotide cleavage junctions, using the pro- S_p oxygen at the scissile phosphate for metal binding.

From fundamental point of view, these two DNazymes (Ce13d and Lu12) provide a useful scaffold for studying DNA-based metal coordination. Lanthanides are useful spectroscopic probes for understanding the binding with DNA. They have high affinity for DNA backbone and bases. Lanthanides can act as efficient co-factors for the reactions catalyzed by DNazymes, and DNazymes can be converted into specific biosensor for the lanthanides detection.

Our ultimate goal is to produce a sensor array that can detect individual lanthanides. Combining the two selected DNazymes (Ce13d and Lu12) resulted in a biosensor that can partially discriminate a few large lanthanides. Future studies can focus on selecting new DNazymes with better selectivity in the group of lanthanide, using different length libraries and/or different conditions. Future studies can also work on selecting specific DNazymes that can work in a broader range of buffer conditions (e.g. temperature and ionic strength), or can compete with many other biopolymers for metals, which are important aspects in environmental and intracellular applications, respectively.³⁷⁴⁻³⁷⁷

In addition to trivalent lanthanides, the group 3A metal ions and trivalent chromium were also used for the *in vitro* selection in this study. However, no new DNazymes were selected with these metal ions, as they interacted differently with DNA molecules and did not show much tendency to bind to DNA backbones, especially for the group 3A metals. Different trivalent metal ions have different properties, and different strategies are required for the *in vitro* selection with different groups of metal ions. Using new buffer conditions by changing pH or salt might be required to obtain new DNazymes active with these metals.

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