An orthogonal ribosome-tRNAs pair by the engineering of peptidyl transferase center

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Abstract

The Watson-Crick base pairs between the 3′-terminal end of tRNAs and ribosomal RNA in the peptidyl transferase center are universally conserved. Here, we report that the introduction of compensatory mutations to *Escherichia coli* RNAs in this site leads to an orthogonal system independent of the wild type counterpart, as demonstrated via the production of two peptide sequences from a single mRNA. This work thus identifies a new way to reprogram the genetic code.

Main Text

The interaction of codon-anticodon base pairs in the mRNA-ribosome complex with peptidyl- and aminoacyl-tRNAs (aa-tRNAs) determines translation of the genetic code. However, in the peptidyl transferase (PT) center, a second critical Watson-Crick base-pairing interaction occurs between the universally conserved 3′ end of tRNAs, C74C75, and *Escherichia coli* (*E. coli*) 23S ribosomal RNA (rRNA) G2251G2252 at the P-site as well as with G2553 at the A-site (Fig. 1)[1-8] that is also required for translational fidelity. Previous work demonstrated that mutation of the ribosome at this site (G2553C) coupled with a paired mutation in a synthetic fragment of tRNA was able to restore activity to the single deactivating ribosomal mutation, suggesting that the compensatory mutation could be tolerated[9]. However, it is yet empirically unknown whether such a mutation(s) accommodates translation in its entirety, including the decoding event, to produce a polypeptide in an mRNA-dependent manner. We here report an engineered system utilizing pairs of ribosomal RNAs and tRNAs with the compensatory mutations described above. This translation machinery specifically uses a genetic code that is programmed distinctly from the naturally occurring genetic code

and so is able to synthesize peptides orthogonally to the wildtype counterpart. By means of these two translation machineries, a single mRNA produces two different peptides according to the artificially programmed genetic codes. This work represents a proof-of-concept of *in vitro* synthetic biology for generating novel translation machineries that function in parallel under their designated genetic codes.

Protein aminoacyl-tRNA synthetases (ARSs) specifically recognize the structure of a cognate tRNA, but most ARSs also interact with the universally conserved CCA-3′ end of tRNAs[10,11], hampering charging of amino acids onto tRNAs mutated in the CCA-3′ end [12-14]. To avoid this problem, we used here the flexible tRNA acylation ribozymes (flexizymes, dFx or eFx) that charge non-aromatic or aromatic sidechain amino acids using 3,5-dinitrobenzyl esters (DBE) or cyanomethyl esters (CME), respectively (Supplementary Results, Supplementary Fig. 1)[15,16], and whose activity is not dependent on the tRNA body sequence[17] (Supplementary Fig. 2). Thus, mutations in the flexizymes allow generation of aa-tRNAs bearing non-CCA-3′ ends[18].

We arbitrarily chose Lys (CUU) and Tyr (GUA) anticodons for generating the 3′ end mutant tRNAs bearing the body sequence of $tRNA^{AsnE2}$ (Supplementary Fig. 3)[19-21]. We *in vitro* transcribed three mutant tRNAs containing a single (C74G or C75G) or double mutations (C74G/C75G) within the Lys or Tyr anticodons and the compensatory C-single or double-mutant flexizymes derived from dFx and eFx (Supplementary Fig. 4). The mutant tRNAs were efficiently aminoacylated by the cognate mutants of dFx and eFx in the presence of Lys-DBE and Tyr-CME, respectively, as opposed to the mispairs of mutant tRNAs and wildtype flexizymes (Supplementary Fig. 4). Several other amino acids could also be charged onto the appropriate mutant tRNAs in comparable yields (Supplementary Figs. 1 and 5).

We next established an assay system to detect translated peptides. Because *E. coli* ARSs do not uniformly charge amino acids onto C74 and/or C75-mutant tRNAs[12-14], a conventional radioisotope-labeling method is unsuitable for a quantitative comparison of the peptide expression level. Instead, we utilized eFx to charge a fluorescently-labeled phenylalanine CME (Fph-CME, **1**) onto the C74C75-mutant $tRNAs^{fMet}_{CAU}$ (Online Methods, Supplementary Figs. 1, 3 and 5). We used an *E. coli* custom-made *in vitro* translation system that was integrated with the flexizyme technology, referred to as FIT system[21], to reassign the initiation codon from fMet to Fph. Lys and Tyr were charged onto $tRNA^{GluE2}_{CUU}$ and $tRNA^{GluE2}_{GUA}$ using dFx and eFx, respectively (Supplementary Fig. 3); and their corresponding C74C75-mutant tRNAs were also charged using the cognate flexizymes. We prepared a DNA template encoding Fph-Lys-Tyr-Lys-Lys-Tyr-Lys (heptapeptide-1), and tested its expression in the FIT system comprising T7 RNA polymerase, ribosomes, protein factors, and other essential components in the presence of the aa-tRNAs. Tricine-SDS-PAGE analysis of the translated product allowed us to visualize a fluorescent band (Fig. 2a) at the expected molecular weight as monitored by MALDI-TOF (Supplementary Fig. 6). We quantified band intensity as a function of time by comparison with a band generated by a known concentration of Fph. The production of peptide-1 plateaued at 30 min, with a final concentration of approximately 3.5 μM (Fig. 2b), thus allowing us to reliably quantify the level of peptide expression. We also examined expression of heptapeptide-1 using $aa\text{-}tRNAs^{AsnE2}$ under the same conditions, but the maximal expression level reached only 1.0 μM (Supplementary Fig. 7a); this lower expression level could be attributed to lower binding ability of $aa\text{-}tRNAs^{AsnE2}$ to EF-Tu than $aa\text{-}tRNA^{GluE2}$ (Supplementary Fig. 8).

We subsequently tested whether three mutant tRNAs bearing GCA-, CGA- or GGA-3′ ends were utilized by the wildtype ribosome for mRNA-dependent translation. To our surprise, the wildtype ribosome expressed the heptapeptide-1 using aa-tRNAs-GCA (Fig. 2a) while synthesis plateaued at a final concentration of 2.7 μM (Fig. 2b). In contrast, tRNAs-CGA and tRNAs-GGA were inactive (Fig. 2a). These results indicate that tRNAs-CGA and tRNAs-GGA but not tRNAs-GCA are orthogonal to the wild type tRNAs-CCA (Supplementary Fig. 9).

We then prepared three mutant ribosomes, G2252C, G2251C/G2553C, and G2251C/G2252C/G2553C using an MS2-tag affinity purification procedure[22]. The mutations of each ribosome were confirmed by the primer extension method[22] (Supplementary Fig. 10), giving the support of a negligible contamination of untagged wildtype ribosome (< 3%). As predicted from our results with the non-cognate pair of the wildtype ribosome and tRNAs-GCA, the G2252C ribosome was also active with both non-cognate tRNAs-CCA and cognate tRNAs-GCA (Fig. 2a and 2b) but not with tRNAs-CGA and tRNAs-GGA (Fig. 2a). The double-mutant ribosome, G2251C/G2553C, was active with its cognate mutant pair, tRNAs-CGA, yielding heptapeptide-1 as a major product (Fig. 2a and Supplementary Fig. 6). In contrast, neither pair of G2251C/G2553C with tRNAs-CCA, tRNAs-GCA nor tRNAs-GGA was sufficiently active, although a very faint band originating from the background crossreaction tolerated by the mispair between G2252 and G74 was detected for tRNAs-GGA. Time-course analysis and quantification of the peptide indicated that expression plateaued at a final concentration of 1.7 μM (Fig. 2b). This suggests that the cognate pair of G2251C/G2553C and tRNAs-CGA is sufficiently active and functions orthogonally to the wildtype pair;

however, it exhibits slower translation rate than the wildtype, resulting in a lower yield of peptide due to competing hydrolytic consumption of the aa-tRNAs.

The triple-mutant ribosome, G2251C/G2252C/G2553C, like the double mutant, was successful in generating heptapeptide-1 only with the cognate tRNAs-GGA (Fig. 2a). However, the rate of translation was diminished to less than 5% of the wildtype pair (Fig. 2b), which was reflected as a barely detectable peak of heptapeptide-1 in MALDI-TOF (Supplementary Fig. 6). This lowered rate indicates that despite the fact that the G-C base pairs occurring at G2251/G2252/G2553 of 23S rRNA and C74C75 of tRNAs are essential for the PT reaction[9], the compensatory mutations did not fully restore the translation activity, and further suggests that these bases play crucial roles in not only the PT reaction but also in other steps of translation, potentially involving interactions with other rRNA nucleotides or protein factors during translocation. Of note, we did confirm that EF-Tu was able to form ternary complexes with GTP and the aa-tRNA mutants (Supplementary Fig. 8). We also examined three other ribosome mutants to test for possible orthogonal function, G2251A/G2553A, G2251C and G2253C, although these ribosomes with the cognate or noncognate tRNAs were insufficiently active to yield the peptides neither for the time course experiment nor MALDI-TOF analysis (Supplementary Fig. 11).

Because the pair of G2251C/G2553C-ribosome and tRNAs-CGA had comparable translational activity and acted as translational machinery orthogonal to the wildtype ribosome and tRNAs-CCA pair, we decided to examine whether two distinct peptides could be expressed from a single mRNA template under artificially programmed genetic codes. We assigned four amino acids to the respective genetic codes as follows: For the wildtype ribosome–tRNAs pair, a genetic code (referred to as WT-code) assigned Fph

(AUG), Lys (AAG), Tyr (UAC), and Asp (GAC); and for the pair of double-mutant ribosome and tRNAs-CGA, an orthogonal genetic code (referred to as OR-code) assigned Fph (AUG), Lys (AAG), L-Anv (UAC), and L-Aly (GAC) (Supplementary Figs. 12a and b). The tRNAs-CCA and tRNAs-CGA charged with the respective amino acids were prepared using wildtype or mutant flexizymes. A DNA template encoding heptapeptide-2 according to the WT-code and heptapeptide-3 according to the OR-code was added to the FIT system comprising the combination of the wildtype, double-mutant ribosomes, and/or the above aa-tRNAs (Fig. 3a).

Translation of an mRNA with the wildtype ribosome and aa-tRNAs-CCA pair yielded the heptapeptide-2 as expected (Fig. 3b-d). Adding the orthogonal tRNAs to the wildtype pair did not change the outcome. Similarly, the FIT system comprising the pair of mutant ribosome and aa-tRNAs-CGA or this pair with the wildtype tRNA yielded heptapeptide-3 (Fig. 3b-d). The decreased yields observed when one ribosome was combined with both tRNAs could not be compensated by increasing EF-Tu concentrations from 10 μM to 20 μM, ruling out EF-Tu sequestration by the mismatched tRNA as an explanation for this effect (Supplementary Fig. 14). Combining both pairs of ribosome–tRNAs yielded the desired two heptapeptides from a single DNA template according the WT- and OR-codes (Fig. 3b-d), while no peptide was detected in the absence of the DNA template (Fig. 3b); being consistent with the observation that translation proceeded in an mRNA-dependent manner. Moreover, the same experiment using a different DNA template produced two other heptapeptides according to the programmed genetic code (Supplementary Fig. 15). Most importantly, we did not detect any hybrid products generated from potential cross-reading(s) of

codons in the non-cognate genetic code, indicating that the two coexisting translational machineries acted orthogonally and utilized only the cognate genetic codes.

Here, we demonstrated that two orthogonal translational machineries could function in parallel, producing two distinct peptides from a single mRNA template according to artificially programmed genetic codes. We also revealed that the wildtype ribosome (and G2252C-ribosome) cross-reacts with cognate tRNAs-CCA and non-cognate tRNAs-GCA indicating that mispairing between G2252 and C74 is tolerated in translation reaction. In contrast, G2251-C75 and G2553-C75 base pairs are more critical for activity, and their mispairing completely abolishes translation. However, the compensatory restoration of these base pairs recovers the translational activity, allowing us to create a ribosome-tRNAs pair that functions orthogonally to the wildtype counterpart and specifically uses an artificially programmed genetic code. This demonstration opens an entrance to a new opportunity of *in-vitro* synthetic biology involving the engineering of the genetic codes and translation machineries.

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Author Contributions

N.T and H.G. conducted biochemical and chemical studies, and T.K. and H.S. supervised the research. All authors contributed to writing the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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Figure legends

Figure 1| Base pair interaction of the 3′ end of tRNAs to 23S rRNA and flexizymes. Interaction between the 3′ end of tRNAs and the peptidyl transferase center in *E. coli* 23S rRNA[7]. Bold lines indicate helix and loop structures. Lines between bases denote the number of hydrogen bonding and dots (•) denote non-Watson-Crick base pairs.

Figure 2| Translational activity of pairs of ribosome and tRNA mutants. (**a**) Tricine-SDS-PAGE analysis of heptapeptide-1 (FphLysTyrLysLysTyrLys) synthesized using the designated pairs of ribosome–tRNA mutants. $tRNAs^{fMet}_{CAU}$ bearing the respective NNA-3' end were used as initiator tRNAs, and $tRNAs^{GluE2}_{NNN}$ (NNN denotes CUU and GUA designating Lys and Tyr, respectively) bearing the respective NNA-3' end were used as elongator tRNAs. Faint bands appeared in lanes 9, 10, 13 and 14 could be ascribed to a peptide originating from the background translation by a trace amount (< 3%) of wildtype ribosome contamination to the respective mutant ribosome, indicated by the experiment shown in Supplementary Figure 10; and a faint band in lane 12 could be a product of cross-reaction of the double mutant ribosome with aa-tRNAs-GGA. However, these bands are too faint to reliably quantify the intensity for the time-course experiment below and determine the product identity by MALDI-TOF analysis. The entire gel image is shown in Supplementary Figure 16. (**b**) Time-course analysis of heptapeptide-1 production by active pairs of ribosomes and tRNAs. We investigated four cognate pairs of the wildtype ribosome–tRNAs-CCA (cyan solid line), G2252C-ribosome–tRNAs-GCA (yellow solid), G2251C/G2553C-ribosome–tRNAs-CGA (red solid), and G2251C/G2252C/G2553C-ribosome–tRNAs-GGA (purple solid), and two non-cognate pairs of the wildtype ribosome–tRNAs-GCA (black dashed)

and G2252C-ribosome–tRNAs-CCA (brown dashed). The data represent the average of three independent reactions. The error bars represent the standard deviation.

Figure 3| Simultaneous expression of two distinct peptides from a single mRNA sequence under two artificially programmed genetic codes. (**a**) Schematic illustration of simultaneous expression. The wildtype ribosome–tRNAs-CCA and G2251C/G2553C-ribosome–tRNA-CGA pairs generated heptapeptide-2 and heptapeptide-3, respectively, according to the WT- and OR-codes. (**b**) Tricine-SDS-PAGE analysis of the respective heptapeptides. The entire gel image is shown in Supplementary Figure 17. (**c**) Time-course analysis of simultaneous expression of heptapeptides. The region of y-axis in 0–0.45 μM of peptide concentration is shown, while the entire region of y-axis in 0–1.0 μM of peptide concentration is shown in Supplementary Figure 13. Heptapeptide-2 expression is seen in lane 1 (black dashed line), lane 2 (purple) and lane 5 (cyan, simultaneous expression); heptapeptide-3 expression is seen in lane 3 (brown dashed line) lane 4 (yellow) and lane 5 (red, simultaneous expression). Lane numbers are those described in Figure 3b. The data represent the average of three independent reactions and error bars represent the standard deviation. (**d**) MALDI-TOF MS analysis of translational products. Calculated (C) and observed mass (O) values are shown in the right panel of the spectra. † and ‡ denote a potassium adduct of heptapeptide-2 and heptapeptide-3, respectively. ¶ denotes heptapeptide-3 whose azide group was reduced to a primary amino group presumably by thiols present in the FIT system[23]. Since other minor unidentified peaks were present in the non-templated translation product, they are likely present in the translation system.

The data shown in Figure 3b and 3d were generated from a sample of the end product of translation reaction.

Methods

Chemical synthesis of L-tyrosine cyanomethyl ester (Tyr-CME), L-aspartic acid 3,5-dinitrobenzyl ester (Asp-DBE), L-lysine 3,5-dinitrobenzyl ester (Lys-DBE), L-acetyllysine 3,5-dinitrobenzyl ester (Aly-DBE), and L-azidonorvaline 3,5-dinitrobenzyl ester (Anv-DBE)

Tyr-CME, Asp-DBE, Lys-DBE, Aly-DBE and Anv-DBE were synthesized as previously described[15,23].

Chemical synthesis of *N*-(5-FAM)-L-phenylalanine-cyanomethyl ester (Fph-CME)

L-phenylalanine-cyanomethyl ester (Phe-CME) was synthesized as previously described[18]. Triethylamine (22 μL, 160 μmol) was added to a mixture of Phe-CME (14.4 mg, 60 μmol) and 5-carboxyfluorescein succinimidyl ester (Lifetechnologies, 19 mg, 40 μmol) in 500 μL of *N*,*N*-dimethylformamide and then the mixture was stirred at room temperature for 13.5 h. After the reaction, ethyl acetate was added, and the solution was treated with 1 M HCl (3 mL × 3) and saturated NaCl (3 mL × 1). The organic layer was dried over Na_2SO_4, and concentrated under reduced pressure. The crude residue was dissolved in methanol and injected into a high-pressure liquid chromatography (HPLC) system equipped with a Cadenza 5CD-18 reverse-phase chromatography column (Code 5CD0Q6). The column was equilibrated with 20% (v/v) acetonitrile in H_2O containing 0.1% (v/v) TFA, and eluted with a 2.2%/min gradient of 99.9% (v/v) acetonitrile in 0.1% (v/v) TFA to 90% (v/v) acetonitrile. The HPLC profile was monitored by measuring the absorbance at 494 nm. The fractions of Fph-CME were

lyophilized. The concentration was determined by absorbance at 494 nm in 500 mM Tris-HCl (pH 8.0).

Fph-CME: ^{1}H NMR (300 MHz, DMSO-d_6, δ): 10.16 (br, 2H), 9.36 (d, J = 7.5 Hz, 1H), 8.45 (d, J = 0.6 Hz, 1H), 8.19 (dd, J = 8.1, 1.5 Hz, 1H), 7.39 (d, J = 8.1 Hz, 1H), 7.36–7.20 (m, 5H), 6.68 (d, J = 2.1 Hz, 2H), 6.61–6.52 (m, 4H), 5.05 (s, 1H), 4.87–4.80 (m, 1H), 3.33–3.14 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6, δ): 171.0, 168.5, 165.4, 160.0, 155.6, 152.2, 137.6, 135.4, 135.3, 129.7, 129.5, 128.8, 127.1, 127.0, 124.9, 123.9, 116.2, 113.1, 109.3, 102.7, 83.8, 54.5, 50.0, 36.2.

The high-resolution mass spectrum (HRMS) was determined using flow injection (direct electrospray ionization (ESI)-MS) in positive mode (Thermo Exactive). Calculated [$(M+H)^+$] *m/z* for $C_{32}H_{23}N_2O_8^+$ = 563.1449, found = 563.1445.

In vitro transcription

The tRNAs, eFxs, and dFxs were prepared using run-off in vitro transcription with T7 RNA polymerase. DNA templates were modified with 2′-*O*-methylation at the second last nucleotide of the 5′ termini to reduce non-templated nucleotide addition by T7 RNA polymerase[24]. The primers for preparing transcription templates are shown in Supplementary Table 1. All non-methylated primers were purchased from Operon Biotechnology (Japan) and methylated primers were purchased from Gene Design (Japan).

Expression and purification of maltose binding protein (MBP) -MS2-His_6

The plasmid pMAL-c2g (Amp^R) encoding His_6-tagged MS2 coat protein (a gift from R. Green, Johns Hopkins University) was used to transform *E. coli* BL21-(DE3) in LB medium with 100 μg/mL carbenicillin. A single colony isolated by streaking was used to inoculate a 30 mL overnight culture in LB supplemented with 100 μg/mL carbenicillin, and 10 mL of this culture was used to inoculate one liter of the same medium. The culture was grown to an OD_{600} of 0.7 at 37°C. The expression of MBP-MS2-His_6 was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were cultured for another 4 h at 37°C. The culture was then centrifuged for 10 min at 4,000× *g*, the pelleted cells were resuspended in 30 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 6 mM β-mercaptoethanol (β-ME), 1 mM phenylmethanesulfonyl fluoride, adjusted to pH 8.0 with NaOH), and were then sonicated. The lysate was clarified twice by centrifugation for 20 min at 15,000× *g* (4°C) and filtered through a Millex-LH 0.45 μm filter unit (Millipore). MBP-MS2-His_6 was purified using a fast protein liquid chromatography (FPLC) system (AKTA Avant, GE Healthcare) with a His-Trap HP column (GE Healthcare). The column was washed with NiNTA buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, 6 mM β-ME, adjusted to pH 8.0 with NaOH) and the protein was eluted with NiNTA buffer containing 250 mM imidazole. Protein-containing fractions were pooled and concentrated using a membrane filter (Amicon Ultra15, 3,000 MWCO, Millipore). The concentrated protein was dialyzed against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20% glycerol, and 1 mM dithiothreitol (DTT). The protein concentration was determined by absorbance at 280 nm, assuming an extinction coefficient of 84,800 cm^{-1} M^{-1} (calculated on: http://web.expasy.org/protparam/).

Expression and purification of tagged ribosomes

The plasmid pcI^{857} (Kan^{R}) encoding a temperature-sensitive mutant of the lambda repressor protein cI (a gift from R. Green, Johns Hopkins University)[22] was used to transform *E. coli* DH5α in LB medium containing 50 μg/mL kanamycin. Any 23S rRNA mutation of interest was introduced into the plasmid p278 MS2 encoding 23S rRNA tagged with the MS2 stem-loop (a gift from R. Green, Johns Hopkins University) by site directed mutagenesis using the Quickchange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). The primers for mutagenesis are shown in Supplementary Table 1. The mutations were confirmed by sequencing. The mutated plasmid p278 MS2 was used to transform DH5α with pcI^{857} in LB medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. A colony isolated by streaking was used to inoculate a 50 mL overnight culture grown in LB supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin at 30°C. One liter of this medium was inoculated with 20 mL of an overnight culture and grown to an OD_{600} of 0.7–0.9 at 42°C. The cultures were centrifuged for 10 min at 4,000× *g* and the pelleted cells were resuspended in 5.5 mL ribosome buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, and 6 mM β-ME). Lysozyme (Nacalai Tesque) was added (final concentration, 0.1 mg/mL) and incubated at 4°C for 30 min. This solution was disrupted twice using a cell disruption vessel (Parr Instrument Company). The lysate was clarified twice by centrifugation for 15 min at 15,000× *g* (4°C), layered onto 10 mL of ribosome buffer D (20 mM Tris-HCl, pH 7.5, 500 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.1 M sucrose) in a 25PC tube (Hitachi), and centrifuged at 104,000× *g* in an S50A rotor (Hitachi) for 18–20 h at 4°C. Ribosome buffer A was used to rinse and then dissolve the pellet, which was then stored at −80°C.

Tagged ribosomes were purified using FPLC (AKTA Avant, GE Healthcare). First, 2 mg MBP-MS2-His_6 was loaded on an MBP Trap HP column (GE Healthcare), and the bound protein was washed with ribosome binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, and 10 mM $MgCl_2$). Crude ribosomes were loaded onto the column and washed with 25 mL of ribosome-binding buffer. Tagged ribosomes bound to MBP-MS2-His_6 were eluted with 20 mL of ribosome-elution buffer (20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 10 mM maltose). Purified tagged ribosomes were concentrated to 10–20 μM, and the buffer was exchanged with RE buffer (20 mM HEPES-KOH, pH 7.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT) using membrane filtration (Amicon Ultra15, 100,000 MWCO, Millipore). The concentration of 70S ribosomes was determined by absorbance at 260 nm using the conversion factor 1 A_{260} = 23 pmol.

Aminoacylation by flexizymes

Aminoacylation reactions of Tyr-CME, Asp-DBE, Lys-DBE, Aly-DBE, and Anv-DBE were performed as previously described[15]. The reaction times were changed to 30 min (Tyr-CME), 9 hours (Asp-DBE), and 3 hours (Lys-DBE, Aly-DBE and Anv-DBE). Aminoacylation of Fph-CME was performed as follows: A mixture of 3 μL of 41.7 μM $tRNA^{fMet}_{CAU}$ and 41.7 μM eFx in 83.3 mM HEPES-KOH (pH 7.5) was heated at 95°C for 2 min and cooled to room temperature over 5 min. One microliter of 3 M $MgCl_2$ was added, and the mixture was transferred to an ice bath, 1 μL of 5 mM Fph-CME was added, and the mixture was incubated on ice for 16 h. After the reaction, aminoacyl-tRNA (aa-tRNA) was precipitated with ethanol as previously described[15].

Analysis of acylation

Ethanol-precipitated aa-tRNA was dissolved in acid-PAGE loading buffer (150 mM NaOAc, pH 5.2, 10 mM EDTA, and 93% (v/v) formamide) and then loaded on acid-urea 12% polyacrylamide gels (8 M urea, 50 mM NaOAc, pH 5.2). Electrophoresis was performed using 300 V (approximately 10 V cm^{-1}) for 21 h. The gels were stained with ethidium bromide and analyzed using an FLA-5100 fluorescent image analyzer (Fujifilm) or PharosFX molecular imager (BIO-RAD). Aminoacylation efficiency was calculated according to the band intensity of aa-tRNA (A) and free tRNA (T) and is presented as (A)/[(A) + (T)]. Because of the fluorescence of Fph-tRNA, the efficiency for Fph-CME was calculated using the expression $1-[(T_{aa+})(F_{aa-})]/[(T_{aa-})(F_{aa+})]$ according to the band intensity of flexizyme in the lane without amino acids (F_{aa-}), flexizyme in the lane with amino acids (F_{aa+}), free tRNA in the lane without amino acids (T_{aa-}), and free tRNA in the lane with amino acids (T_{aa+}). Values reported are the average of three independent reactions, and error bars represent the standard deviation.

DNA templates for translation

The DNA templates for translation were prepared using the polymerase chain reaction (PCR), and the amplicons were extracted with phenol-chloroform mixture and precipitated with ethanol. The DNA templates were purified using 12% native-PAGE, and the concentrations were determined by absorbance at 260 nm. The primers for preparing DNA templates for translation are shown in Supplementary Table 1.

***In vitro* translation using the FIT system**

The FIT system comprised a mixture of all desired components for translation[21]. The ribosomes and aa-tRNAs were added as necessary in each experiment. The composition of the FIT system was as follows: 50 mM HEPES-KOH (pH 7.6), 12 mM magnesium acetate, 100 mM potassium acetate, 2 mM spermidine, 20 mM creatine phosphate, 2 mM DTT, 2 mM ATP, 2 mM GTP, 1 mM CTP, 1 mM UTP, 0.6 μM MTF, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 0.26 μM EF-G, 10 μM EF-Tu, 10 μM EF-Ts, 0.25 μM RF1, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 0.1 μM T7 RNA polymerase, 4 μg/mL creatine kinase, 3 μg/mL myokinase, 0.1 μM pyrophosphatase, 0.1 μM nucleotide-diphosphatase kinase, and 400 nM DNA template. The concentration of EF-Tu and EF-Ts only in the experiment shown in Supplementary Figure 14 was increased to 20μM. Translation reactions were started by adding aa-tRNAs at 37°C after a 5 min incubation. Translation reactions were terminated by adding an equal volume of 2× Tricine-SDS-PAGE loading buffer (900 mM Tris-HCl (pH 8.45), 8% (w/v) SDS, 30% (v/v) glycerol).

In the experiment shown in Figure 2 and Supplementary Figure 6 and 11, the concentration of ribosomes, Fph-tRNA$^{fMet}_{CAU,}$ Lys-tRNA$^{GluE2}_{CUU}$, and Tyr-tRNA$^{GluE2}_{GUA}$ were 2 μM, 5 μM, 20 μM, 10 μM, respectively. The sequence of the DNA template was 5′-GGCGT AATAC GACTC ACTAT AGGGC TTTAA TAAGG AGAAA AACAT GAAGT ACAAG AAGTA CAAGT GAGCT TCG-3′.

In the experiment shown in Figure 3, and Supplementary Figures 13 and 14, the concentration of the wildtype ribosomes, G2251C/G2553C ribosomes, Fph-tRNA$^{fMet}_{CAU}$-CCA, Fph-tRNA$^{fMet}_{CAU}$-CGA, Lys-tRNA$^{Lys}_{CUU}$-CCA,

Lys-tRNA$^{Lys}_{CUU}$-CGA, Asp-tRNA$^{Asp}_{GUC}$-CCA, Aly-tRNA$^{GluE2}_{GUC}$-CGA, Tyr-tRNA$^{Tyr}_{GUA}$-CCA, and Anv-tRNA$^{GluE2}_{GUA}$-CGA were 0.1 μM, 2 μM, 5 μM, 1.5 μM, 10 μM, 3 μM, 10 μM, 3 μM, 10 μM and 3 μM, respectively. The sequence of the DNA template was 5′-GGCGT AATAC GACTC ACTAT AGGGC TTTAA TAAGG AGAAA AACAT GAAGT ACGAC AAGTA CGACT GAGCT TCG-3′.

In the experiment shown in Supplementary Figure 7, the concentration of ribosomes, Fph-tRNA$^{fMet}_{CAU,}$ Lys-tRNA$^{AsnE2}_{CUU}$, and Tyr-tRNA$^{AsnE2}_{GUA}$ were 2 μM, 5 μM, 20 μM, 10 μM, respectively. The sequence of the DNA template was 5′-GGCGT AATAC GACTC ACTAT AGGGC TTTAA TAAGG AGAAA AACAT GAAGT ACAAG AAGTA CAAGT GAGCT TCG-3′.

In the experiment shown in Supplementary Figure 15, the concentration of the wildtype ribosomes, G2251C/G2553C ribosomes, Fph-tRNA$^{fMet}_{CAU}$-CCA, Fph-tRNA$^{fMet}_{CAU}$-CGA, Lys-tRNA$^{Lys}_{CUU}$-CCA, Lys-tRNA$^{Lys}_{CUU}$-CGA, Asp-tRNA$^{Asp}_{GUC}$-CCA, and Aly-tRNA$^{GluE2}_{GUC}$-CGA were 0.3 μM, 2 μM, 5 μM, 1.5 μM, 20 μM, 6 μM, 10 μM, 3 μM, respectively. The sequence of the DNA template was 5′-GGCGT AATAC GACTC ACTAT AGGGC TTTAA TAAGG AGAAA AACAT GAAGG ACAAG AAGGA CAAGT GAGCT TCG-3′.

The aa-tRNAs were prepared by aminoacylation of tRNAs using cognate flexizymes. The concentration of aa-tRNA was calculated from acylation efficiency shown in Supplementary Figure 5.

MALDI-TOF analysis

After incubation, the *in vitro* translation mixtures were desalted using a *C*18-tip (Nikkyo Technos) and analyzed using MALDI-TOF mass in linear positive modes. All MALDI-TOF analysis was performed using an Autoflex II (Bruker Daltonics) or ultrafleXtreme (Bruker Daltonics) with external calibration (Peptide Calibration Standard II, Bruker Daltonics).

Tricine-SDS-PAGE analysis of translation products

The quantity of Fph incorporated into peptides was determined using Tricine-SDS-PAGE as previously described[21]. The concentration of acrylamide in separation gel was 12% for the experiment shown in Figure 2a and Supplementary Figure 11a, 19% for Figure 3b and 20% for Supplementary figure 15b. The fluorescence of the peptides was determined using the FLA-5100 (Fujifilim) or PharosFX molecular imager (BIO-RAD), and the data were fit to an exponential curve using KaleidaGraph (Synergy Software). The amounts of peptides were quantified according to that of a fluorescent band generated by a known concentration (0 μM to 4 μM) of Fph. Values reported are the average of three independent reactions, and error bars represent the standard deviation.

Quantification of the abundance of tagged ribosome by primer extension

Primer extension analysis was performed mainly following the previous report[22]. Primer extension using a primer that is complementary to the bases 2254-2273 in 23S rRNA produced a dideoxyguanosine stop at +2 for tagged G2252C , +3 for tagged G2251C, or +6 for untagged wildtype ribosomes, or a dideoxythymidine stop at +3 for

tagged G2251A or +7 for untagged wildtype ribosomes. Primer extension using a primer that is complementary to the bases 2556-2575 in 23S rRNA produced a dideoxyguanosine stop at +3 for tagged G2553C or +5 for untagged wildtype ribosomes, or a dideoxythymidine stop at +3 for tagged G2553A or +9 for untagged wildtype ribosomes. Ribosomal RNAs were extracted from purified ribosomes by phenol-chloroform extraction and ethanol precipitation. Primer extension was carried out as follows: A mixture of 7 μL of 57.1 nM rRNA, 171.4 nM [^{32}P] 5′-end labeled primer, 7.14 mM each dATP/dCTP/dTTP/ddGTP or dATP/dGTP/dCTP/ddTTP was heated at 65°C for 5 min and cooled on ice 1 min. Two microliter of 5× FS buffer (Lifetechnologies), 0.5 μL of 0.1 M DTT and 0.5 μL of SuperScript™ III (Lifetechnologies) were added, and the mixture were incubated at 55°C for 1 hour. Primer extension products were resolved on a 15% denaturing polyacrylamide gel (8M urea, 1×TBE) and the intensities of the bands were quantified using FLA-5100 (Fujifilim).

Gel-shift analysis of ternary complex of EF-Tu, GTP and aa-tRNA

Binding of EF-Tu to aa-tRNAs was investigated by following previous report[25]. First, 10 μM EF-Tu was preincubated with 1 mM GTP at 37°C for 15 min in 5 μL total volume containing 70 mM HEPES-KOH (pH 7.6), 52 mM NH_4OAc, 8 mM $Mg(OAc)_2$, 30 mM KCl, 0.8 mM DTT, 1.6 μM GDP, 6% glycerol, 10 mM phosphoenolpyruvate, and 0.08 U/μL pyruvate kinase. After aminoacylation reaction by flexizyme, the ethanol precipitated mixtures which contain flexizymes, aa-tRNAs and tRNAs were resuspended in 6 mM KOAc at 8.3 μM final concentration of aa-tRNA. The concentrations of aa-tRNAs were calculated from acylaton efficiency shown in

Supplementary Fig. 3. This 3 μL aa-tRNA solution and 2 μL of ternary complex buffer, containing 150 mM HEPES-KOH (pH 7.6), 195 mM NH_4OAc, and 30 mM $Mg(OAc)_2$ were added to the preincubated EF-Tu solution. The mixture was incubated at 37°C for 10 min. Native-PAGE was performed using 8% polyacrylamide gels at 4°C in a running buffer containing 50 mM Tris-HCl (pH 6.8), 65 mM NH_4OAc, and 10 mM $Mg(OAc)_2$. Gels were stained by SimplyBlue^{TM} SafeStain (Lifetechnologies). Intensities of bands were analyzed by Multi Gauge (Fujifilim).

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