Doctorate Dissertation (Censored)
博士論文 (要約)

In vitro selection and studies on tRNA-recognizing ribozymes derived from T-box motifs

（T-box モチーフを基にした tRNA 認識リボザイムの試験管内選択およびその解析）

A Dissertation Submitted for Degree of Doctor of Philosophy
December 2018

平成 30 年 12 月博士（理学）申請

Department of Chemistry, Graduate School of Science,
The University of Tokyo
東京大学大学院理学系研究科化学専攻

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Abstract

Aminoacylation reaction is a key step that bridges RNA to protein. This reaction is solely conducted by protein enzymes called aminoacyl-tRNA synthetases (ARSs) in the current world. The high specificity toward both tRNA and amino acid of ARSs ensures the right connection between anticodon and amino acid thus accurate translation of mRNA to protein is achieved. It is thought that in the early stage of life, RNA was responsible not only for genomic storage but also for catalyzing chemical reaction and this hypothesis is called the RNA world. In the transition era between the RNA world and the current world, the aminoacylation reaction is possibly conducted by RNA. Such RNA responsible for aminoacylation would be called aminoacylation ribozyme. This ribozyme is expected to have the characteristics of natural ARS that is the specificity toward both amino acid and tRNA. Since no such ribozyme has yet been discovered, the aim of this thesis is to discovery such ARS-like ribozyme.

Chapter 1 describes the general introduction of this thesis. The RNA world hypothesis is explained in detail and the riboswitch which is a possible relic of RNA world is explained as well. Also, the history of aminoacylation ribozyme discovery is presented.

In chapter 2, the discovery of aminoacylation ribozyme using T-box riboswitch as starting structure is described. A ribozyme which has specificity toward both amino acid and tRNA was obtained. The detailed characterization of this ribozyme is also explained. This aminoacylation ribozyme was coupled to the *in vitro* translation mixture to demonstrate the feasibility of this ribozyme.

In chapter 3, the amino acid specificity against other amino acid was attempted using the similar approach as chapter 2.

In the last chapter, the achievement of this thesis is discussed. The implication of the discovery of aminoacylation ribozyme from riboswitch is also pointed out.

Contents

Chapter 1

General introduction

1.1 RNA world hypothesis

In the current world, life is governed by three types of biomolecules; DNA, RNA and protein. Genetic information is stored in the form of DNA which is transcribed into mRNA and then translated into protein and all of these processes are done with the help of protein enzymes (Figure 1.1a). It is unlikely that the complicated biological system nowadays suddenly appeared and it is thought that the life on early earth was using a different system. The idea that an RNA-based system was predominant before the rise of the current DNA/RNA/protein based system is a popular hypothesis which is also called the RNA world hypothesis. In this hypothesis, RNA was responsible for both genomic storage and chemical reaction catalysis (Figure 1.1b). This hypothesis was coined by Walter Gilbert in 1986[3]. The discovery of RNA bearing catalytic activity, ribozyme, in 1980s have enhanced the plausibility of this hypothetical RNA world. These ribozymes include the self-splicing RNA found in *Tereahymena thermophila*[4] and the M1 RNA which is the RNA component of bacterial RNase P responsible for tRNA maturation[5]. It is important to note that these ribozymes have catalytic activity even in the absence of protein. Since then many RNAs bearing catalytic function were both discovered in nature or artificially engineered in laboratory. Especially the engineering of ribozyme was boosted by the development of a methodology to selecting RNAs from large random libraries also known as Systematic Evolution of Ligands by Exponential enrichment (SELEX)[6,7].

Another evidence of the possible RNA world is the fact that the ribosome which plays a critical role in the modern translation system is also a ribozyme. Although ribosome is a ribonucleoprotein which is composed of both ribosomal RNAs and ribosomal proteins, the central core; peptidyl transfer center (PTC) is composed only of RNAs which was indisputably determined by X-ray structure[8,9]. These findings pose the possibility that the primitive ribosome could have been solely composed of RNA as coined by Crick[10].

In the course of evolution, the hypothetical ribozymes present in the RNA world would have been taken over by protein enzymes, and it is unlikely to find ancient ribozymes in the contemporary world. However, as the current ribosome is regarded as a ribozyme, the relic of the ancient ribozymes could be present or even experimentally engineered from random RNA library. Such ribozymes include the RNA polymerase ribozyme[11] which is able to replicate RNA in a protein-free fashion. Since replication of RNA in the absence of protein enzymes is an important feature in the RNA world, the successful engineering of such ribozyme, although not found in nature, is an evidence that supports the RNA world hypothesis.

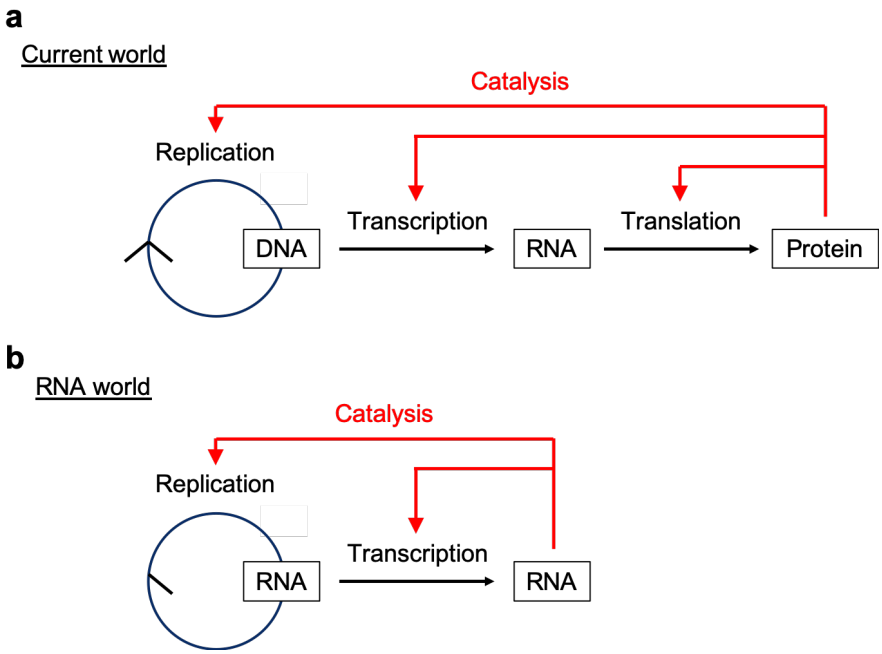

Figure 1.1 | Schematic illustration of the central dogma
(a) The interplay of DNA, RNA and protein in the current world. (b) The schematic image of how RNA functioned in the RNA world.

1.2 Riboswitch

Riboswitches are class of RNA motif that regulates the expression of target gene in the presence of its ligand. Riboswitches reside upstream of the gene they regulate and commonly found in 5'-untranslated region (UTR) of bacteria mRNA. Although most of the riboswitches are discovered in bacteria, some are spread in all three domains of life and there are variety of ligands that function as a gene expression switch for the riboswitch. These ligands include; coenzymes[12-15], nucleotides[16-19], amino acids[20-22], sugar[23], ions[24-26] or even RNA[27-29]. Upon binding of its ligand, the riboswitch undergoes a structural change and controls the expression of the target gene at the level of either transcription attenuation or translation initiation.

In most cases, riboswitch comprises two domains, where one domain is responsible for ligand sensing and the other domain is responsible for gene regulation. The sequence overlapping between these two domains contributes to the switching of riboswitch upon ligand binding. In terms of transcriptional attenuation, the gene regulation domain commonly forms a helix, followed by poly-uridine sequence. The weak interaction with poly-uridine sequence and DNA results in the release of RNA polymerase thus transcription termination is achieved. As for translation initiation, the gene regulation domain forms a helix that include the Shine-Dalgarno sequence. This would prevent ribosomal binding which hinders translation initiation. Ligand binding will either induce or prevent this helix formation and translation initiation is regulated.

The intriguing fact about the discovery of riboswitch is that RNA alone can sense the surrounding environment and undergoes structural change as well as has the ability to selectively bind to metabolites or even other RNAs. This has led us the possibility that RNAs in the possible RNA world could have been using a similar recognition mechanism

as seen in current riboswitches or riboswitches could be the reminiscent of ancestral ribozymes present in the RNA world[30].

1.3 Strategy to obtain functional oligonucleotides

SELEX or also known as *in vitro* selection is an established screening method for enriching nucleotides from a pool of random library based on desired functionality. This method was first reported from two different groups in 1990 and they succeeded in identifying specific RNA sequences that bind to T4 DNA polymerase[31] and organic dyes[7] respectively. The establishment of this method in 1990s has boosted the discovery of numerous ribozymes and RNA sequences binding to specific target compounds (aptamers). *In vitro* selection of functional RNA comprises of mainly four steps (Figure 1.2). First, DNA library is constructed where T7 promoter domain for transcription and constant regions at both 5' and 3' end are included. These constant regions are necessary for PCR and reverse transcription in the downstream procedure. The prepared DNA library was then *in vitro* transcribed and usually gel purified. The obtained library was then applied to screening based on affinity or catalytic activity. The active species that are selectively recovered from the random pool are then reverse transcribed followed by PCR amplification to obtain another set of a DNA library. By repeating the above cycle for multiple times, RNA sequence bearing the desired functionality would be enriched from a random library. Using this method, ribozymes catalyzing various chemical reactions have been obtained. These ribozymes, include the aforementioned RNA polymerase ribozyme[11,32,33], alcohol dehydrogenase ribozyme[34], ribozyme that catalyzes Diel-Alder reaction[35,36] or aminoacylation ribozymes[2,37-41].

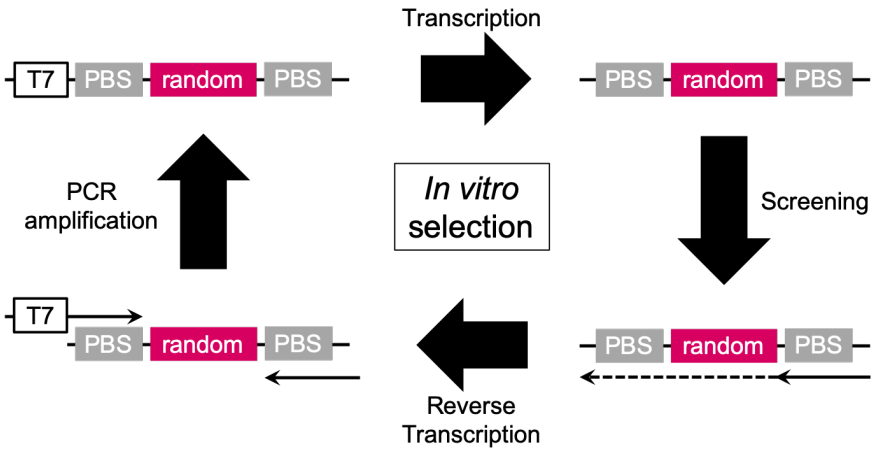

Figure 1.2 | Schematic illustration of the *in vitro* selection
PBS denotes primer binding site circled with gray. T7 denotes T7 promoter sequence. Random region is colored magenta.

1.4 History of Aminoacylation ribozyme

1.4.1 Aminoacy-tRNA synthetase (aaRs)

Aminoacylation is a key step that bridges amino acid and RNA in the current world. This reaction is catalyzed by protein enzymes called aminoacyl-tRNA synthetases (aaRs). It is proposed that this reaction could have been catalyzed by RNA especially in the transition era between the RNA world and the current RNA/DNA/protein world. The reaction catalyzed by aaRs has two steps; (i) amino acid activation and (ii) aminoacyl transfer (Figure 1.3). The activation of amino acid is achieved by adenylation of the carboxylic acid using ATP as substrate. The adenylated amino acid is reacted with the substrate tRNA and 3′ end of tRNA is aminoacylated. Many researchers have attempted to engineer aminoacylation ribozymes that catalyze the above reaction but all of them can only catalyze either of the two reactions.

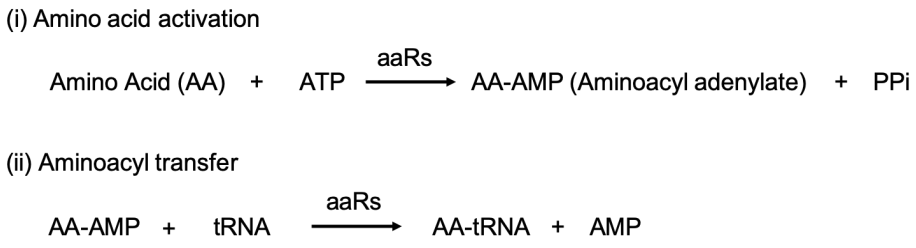

Figure 1.3 | Two reactions carried out by aminoacyl-tRNA synthetases (aaRs)
(i) Amino acid activation is achieved with the help of ATP and aminoacyl adenylate (AA-AMP) is formed while releasing pyrophosphate (PPi) after activation. (ii) aaRs recognizes the substrate tRNA and transfers AA-AMP to the 3′-end of tRNA.

1.4.2 Amino acid activating ribozyme

In 2001, Yarus group succeeded in developing an amino acid activation ribozyme, KK13[1]. This ribozyme utilizes the 5′-triphospahte of its own sequence to activate amino acid. The selection strategy employed in the discovery of KK13 is shown in Figure 1.4. They used 3-mercapto-propionic acid as the initial substrate and mixed it with the RNA pool having 5′-triphospate. After incubation, the reacted RNA was treated with thiopropyl

sepharose 6B matrix to selectively capture active RNAs. The active RNAs were eluted using DTT, ethanol precipitated, reverse transcribed, PCR amplified, and transcribed. After repeating this step for 8 times, they succeeded in identifying several sequences and among them, KK13 was further characterized and activation using amino acid substrates was confirmed. The secondary structure of KK13 is shown in Figure 1.5. The 5′-terminal triphosphate was necessary for activation since 5′-pKK13 and 5′-OH KK13 did not react with the substrate. However, this ribozyme functions best at pH 4-4.5 due to the instability of the product.

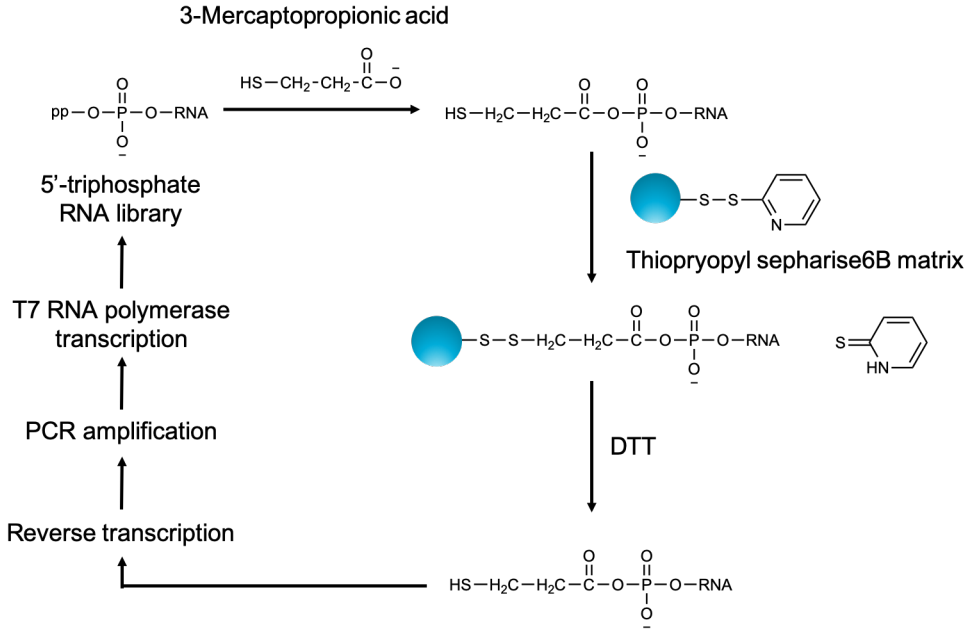

Figure 1.4 | Schematic procedure of selecting carboxyl-activating ribozymes.
This figure is adapted from previous paper[1]. 3-mercaptopropionic acid was used as the substrate and subjected to incubation with the RNA library. The active species were selectively recovered by the thiopyropyl sepharose6B matrix.

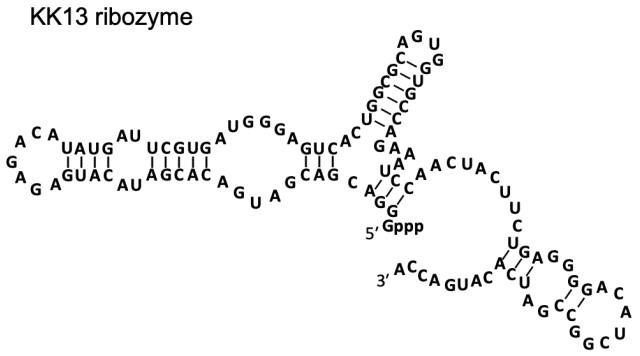

Figure 1.5 | Secondary structure of KK13
This figure is adapted from previous paper[1]. The 5′-terminal triphosphate attacked by the carboxylate oxygen to give activated amino acid.

1.4.3 Aminoacyl transfer ribozyme

All aminoacyl transfer ribozymes discovered so far utilizes an already activated amino acid. The activated amino acid could be either aminoacyl adenylate which the current aaRs utilizes or other artificially activated amino acid such as cyanomethyl esters (CME). The first aminoacylation ribozyme was discovered by Yarus group in 1995 using Phe-AMP as substrate[2]. The selection procedure employed is shown in Figure 1.6. The RNA library was mixed with Phe-AMP and then reacted with naphthoxyacetyl-N-hydroxysuccinimidyl ester to convert the active RNA more hydrophobic and purifiable using reverse-phase HPLC. The selection resulted in isolating isolate #29 RNA which aminoacylate Phe-AMP at the 3′-teminal guanosine. They also succeeded in minimizing isolate #29 RNA sequence (95 mer) to 29-mer (Figure 1.7a,b)[39]. Both the minimized isolate #29 or original isolate #29 RNA had no amino acid specificity and required both Mg^{2+} and Ca^{2+} for their activity. Yarus group also engineered a tiny aminoacylation ribozyme called C3 ribozyme (Figure 1.7c)[41]. This ribozyme was engineered utilizing an RNA library that includes hammerhead ribozyme (HDV) at the 3′-end. Since the HDV does not have any requirement for the upstream nucleotide, the 3′-end nucleotide for the aminoacylation site can be anything as opposed to the previous selection, where 3′-end nucleotide

was restricted because it was included in the primer binding site. This C3 ribozyme, as compared to #29 ribozyme, does not need divalent ions such as Mg^{2+} or Ca^{2+} for reactivity, and highly specific for L-amino acids.

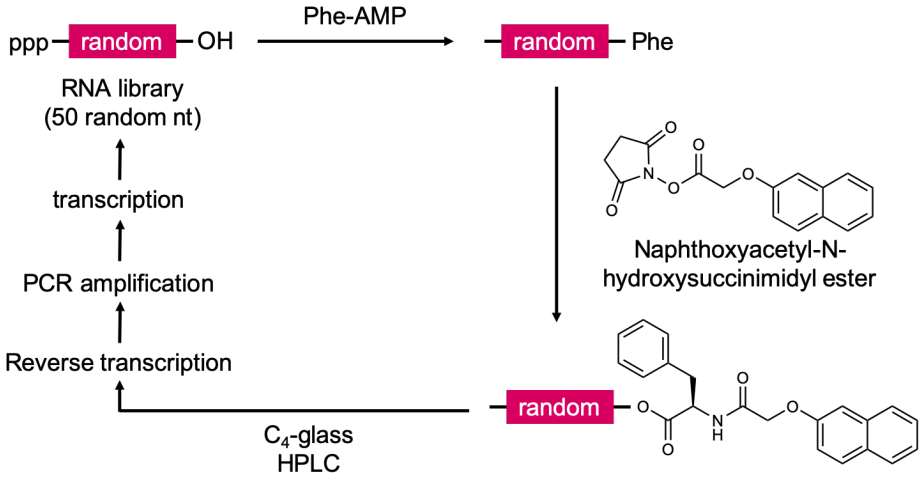

Figure 1.6 | Selection scheme for discovering isolate #29 ribozyme
This figure is adapted from previous paper[2]. The RNA pool was reacted with Phe-AMP followed by naphthoxyacetyl-N-hydroxysuccinimidyl ester mixing. Beacuase of the hydrophobilicy of naphthalene moiety, this resulted in retention time diference compared to unreacted RNA in the HPLC purification.

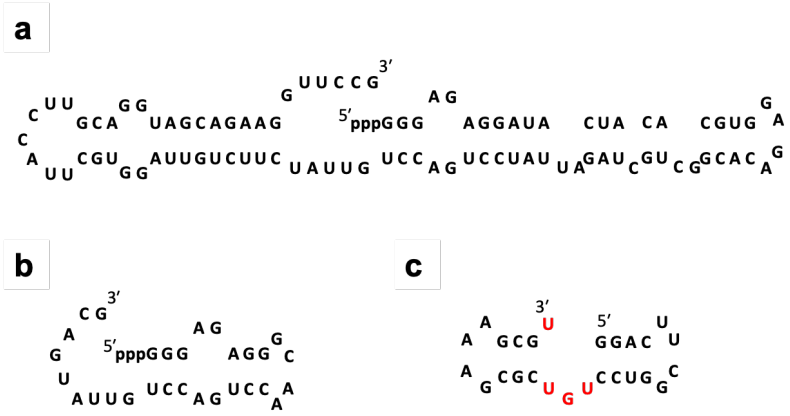

Figure 1.7 | Secondary structures of isolate #29 RNA derivative and C3 ribozyme.
(a) Secondary structure of isolate #29 RNA. (b) Secondary structure of minimized isolate #29 RNA. (c) Secondary structure of C3 ribozyme. Red nucleotides are critical for its activity.

Several aminoacylation ribozymes have been developed that utilizes artificially activated amino acids as well. The first of such aminoacylation ribozyme is ATRib which was developed in Szostak's group (Figure 1.8a)[42,43]. ATRib uses aminoacylated hexanucleotide as an aminoacyl donor and self-aminoacylates itself. The selection strategy was using Biotin-Met as an amino acid substrate and recovering active species through streptavidin agarose. Using ATRib, aminoacylation to tRNA was also achieved using a ping-pong like process (Figure 1.8b). Briefly speaking, ATRiB first binds to aminoacylated hexanucleotide and the amino acid is tranfered to ATRiB. The free-hexanucleotide is detached and tRNA-3′-end binds to the same site as hexanuleotide. The amino acids that was attached to ATRib itself then transfers to the bound tRNA which will result in the release of aminoacylated tRNA. Although all of these processes are in equilibrium, they succeeded in obtaining 30-40% of aminoacylated tRNA in 2hrs. Also, another *in vitro* selection was conducted to give ATRib additional tRNA specificity, which resulted in the

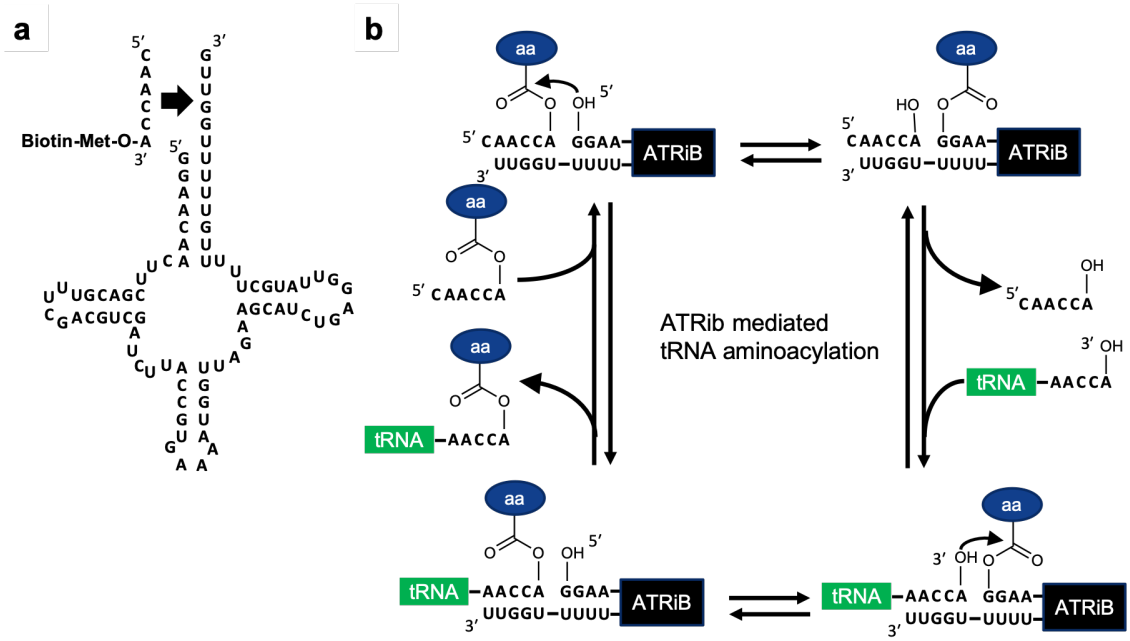

Figure 1.8 | Characterization of ATRib and ATRib mediated aminoacylation
(a) Secondary structure of ATRib. (b) Schematic illustration of ATRib mediated tRNA aminoacylation.

engineering of aminoacylation ribozyme BC28[44]. This ribozyme has an accessory domain to ATRib that recognizes tRNA anticodon.

Nearly two decades ago, our lab also developed an aminoacylation ribozyme based on the hypothesis that 5′-leader sequence of tRNA before maturation by RNaseP could have been responsible for aminoacylating the downstream tRNA[38]. An RNA library having 70-nt random sequence upstream of tRNA was prepared and those that can self-aminoacylate biotin-Phe-CME was selectively recovered through streptavidin agarose. Total of 17 rounds of screening was conducted, which eventually resulted in obtaining self-aminoacylating ribozyme, pre-24. This ribozyme also retained its activity when treated with RNaseP which means that this ribozyme functions *in trans* as well. Minimization and further engineering of r-24 (tRNA deleted version of pre-24) resulted in an aminoacylation ribozyme Fx3[45]. This ribozyme base-paris with the CCA-end of tRNA and could accept Phe derivative amino acids. Fx3 was further engineered to accept more variety of amino acid substrates as well as enhanced activity and these ribozymes were called flexizymes[40,46] (Figure 1.9). eFx accepts aromatic amino acids activated with cyanomethyl esters or chlorobenzyl thioester. dFx accepts non-aromatic amino acids that are activated with 3,5-dinitrobenzyl ester and aFx accepts amino acids with (2-aminoethyl)- amidocarboxybenzyl thioester. The versatility of flexizymes enabled us to mischarge any tRNA of interest with non-natural amino acids which lead to studies on genetic code reprogramming[47-50] or even discovery of functional non-natural peptides binding to specific protein(s) of interest[51-59].

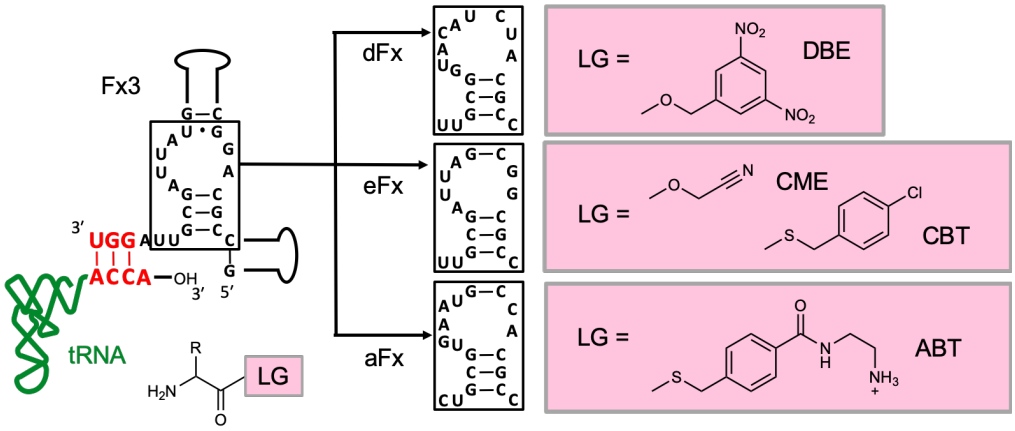

Figure 1.9 | Schematic illustration of Fx3 and flexizymes.
Red letters represent the positions of base-pairs between Fx3 and substrate tRNA. LG denotes leaving group. DBE denotes 3,5-dinitrobenzyl-ester, CME denotes cyanomethyl-ester, CBT denotes chlorobenzyl-thioester and ABT denotes (2-aminoethyl)- amidocarboxybenzyl thioester.

Chapter 2

In vitro selection of aminoacylation ribozyme using T-box motif

Chapter 2 was omitted due to publication and patent application.

Chapter 3

In vitro selection of T-box motif derived aminoacylation ribozyme using various substrates

Chapter 3 was omitted due to patent application.

Chapter 4

General conclusion

In the current world, the aminoacylation reaction is conducted solely by aminoacyl-tRNA synthetases (ARS) which recognizes its corresponding tRNA and amino acid specifically. The defined correspondence between tRNAs and acids defines the genetic code, which is essential for accurate translation of mRNAs to proteins in living organism. In the transition era between the current world and the possible RNA world, such aminoacylation reaction could be conducted by ribozyme. It is possible that ribozyme specifically recognizing both the amino acid and tRNA could have been present. In this thesis the recreation of such ribozyme was attempted.

In chapter 2, with the use of T-box riboswitch, a novel ribozyme named Tx2.1 was discovered. This ribozyme has the ability to specifically recognize biotin-^{L}Phe-CME for its amino acid substrate. Furthermore, Tx2.1 could distinguish the tRNA anticodon as well. The similar characteristics between natural ARS and Tx2.1 presents us the possibility that such ribozyme similar to Tx2.1 could have been present in the ancient time. Furthermore, the demonstration that Tx2.1 was able to function within the in vitro translation mixture was presented. This is to my knowledge the very first case to show that aminoacylation ribozyme functions in parallel with the translation mixture. This approach to discover ribozymes from existing riboswitch could be applicable to other riboswitches as well and those engineered ribozymes could also be resembling a possible ancient ribozyme existed in the RNA world. Since there is still room for improvement in efficiency, the discovery of Tx2.1 presents us with new apparatus for *in situ* aminoacylation for genetic code reprogramming as well.

In chapter 3, similar approach to chapter 2 was conducted against different amino acid substrates and His-DBE and Hbi-DBE was chosen as amino acid substrates. In the case

of His-DBE selection, the identified RNA sequence turned out to be biotinylation ribozyme instead of aminoacylation ribozyme. The study indicates the possible need of biotin residue directly attached to the amino acid substrate rather than conjugating afterwards to remove the risk of obtaining biotinylation ribozyme. The reduction of reaction time for biotinylation could also be applied to selectively recover aminoacylating ribozymes. For Hbi-DBE selection, ribozymes having selfaminoacyalation activity was obtained. These results present us the benefit of using T-box riboswitch as base structure rather than from totally random RNA sequence and that this T-box riboswitch approach to obtain aminoacylation ribozyme is to some extent feasible.

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List of accomplishments

【Publication(s) related to the thesis】

1. “Ribosomal Synthesis of Norbornene Containing Peptides for Peptide Hetero-Dimerization”, Satoshi Ishida, Takayuki Katoh, Hiroaki Suga, Peptide Science 2017, 2018, 66-67

2. “An aminoacylation ribozyme evolved from a natural tRNA-sensing T-box riboswitch”, Satoshi Ishida, Naohiro Terasaka, Takayuki Katoh, Hiroaki Suga, *accepted in principle*

【Oral presentation】

1. “Ribozymes with tRNA recognition and aminoacylation properties”, Satoshi Ishida, Naohiro Terasaka, Takayuki Katoh, Hiroaki Suga, 26th tRNA conference, Jeju, Korea, September 2016

2. “An aminoacylation ribozyme evolved from a natural tRNA sensing T-box riboswitch”, Satoshi Ishida, Naohiro Terasaka, Takayuki Katoh, Hiroaki Suga, RNA2019 Tokyo, Tokyo, Japan, July 2019

【Poster presentation】

1. “In vitro selection of tRNA-recognizing aminoacylation ribozyme derived from a T-box motif.”, Satoshi Ishida, Naohiro Terasaka, Takayuki Katoh, Hiroaki Suga, RNA 2016, Kyoto, Japan, April 2016

2. “T-box リボスイッチに基づいた tRNA 認識アミノアシル化リボザイムの開発”, 石田 啓, 寺坂 尚紘, 加藤 敬行, 菅 裕明, 第 5 回バイオ関連化学シンポジウム若手フォーラム, 東京, 2017 年 9 月

3. “*In vitro* selection 法による tRNA 認識とアミノアシル活性を併せ持ったリボザイムの開発”, 石田 啓, 寺坂 尚紘, 加藤 敬行, 菅 裕明, 第 11 回バイオ関連化学シンポジウム, 東京, 2017 年 9 月

4. “Construction of a hetero-dimeric macrocyclic pepetide library for the discovery of pepetide ligands that induce IL28RA-IL10R2 hetero-dimerization” ,Satoshi Ishida, Takayuki Katoh, Hiroaki Suga, 第 54 回ペプチド討論会, 大阪, 2017 年 11 月

5. “Preparing hetero-dimeric library for the discovery of peptide ligands that induce IL28RA-IL10RB hetero-dimerization.”, Satoshi Ishida, Takayuki Katoh, Hiroaki Suga, Gordon Research Conference, Ventura, California, February 2018

6. “Toward the discovery of heterodimeric-macrocylic peptides that induce IL28RA activation.”, Satoshi Ishida, Takayuki Katoh, Hiroaki Suga, 10th International peptide symposium, Kyoto, Japan, December 2018

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1. Advanced Leading Graduate Course for Photon Science (ALPS) (2015-2019)

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