論文の内容の要旨

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)

氏名 石田 啓

Introduction

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. Before the emergence of such complicated biological system, it is proposed that early life form could have been relying on RNA based system where RNA was responsible for both genomic storage and chemical reaction catalysis. This hypothesis is called the RNA world hypothesis which is one of the most popular hypotheses of life on early earth. The plausibility of this hypothesis was enhanced by the discovery of catalytic RNAs such as RNase P or self-splicing RNA in nature or the obtainment of artificial ribozymes with the use of SELEX (Systematic Evolution of Ligands by EXponential enrichment). Aminoacylation ribozymes are particular of interest since they provide the bridge between RNA world and the modern DNA/RNA/protein world. Several aminoacylation ribozymes have been developed in the lab but their sequences were not related to the currently existing RNA molecular fossils, i.e. not to be a part of reminiscence of the RNA world.

In this study I have aimed at discovering aminoacylation ribozymes from a library consisting of a small random sequence domain embedded in a molecular fossil of the RNA world found in contemporary life. To accomplish this, I have focused on a specific riboswitch called the T-box riboswitch given that some modern

riboswitches are considered the biological fossils of ancient ribozymes existed in the RNA world.

***In vitro* selection of aminoacylation ribozyme using T-box motif**

T-box riboswitch is an RNA motif that is found in gram-positive bacteria. It resides in the upstream of the gene it regulates, and monitors amino acid status by monitoring the aminoacylation status of the cognate tRNA. When a specific tRNA is charged with the cor-responding amino acid, there is no change in the T-box riboswitch structure. However, when a specific uncharged tRNA is present, T-box binds with this tRNA, undergoes a structural change and starts the transcription of the downstream gene. I focused on this tRNA recognition property of T-box riboswitch and randomized a portion of T-box riboswitch in the hope of given additional aminoacylation activity. In vitro selection was conducted to fish out the desired species from the partially randomized T-box library. Bio-L-Phe-CME was used as the amino acid substrate and active species were selectively recovered through streptavidin coated magnetic beads. Through 7 rounds of screening process, enrichment of active species was observed and ribozyme named Tx2.1 was identified which has both self-aminoacylation (in *cis*) and transaminoacylation activity. Further characterization of Tx2.1 revealed that this ribozyme was specific to bio-L-Phe-CME suggesting that this ribozyme recognizes both the biotin residue and the side chain residue (Figure 1a). The natural T-box forms base-paring with the tRNA anticodon and this property was maintained for Tx2.1. This ribozyme recognizes the tRNA anticodon and the activity is abolished when tRNA bearing anticodon that forms no-base paring was treated with Tx2.1 (Figure 1b). The lost activity was recovered when compensatory mutastion was introduced to the Tx2.1 specifier sequence. Not only the anticodon interaction but tRNA D-loop interaction with the T-box apical loop was also observed for natural T-box and similar interaction could be observed by monitoring the aminoacylation yield of tRNA mutants with different D-loop size (Figure 1c). The larger the loop size was, the less the aminoacylation activity became. The secondary structure of Tx2.1 was validated by deep mutational scanning of the Tx2.1 random domain. Figure 2a shows the heat map representing the effect of each mutation to the aminoacylation activity. This heat map along with other aminoacylation

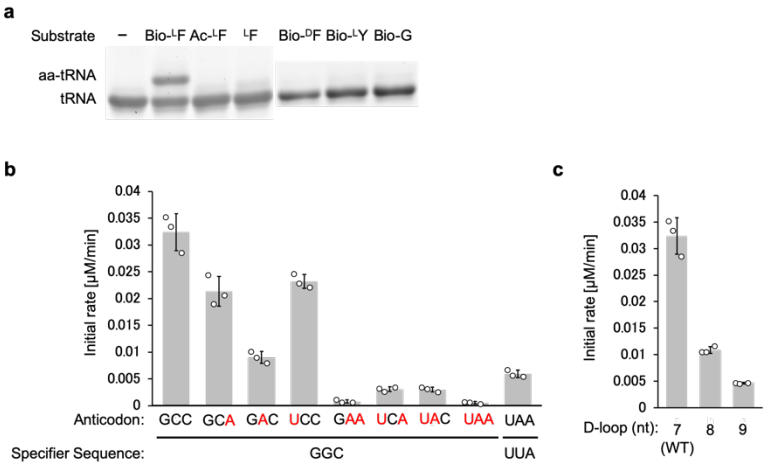

Figure 1. Characteristics of Tx2.1 (a) The amino acid selectivity of Tx2.1 (b) Apparent initial rates for transaminoacyaltion acitivity against various anticodon mutant tRNAs. (c) Transaminoacylation acitivity of Tx2.1 against various D-loop mutants.

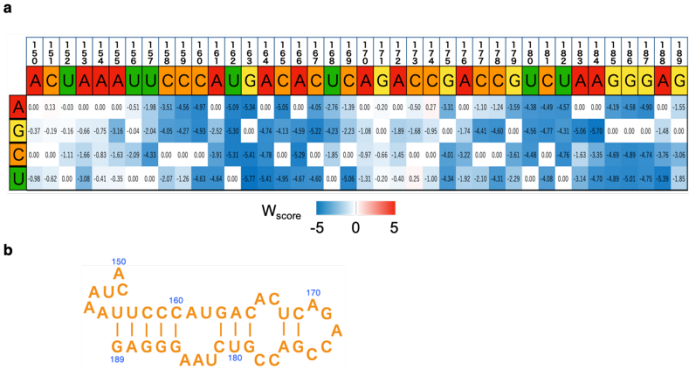

Figure 2. (a) Heat map of mutational scanning of Tx2.1 random domain. (b) Secondary structure of Tx2.1 revealed by deep mutational scanning experiment.

activity measurement toward Tx2.1 mutant revealed the most plausible secondary structure of Tx2.1 (Figure 2b).

With the tRNA recognizing aminoacylation ribozyme in hand, I also checked whether aminoacylation by Tx2.1 can function in parallel with translation. The expression of a model peptide was evaluated in the presence of Tx2.1, $tRNA^{Gly}_{GCC}$, Bio-L-Phe-CME, and a DNA template within our FIT (Flexible In-vitro Translation) cock-tail. If Tx2.1 can aminoacylate $tRNA^{Gly}_{GCC}$ using Bio-L-Phe-CME in the FIT cocktail, a model peptide initiating with Bio-L-Phe should be observed. Since Tx2.1 was most active toward tRNA bearing GCC anticodon, the start codon was engineered to GGC rather than the canonical AUG to initiate the translation with Bio- L-Phe (Figure 3a). The expression of model peptide was measured by Tricine SDS-PAGE and a clear band corresponding to the full-length product was observed only when all of the Tx2.1 aminoacylation component was added to the translation mixture. The identity of the band was confirmed by MALDI-TOF MS showing the correct mass corresponding to the Bio-L-Phe initiated peptide. These results indicate that Tx2.1 indeed function within the translation mixture and the aminoacyl-tRNA produced was accepted by ribosome for translation.

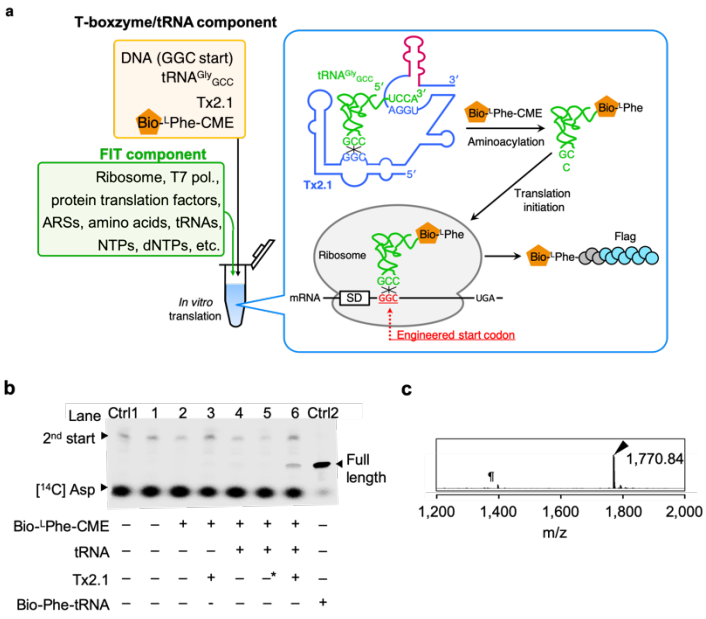

Figure 3. (a) Schematic representation of the in situ aminoacylation and translation experiment conducted in this study. (b) Tricine-SDS PAGE gel image of in vitro translation experiments under various conditions. Ctrl1 indicates the control experiment of translated peptide initiating from the second amino acid. Ctrl2 indicates the control experiment of full-length peptide by adding pre-charged Bio-L-Phe-tRNA prepared by eFx. In lane 5, the glyQS T-box riboswitch was added instead of Tx2.1, which is represented as –*. (c) MALDI-TOF-MS spectrum of translation product from lane 6 of (a). Black triangle indicates the full-length product. (calculated m/z = 1770.84). ¶ denotes the peptide initiating from the second amino acid.

Since Tx2.1 was specific for Bio-L-Phe-CME, I have also attempted to change the specificity of the aminoacylation ribozyme. Conducting the same in vitro selection using the partially randomized T-box riboswitch with different amino acids, I also succeeded in obtaining aminoacylation ribozymes which accept amino acid substrate having biotin residue on the side chain or biotinylation ribozymes.

Conclusion

Using T-box sequence as the starting structure, I have succeeded in discovering aminoacylation ribozymes that function either in cis or also in trans. The trans acting ribozyme Tx2.1 inherited the tRNA specificity nature and also possessed amino acid specificity which is a similar function to the protein aminoacyl-tRNA syntheatse. I also succeeded in coupling aminoacylation ribozyme Tx2.1 with the in vitro translation mixture demonstrating for the first time that aminoacylation ribozyme can function within the translation machinery. This discovery of aminoacylation ribozyme from possible relic of RNA world presents us the link between RNA world and the protein world governed by the genetic code.