論文の内容の要旨

論文題目

Applications of aminoacylation ribozymes that recognize the 3'-end of tRNA via two consecutive base pairs

（tRNA の 3'末端を塩基対合によって認識する アミノアシル化リボザイムの応用）

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Introduction

Ribozyme is an enzyme whose catalytic core consists of RNA. Many natural ribozymes have been discovered and various artificial ribozymes have been obtained by SELEX (Systematic Evolution of Ligands by EXponential enrichment). Among the various ribozymes, aminoacylation ribozymes are particularly important because they provide a potential link between the ancient RNA world and the modern protein world. In addition to this scientific importance, aminoacylation ribozymes have potential to be applied for other RNA research. Although several aminoacylation ribozymes have been developed, I focused attention on flexizyme having unique characteristics; (i) flexizyme recognizes substrate tRNA by two consecutive base pairs between 3′ end of substrate tRNA and 3′-end of flexizyme, (ii) these base pairs can be substituted with other base pairs and (iii) various activated amino acids can be used as substrates including both proteinogenic and nonproteinogenic amino acids[1,2] (Fig. 1). These characteristics are distinct from natural aminoacyl-tRNA synthetase (ARS) and other artificial aminoacylation ribozymes. In Ph.D. course, first, I discovered human microRNA precursor binding to folic acid by small RNA transcriptomic SELEX from tRNA-depleted human small RNA library prepared using flexizyme. Second, the method to easily aminoacylate CCA-3′ end mutated tRNA using compensatory mutated flexizymes were developed and an orthogonal translation machinery independent of the wild-type counterpart were constructed.

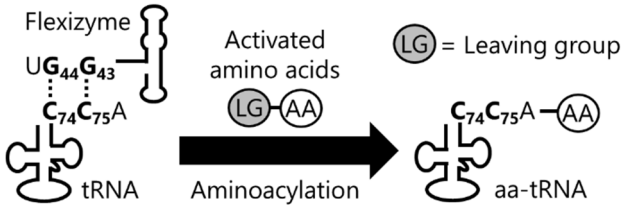

Figure 1 | Schematic illustration of aminoacylation reaction catalyzed by flexizyme.

Discovery of human microRNA precursor binding to folic acid by small RNA transcriptomic SELEX

Micro RNA (miRNA) is about 22 nt single strand RNA, which is one of the small ncRNAs. miRNAs are loaded into an Argonaute protein to form an RNA-induced silencing complex (RISC) and a RISC binds to a mRNA via base paring with miRNA to regulate the expression of target gene. miRNAs are transcribed from genome as part of a long primary transcripts (pri-miRNAs) and then pri-miRNAs are cleaved to produce precursor miRNAs (pre-miRNAs) which form hairpin structure. Pre-miRNAs are exported to the cytoplasm and they are cleaved to mature miRNA. Biogenesis and functions of miRNAs are regulated by proteins and RNAs in many steps. Recently, regulation of miRNA functions by binding with small molecules were discovered in human. This discovery suggests the existence of RNA aptamer elements binding to small molecules with specific biochemical activities in other human miRNAs. In my master course, tRNA-depleted human small RNA library was constructed by aminoacylating tRNAs with biotin-conjugated phenylalanine using flexizyme because tRNAs are much abundant in the small RNA fraction. Then, SELEX to folic acid (Fig. 2a) were performed using this library and three kinds of human small RNAs; precursor microRNA 125a (pre-miR125a), FA1: fragment of $tRNA^{Gly}_{GCC}$, FA2: antisense of mitochondrial mRNA were obtained.

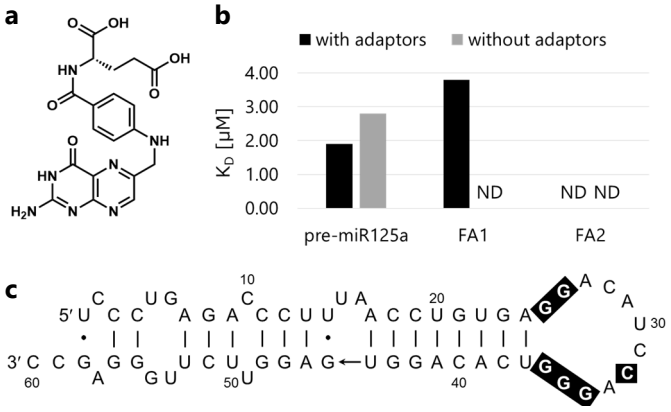

Figure 2 | (**a**) Chemical structure of folic acid. (**b**) Dissociation constants (K_D) of RNAs obtained from SELEX. ND indicates "not determined". (**c**) Secondary structure of human pre-miR125a. Highlighted bases are essential for biding to folic acid.

In my Ph.D. course, interaction between RNAs and folic acid were measured by bio-layer interferometry. Because adaptor sequences were ligated with both 5′- and 3′-end of natural RNAs to perform reverse transcription and PCR during SELEX, dissociation constant (K_D) of these RNAs with or without adaptors were measured (Fig. 2b). Consequently, only pre-miR125a bound to folic acid without adaptors. FA1 did not bind to folic acid without adaptors probably because ligation of adaptors may dramatically change the secondary structure, which is predicted by software. Next, K_D value of mutants of pre-miR125a were measured. This mutation study revealed that essential motif of pre-miR125a to bind to folic acid (Figure 2c). Among the human miRNA precursors including both pri- and pre-miRNAs, this motif was only observed in miR125a precursors and this motif is conserved in mammalian miR125a precursors. This is the first report that precursor microRNA binds to metabolites, which suggests this interaction play some biological roles in miRNA processing pathway.

Orthogonal ribosome-tRNAs pair by engineering of peptidyl transferase center

The CCA-3′ sequence of tRNA is conserved among all organisms. In bacteria, the CCA end makes Watson-Crick base pairs with 23S rRNA in both A and P sites in the classical state during translation, which are revealed by biochemical analysis and crystal structural analysis (Fig. 3). In addition, biochemical analysis indicated that these bases also make base pairs during translocation. These base pairs are important for translation activity, and the compensatory mutations in these base pairs are tolerated during peptidyl transfer reaction and translocation. However, it is yet empirically unknown whether such mutations accommodates translation in its entirety. In this

study, I evaluated the translational activity of mutant ribosomes and CCA-mutated tRNAs pairs using a reconstituted *in vitro* translation system named as FIT (Flexible In vitro Translation) system, and discovered a certain compensatory mutant ribosome-tRNAs pair which works independently of the wild type ribosome-tRNAs pair in one pot.

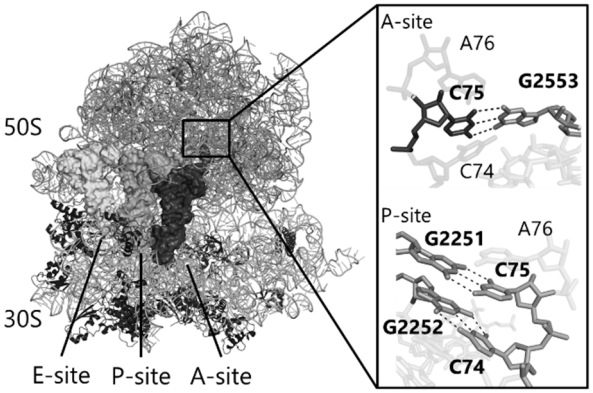

Figure 3 | Crystal structure of classical state of bacterial ribosome-tRNAs-mRNA complex. This figure was prepared based on the previously reported structure (PDB; 2WDK/2WDL).

In natural, ARSs specifically recognize the structure of a cognate tRNA, but most ARSs also interact with the universally conserved CCA end of tRNAs hampering aminoacylation onto CCA-mutated tRNAs. To avoid this problem, I utilized flexizyme which recognizes tRNA by two consecutive base pairs (Fig. 1). Thus, I considered compensatory mutations in the flexizymes allow aminoacylation of CCA-mutated tRNAs (termed as tRNAs-NNA). Three mutant tRNAs (C74G, C75G, C74G/C75G) and compensatory mutant flexizymes were prepared by *in vitro* transcription, and aminoacylation efficiency were measured using cyanomethyl esterified tyrosine (Tyr-CME) as a substrate (Fig. 4). The mutant tRNAs were efficiently aminoacylated by the cognate mutant flexizymes respectively, in contrast with the mispairs of mutant tRNAs and wild-type flexizymes. Several other amino acids could also be charged onto the appropriate mutant tRNAs in comparable yields.

Because *Escherichia coli* ARSs do not uniformly charge amino acids onto tRNAs-NNA, a conventional methods using radioisotope-labeling amino acids is unsuitable. Therefore, an assay system to detect translated peptides were established using FIT system and fluorescently labeled phenylalanine (Fph). A DNA template encoding Fph-Lys-Tyr-Lys-Lys-Tyr-Lys (peptide-1), aminoaclyl-tRNAs (aa-tRNAs) prepared by flexizymes and MS2-tag affinity purified ribosomes were mixed within FIT system, and the production of peptide-1 was measured by tricine-SDS-PAGE (Fig. 5). Consequently, both tRNAs-CCA and tRNAs-GCA could be utilized at almost the same efficiency by both wild-type and G2252C ribosomes. In contrast, tRNAs-CGA and tRNAs-GGA could be utilized only by each cognate ribosomes (G2251C/G2553C and G2251C/G2252C/G2553C) at the lower efficiency than wild-type pair. These results indicate that tRNAs-CGA and tRNAs-GGA but not tRNAs-GCA are orthogonal to the wild-type tRNAs-CCA.

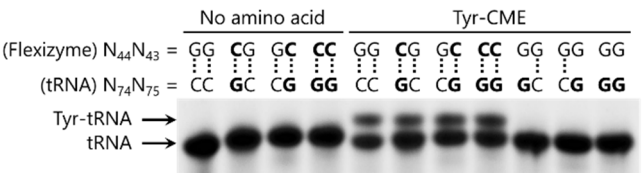

Figure 4 | Aminoacylation of CCA mutated tRNAs by engineered flexizymes[2].

Because the pair of G2251C/G2553C ribosome and tRNAs-CGA had comparable translational activity and acted as translational machinery orthogonal to the wild-type pair of ribosome and tRNAs-CCA, I examined whether two different peptides could be expressed from a single mRNA template in one pot. For the wild-type pair, Fph, Lys, Tyr and Asp were charged onto corresponding tRNAs, and for the mutant pair Fph, Lys, Anv (azidonorvaline) and Aly

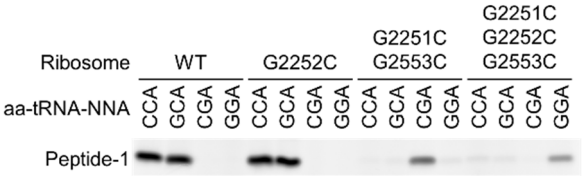

Figure 5 | Tricine-SDS-PAGE analysis of peptides translated by wild-type and mutant ribosome-tRNAs pairs[2].

(acetyllysine) were charged (Fig. 6a and b). These aa-tRNAs, ribosomes and a DNA template encoding peptide-2 according to the WT pair and peptide-3 according to the mutant pair was added to the FIT system to detect the translated peptides by MALDI-TOF-MS and tricine-SDS-PAGE (Fig. 6c and d). In the presence of only wild-type ribosome, only peptide-2 was detected by PAGE and MS analysis as expected. Adding the tRNAs-CGA to the wild-type ribosome-tRNAs pair did not change the translation product. Similarly, in the presence of only mutant ribosome, only peptide-3 was detected. Combining both wild-type and mutant ribosome-tRNAs pairs yielded the desired two peptides from a single DNA template according the wild-type and mutant pairs without any hybrid products generated from potential cross-reading(s) by non-cognate aa-tRNAs. This indicates that two coexisting translation machineries acted orthogonally. This study demonstrated a new way to reprogram the genetic code[2].

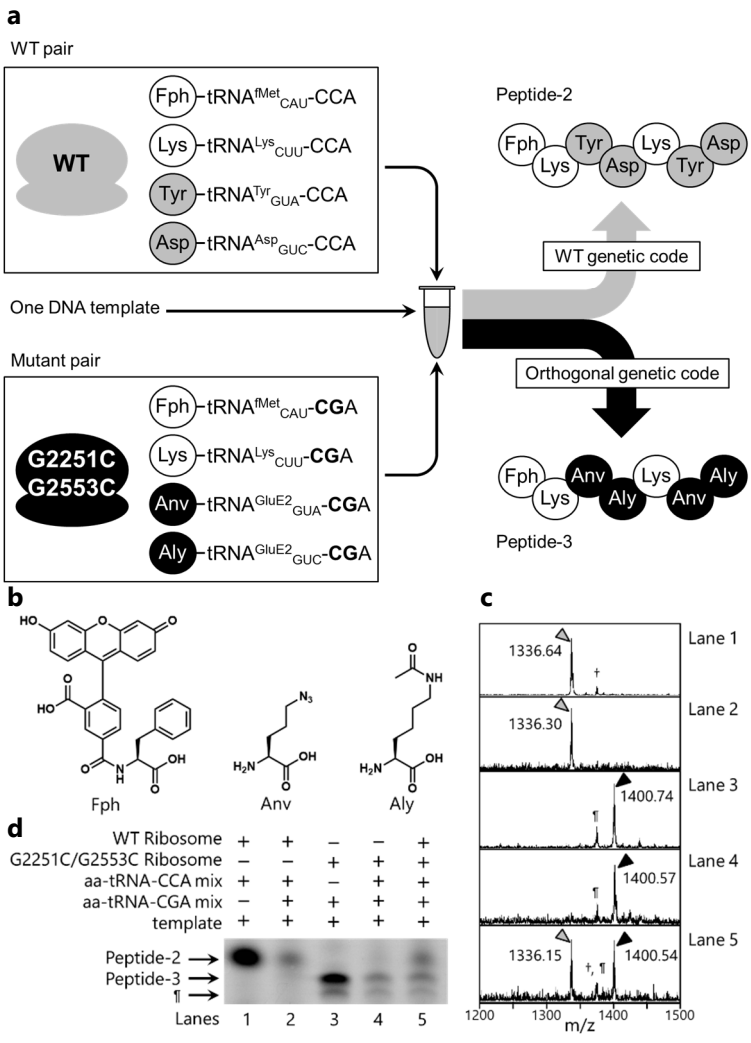

Figure 6 | Simultaneous expression of two different peptides from one mRNA[2]. (**a**) Schematic illustration of simultaneous expression. (**b**) Chemical structure of non-natural amino acids. (**c**) MALDI-TOF-MS analysis. Gray triangle: peptide-2 (calculated m/z = 1336.50), black triangle: peptide-3 (calculated m/z = 1400.67), †: K^+ adduct of peptide-2, ¶: Peptide-3 whose azide group was reduced to amine group. Numbers below the triangles indicate observed m/z. (**d**) Tricine-SDS-PAGE analysis.

Conclusion

In the first study, flexizyme was used as a tool to label and remove tRNAs using nonproteinogenic amino acid conjugated with biotin, and successfully discovered the novel interaction between folic acid and human pre-miR125a. In the second study, orthogonal translation machinery was developed using CCA-mutated aa-tRNAs prepared by compensatory mutated flexizymes. Because flexizyme only recognize 3′-end of substrate RNAs, any RNAs bearing NNA-3′ ends can be aminoacylated by compensatory mutated fleixyzmes with various amino acids having a variety of functional groups. These results demonstrated a powerful potential of flexizyme as a tool used for various RNA related research fields.

References

1 Terasaka, N. & Suga, H. *Chem. Lett.* **43**, 11-19 (2014).

2 Terasaka, N., Hayashi, G., Katoh, T. & Suga, H. *Nat. Chem. Biol.* **10**, 555-557 (2014).