Uniform affinity-tuning of N-methyl-aminoacyl-tRNAs to EF-Tu enhances their multiple incorporation

Yoshihiko Iwane†, Hiroyuki Kimura †, Takayuki Katoh and Hiroaki Suga *

Department of Chemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received December 20, 2020; Revised April 05, 2021; Editorial Decision April 07, 2021; Accepted May 12, 2021

ABSTRACT

In ribosomal translation, the accommodation of aminoacyl-tRNAs into the ribosome is mediated by elongation factor thermo unstable (EF-Tu). The structures of proteinogenic aminoacyl-tRNAs (pAA-tRNAs) are fine-tuned to have uniform binding affinities to EF-Tu in order that all proteinogenic amino acids can be incorporated into the nascent peptide chain with similar efficiencies. Although genetic code reprogramming has enabled the incorporation of non-proteinogenic amino acids (npAAs) into the nascent peptide chain, the incorporation of some npAAs, such as N-methyl-amino acids (MeAAs), is less efficient, especially when MeAAs frequently and/or consecutively appear in a peptide sequence. Such poor incorporation efficiencies can be attributed to inadequate affinities of MeAA-tRNAs to EF-Tu. Taking advantage of flexizymes, here we have experimentally verified that the affinities of MeAA-tRNAs to EF-Tu are indeed weaker than those of pAA-tRNAs. Since the T-stem of tRNA plays a major role in interacting with EF-Tu, we have engineered the T-stem sequence to tune the affinity of MeAA-tRNAs to EF-Tu. The uniform affinity-tuning of the individual pairs has successfully enhanced the incorporation of MeAAs, achieving the incorporation of nine distinct MeAAs into both linear and thioether-macrocyclic peptide scaffolds.

INTRODUCTION

In prokaryotic translation machinery, all 20 proteinogenic amino acids (pAAs) are charged onto their corresponding tRNAs, and the resulting aminoacyl-tRNAs (pAA-tRNAs) are subsequently accommodated into the ribosomal A site by elongation factor thermo unstable, EF-Tu (Figure 1A) (1,2). EF-Tu recognizes two distinct regions of pAA-tRNAs: the amino acid moiety and the T-stem region of the tRNA (3–5). The binding affinities between EF-Tu and pAA-tRNAs are determined by the sum of these two interactions. Uhlenbeck and colleagues have demonstrated that twenty distinct pAA-tRNAs bind to EF-Tu with near-uniform affinities from $-9.5$ to $-10.5$ kcal/mol (6). This is achieved by a compensatory relationship between the two binding regions (6–11). In the case of Glu-tRNA$^{\text{Glu}}$, for example, the intrinsic weak affinity of Glu to EF-Tu is compensated by the strong affinity of the T-stem region of tRNA$^{\text{Glu}}$. Such a narrow range of uniform affinities serves as a threshold for excluding mischarged pAA-tRNAs, for example, Glu-tRNA$^{\text{Gln}}$ has an insufficient EF-Tu affinity, and is thus poorly accommodated into the ribosome A site, resulting in failure of efficient peptide elongation (12).

Diverse non-proteinogenic amino acids (npAAs) can be also ribosomally incorporated into nascent peptides by means of genetic code manipulation methodologies (13–16). However, ribosomal incorporation of npAAs often suffers from poorer efficiency than the pAA incorporation. The Schultz group has conducted in vitro evolution of amber suppressor tRNAs from semi-random sequences based on Methanocaldococcus jannaschii tRNA$^{\text{Phe}}$ and obtained those having improved efficiencies for the incorporation of bulky phenylalanine (Phe) analogs into proteins in Escherichia coli (17). Although the fidelity of their incorporation was not clearly defined in their work, the expression level was improved by 2–20-fold likely owing to an affinity enhancement of the npAA-tRNA to EF-Tu. Alternatively, EF-Tu mutants were also engineered to accept certain Phe analogs or O-phosphoserine when they were charged onto cognate tRNAs, where the expression of designated protein containing such npAAs was enhanced (18–20).

1To whom correspondence should be addressed. Tel: +81 3 5841 8372; Fax: +81 3 5841 8372; Email: hsuga@chem.s.u-tokyo.ac.jp
2The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

© The Author(s) 2021. Published by Oxford University Press on behalf of Nucleic Acids Research. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Our group logically devised a novel chimeric tRNA based on D-arm of tRNA^Phe^ and T-stem of tRNA^Glu^, referred to as tRNA^Phe^E^-2^, which were designed to effectively recruit EF-P and EF-Tu, respectively (27). The use of such designer tRNAs has made remarkable improvements of npAA incorporation by the assist of EF-P in not only single but also multiple and consecutive manners. When certain D-amino acids, β-amino acids, or cyclic γ-amino acids were charged on to this series of tRNAs, their incorporation into nascent peptide chain was improved by up to 13-fold that is a comparable level to their natural counterparts; and in some cases improved from an undetectable expression level to a detectable level (27–32). These results indicate that appropriately designed tRNAs could improve the overall translation efficiency of exotic peptides containing various npAAs with wildtype protein factors, EF-P and EF-Tu.

MeAAs are invaluable building blocks to install druglike properties to the peptides, for example, peptidase resistance and membrane-permeability (33–38). Our previous study reported that when MeAAs were charged onto tRNA^Asn^E^-2^ (our standard suppressor tRNA for various npAA incorporations), only five MeAAs (MeG, MeA, MeS, MeF and MeY) showed good incorporation efficiencies (>80% relative to a pAA control); six MeAAs (MeT, MeC, MeQ, MeM, MeH and MeW) were moderate (10–80%); and the other eight MeAAs (MeV, MeL, MeI, MeN, MeD, MeE, MeR and MeK) were poor or undetectable (<10%) (39). It should be noted that the above observation was made by the study of a single incorporation of MeAA into nascent peptide chain (39,40). However, when the frequency of incorporating MeAA residues in nascent peptide chain increases, the expression level as well as fidelity detrimentally decrease (39). Moreover, the consecutive incorporation of MeAAs or even alternating incorporation of MeAA and pAA (e.g. MeAA-pAA-MeAA) is far less efficient when compared to pAA elongation in such peptide sequences. Thus, there remains an important challenge to incorporate not only a greater number of distinct MeAAs but also consecutively and/or alternately into the nascent peptide chain.

Here, we have aimed at improving the incorporation efficiencies of MeAAs by tuning the affinity between MeAA-tRNA and EF-Tu. According to the crystal structure of the ternary complex of EF-Tu-Phe-tRNA^Phe^-GTP, the N-methyl modification on the amino acid would hamper the interaction of EF-Tu with MeAA-tRNA (Figure 1B) (3), which could weaken their affinity, thereby resulting in reduced incorporation efficiency of MeAA (Figure 1C). Since the T-stem of tRNA plays a critical role in tuning the affinity between AA-tRNA and EF-Tu, we have hypothesized that an appropriate engineering of tRNA T-stem would allow us to uniformly tune the affinity between the corresponding MeAA-tRNA and EF-Tu similar to that of pAA-tRNAs and EF-Tu, and thereby the MeAA incorporation efficiencies into nascent peptide chain could be improved (Figure 1D).

**MATERIALS AND METHODS**

**Preparation of tRNAs, flexizymes, mDNAs and mRNAs**

All oligonucleotides listed in Supplementary Table S1 were purchased from Eurofins, Japan. In the nucleotide se-
quences, N(Me) indicates a 2'-O-methylated nucleotide, which aims at suppressing undesired addition of extra nucleotides at 3' end of RNA in transcription (41). The sequences of tRNA transcripts prepared in this study are listed in Supplementary Table S2. Note that tRNA bearing GAA anticodon (decoding Phe codon) has a U20A substitution in order to avoid undesired Phe-charging catalyzed by native PhoRS (42). Double-stranded DNA templates that encode tRNAs were prepared by primer extension followed by PCR as follows: Appropriate forward and reverse primers (1 µM each, see Supplementary Table S3 for the primers used) were mixed in 100 µl PCR mixture [10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl2, 0.25 mM each dNTPs, 0.1% (v/v) Triton X-100, and 45 nM Taq DNA polymerase]. Primer extension was conducted by denaturing (95°C for 1 min) followed by 5 cycles of annealing (50°C for 1 min) and extending (72°C for 1 min). 1 µl of the resulting reaction mixture was diluted 200-fold with 199 µl of PCR mixture containing appropriate forward and reverse primers (0.5 µM each, see Supplementary Table S3 for the primers used), and PCR was conducted for 12 cycles of denaturing (95°C for 40 s), annealing (50°C for 40 s), and extending (72°C for 40 s). Amplification of the PCR product was confirmed by 3% agarose gel electrophoresis and ethidium bromide staining. The resulting DNA was purified by phenol/chloroform/isooamyl alcohol extraction, phenol/chloroform extraction, and ethanol precipitation. The DNA pellet was dissolved in 20 µl water.

In vitro transcription reaction for tRNA was conducted by incubating 200 µl transcription mixture [40 mM Tris-HCl (pH 8.0), 1 mM spermidine, 0.01% (v/v) Triton X-100, 10 mM DTT, 22.5 mM MgCl2, 3.75 mM each NTPs, 5 mM GMP, 22.5 mM KOH, 10% (v/v) DNA template prepared as described above], and 120 nM T7 RNA polymerase at 37°C overnight. The transcription mixture was mixed with MnCl2 (100 mM, 4 µl) and RQ1 RNase-Free DNase (1 U/µl, 1 µl, Promega), and incubated at 37°C for 30 min. The resultant DNA transcript was precipitated by isopropanol and dissolved in water. The tRNA transcript was measured by 8% native PAGE and ethanol precipitation, and then dissolved in 10 µl water. The concentrations of mRNA were measured by A260 of a 10-fold diluted solution.

**Synthesis of aminoacyl-tRNAs by flexizymes**

Amino acids activated with an appropriate ester group (Phe-CME, Tyr-CME, Ser-DBE, MeG-DBE, MeS-DBE, MeA-DBE, MeF-CME, MeL-DBE, MeM-DBE, MeT-DBE, MeY-CME, MeD-DBE, MeV-DBE, MeNi-DBE, MeYm-CME, MeNv-DBE, AcK-CME, and ClAcY-CME; C: cyanoethyl ester; DBE: 3,5-dinitrobenzyl ester) were synthesized as previously reported (39,43,44).

Aminoacyl-tRNAs were prepared by the following procedure: 12 µl HEPES–KOH buffer (pH 7.5, 83 mM) containing 42 µM tRNA and 42 µM flexizyme (eFx for CME-activated amino acids and dFx for DBE-activated amino acids) was heated at 95°C for 2 min and cooled to 25°C over 5 min. MgCl2 (3 M, 4 µl) was added and the mixture was incubated at 25°C for 5 min. The reaction was initiated by addition of each activated amino acid substrate in DMSO (25 mM, 4 µl) and incubated on ice for acylation (incubation time: 2 h for Phe-CME, Tyr-CME, MeG-DBE, MeA-DBE, MeF-CME, and ClAcY-CME; 6 h for Ser-DBE, MeS-DBE, MeL-DBE, MeV-DBE, and MeNi-DBE; 10 h for MeY-CME; and 24 h for MeT-DBE, MeD-DBE, MeV-DBE, MeNi-DBE, and MeNv-DBE). After acylation, the reaction was quenched by addition of 80 µl of 0.3 M sodium acetate (pH 5.2), and the RNA was precipitated by ethanol. The pellet was rinsed twice with 70% ethanol containing 0.1 M sodium acetate (pH 5.2), and once with 100% ethanol. The resulting aminoacyl-tRNA was dissolved in 1 mM sodium acetate (pH 5.2) just before addition to in vitro translation reaction mixture.

For translation of mRNA2 and 3 (Figure 4 and 5), the method to prepare multiple MeAA-tRNAs was changed as follows: after quenching the flexizyme-catalyzed acylation with 0.3 M sodium acetate (pH 5.2), all reaction solutions were mixed into one tube and precipitated by ethanol. The pellet was rinsed twice with 70% ethanol containing 0.1 M sodium acetate (pH 5.2), and once with 100% ethanol. The resulting aminoacyl-tRNA was dissolved in 1 mM sodium acetate (pH 5.2) just before addition to in vitro translation reaction mixture.

For translation of mRNA2 and 3 (Figure 4 and 5), the method to prepare multiple MeAA-tRNAs was changed as follows: after quenching the flexizyme-catalyzed acylation with 0.3 M sodium acetate (pH 5.2), all reaction solutions were mixed into one tube and precipitated by ethanol. The pellet was rinsed twice with 70% ethanol containing 0.1 M sodium acetate (pH 5.2), and once with 100% ethanol. The resulting aminoacyl-tRNA was dissolved in 1 mM sodium acetate (pH 5.2) just before addition to in vitro translation reaction mixture.

The concentration of aminoacyl-tRNA was adjusted as follows: 0.25 µl of the aminoacyl-tRNA solution was diluted 5000-fold with 1250 µl of 1 M sodium acetate (pH 5.2) and the concentrations of the total RNA including tRNA and flexizyme were measured by A260 of the diluted solution. The concentration of aminoacyl-tRNA was calculated based on the total RNA concentration and the flexizyme-catalyzed acylation efficiencies (Supplementary Table S6), the concentration of each MeAA-tRNA was adjusted to 10 µM. For the synthesis of a macrocyclic peptide, ClAcY-tRNA was prepared separately and its con-
centration was adjusted to 25 μM without considering the flexizyme-catalyzed acylation efficiency.

Measurement of the efficiency of flexizyme-catalyzed aminoacylation

The pellet of ethanol-precipitated 50 pmol tRNA pre-charged with pAA or npAA was dissolved in 0.52 μl of 10 mM sodium acetate (pH 5.2) and mixed with 5.0 μl of acid PAGE loading buffer [83% (v/v) formamide, 150 mM sodium acetate (pH 5.2), and 10 mM EDTA]. The solution was loaded on an acid denaturing polyacrylamide gel [12% (w/v) acrylamide/bisacrylamide (19:1), 8M urea, and 50 mM sodium acetate (pH 5.2)] and electrophoresis was conducted at 300 V (approximately 10 V/cm) for 20 h. The gel was stained with ethidium bromide and analyzed using a Typhoon FLA 7000 (GE Healthcare). Aminoacylation efficiency was calculated based on the band intensities of aminoacyl-tRNA (A) and free tRNA (T) and is presented as (A)/(A + T). As flexizymes recognize only the 3′-terminal CCA consensus sequence, the observed acylation efficiencies were similar independent of the tRNA species (45). The mean acylation values for each amino acid are listed in Supplementary Table S6.

Purification of EF-Tu

BL21(DE3) pLysS cells were transformed with pET21a-tufA and grown in 6 l of LB medium containing 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 5% (v/v) glycerol at 37°C until the OD600 reached 0.4. The expression of EF-Tu was induced with 0.5 mM IPTG and the cells were incubated at 37°C for additional 3 h. The cells were harvested and resuspended in 100 ml of buffer A [20 mM Tris–HCl (pH 7.6), 20 mM imidazole, 300 mM NaCl, 10 μM GTP, 1 mM β-mercaptoethanol] with 0.1 mg/ml PMSF. The cells were sonicated on ice for 10 min and the lysate was centrifuged (13 000 rpm, 4°C, for 15 min, CR22GIII equipped with an R15A rotor, Hitachi Koki). The supernatant was filtered through Minisart prefilter and Minisart 0.45 μm syringe filters (Sartorius) and then applied to a 5 ml HisTrap HP column at 4°C (GE Healthcare) equipped on an AKTA avant 25 (GE Healthcare). The column was washed with 20 column volumes of buffer A, and the protein was eluted with a linear gradient from buffer A to buffer B [20 mM Tris–HCl (pH 7.6), 250 mM imidazole, 150 mM NaCl, 50 mM KCl, 10 μM GTP, 1 mM β-mercaptoethanol] over 20 column volumes. The fractions containing the protein were combined and dialyzed in 2 l of buffer C [10 mM Tris–HCl (pH 7.6), 50 mM KCl, 10 μM GTP, 1 mM DTT] with 3.5K MWCO membrane. The concentration of EF-Tu was measured by protein assay kit.

Radiolabeling of 3′-terminus of tRNA

The 3′-terminus of a tRNA was radiolabeled as follows: 7.5 μl of 16.7 μM tRNA lacking 3′-terminal adenine was incubated at 80°C for 3 min, and then put on ice for 10 min. The 25 μl of CCA-adding reaction mixture [120 mM Gly-NaOH (pH 9.0), 75 mM MgCl2, 30 mM DTT, 5 μM tRNA lacking 3′-terminal adenine prepared above, 10 mM non-radiolabeled ATP, 0.67 μM [α-32P]-ATP and 200 nM CCA-adding enzyme] was incubated at 37°C for 20 min. The tRNA was purified by phenol/chloroform extraction, Micro Bio-Spin 30 column (Bio-Rad), and ethanol precipitation. The tRNA pellet was dissolved in 5 μl water and the concentrations of tRNA were measured by A260 of a 100-fold diluted solution.

Quantification of the affinity between EF-Tu and aminoacyl-tRNA (RNase A protection assay)

Aminoacyl-tRNA was prepared as described above except that the acylation reaction was conducted using a mixture of 3′-radiolabeled tRNA (10%) and non-radiolabeled tRNA (90%). The resulting pellet of aminoacyl-tRNA was dissolved in 3.2 μl of 10 mM sodium acetate (pH 5.2). The concentration of aminoacyl-tRNA was adjusted as follows: 0.7 μl of the aminoacyl-tRNA solution was diluted 100-fold with 69.3 μl of 10 mM sodium acetate (pH 5.2) and the concentrations of the total RNA including tRNA and flexizyme were measured by A260 of the diluted solution. The concentration of aminoacyl-tRNA was calculated based on the total RNA concentration and the flexizyme-catalyzed acylation efficiency (Supplementary Table S6). The concentration of aminoacyl-tRNA was adjusted to 2.0 μM by adding an appropriate volume of 10 mM sodium acetate (pH 5.2). Just prior to use, the concentration of aminoacyl-tRNA was adjusted to 100 nM by mixing 2.0 μM aminoacyl-tRNA solution with buffer D [50 mM HEPEs–KOAc (pH 7.6), 100 mM KOAc, 12 mM Mg(OAc)2, 1 mM GTP, 1 mM DTT, 20 mM creatine phosphate, 2 mM spermidine, 3 mM phosphoenol pyruvate and 0.1 μg/μl pyruvate kinase from rabbit muscle (Sigma)].

EF-Tu was incubated in buffer D at 37°C for 30 min in order to be fully converted to a GTP-bound form. 12 μl samples containing different concentrations of EF-Tu, typically ranging from 1.5 nM to 25 μM, were prepared by twofold serial dilution on ice. 9.6 μl of the EF-Tu solution was mixed with 2.4 μl of 100 nM aminoacyl-tRNA and incubated on ice for 20 min. Under equilibrium binding condition, 10 μl of each solution was mixed with 1 μl of 1 mg/ml RNase A (Sigma) on ice to digest the tRNA not bound to EF-Tu. After 20 s, the digestion was quenched by addition of 50 μl of 10% (v/v) trichloroacetic acid (TCA) containing 0.1 mg/ml unfractionated yeast tRNA on ice. The precipitate was filtered using 0.45 μm pore-size nitrocellulose membrane assembled in Bio-Dot microfiltration apparatus (Bio-Rad), and washed by six times of 200 μl each 5% (v/v) TCA. The membrane was soaked in 95% (v/v) ethanol for 5 min and dried. The dried membrane was analyzed by autoradiography using a Typhoon FLA 7000 (GE Healthcare). To correct for the background signal derived from any aminoacyl-tRNA that may remain after the 20 s treatment with RNase A, a no EF-Tu control was analyzed in parallel and its radioactivity was subtracted from the experimental data. The resultant radioactivity was converted to the concentration of ternary complex using the conversion factor determined by the calibration aminoacyl-tRNA samples in buffer D containing 20, 4, 0.8 and 0.16 nM tRNA. Equilibrium dissociation constants were deter-
determined by fitting the binding data to the following equation (1) using KaleidaGraph program (Hulinks). As the concentration of aminoacyl-tRNA varied slightly in every experiment due to its multistep preparation, both \( K_D \) and the aminoacyl-tRNA concentration were set as variables in the fitting analysis.

The \( K_D \) equation is defined as follows, where atatRNA, EF-Tu, and atatRNA-EF-Tu denote aminoacyl-tRNA, EF-Tu, and their complex, respectively.

\[
K_D = \frac{[\text{atatRNA}]_{\text{eq}} \times [\text{EF-Tu}]_{\text{eq}}}{[\text{atatRNA} \cdot \text{EF-Tu}]_{\text{eq}}}
\]

\[
K_D = \frac{[\text{atatRNA}]_{\text{eq}} - [\text{atatRNA} \cdot \text{EF-Tu}]_{\text{eq}}}{[\text{atatRNA}]_{\text{eq}} - [\text{atatRNA}]_{\text{eq}}} \times \frac{([\text{atatRNA}]_{\text{eq}} - [\text{atatRNA} \cdot \text{EF-Tu}]_{\text{eq}}) + [\text{EF-Tu}]_{\text{eq}}}{[\text{EF-Tu}]_{\text{eq}}}
\]

The \( \Delta G \) value was calculated based on determined \( K_D \) value using the following equation (2).

\[
\Delta G = RT \ln K_D
\]

**In vitro translation**

The reconstituted cell-free translation system (46) contained all necessary components for translation except for RF1. Concentrations of translation components were optimized in our previous studies as follows (25): 50 mM HEPES–KOH (pH 7.6), 100 mM KOAc, 2 mM GTP, 2 mM ATP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate, 12–15 mM Mg(OAc)\(_2\), 2 mM spermidine, 1 mM DTT, 100 mM HEPES–KOH (pH 7.6), 100 mM KOAc, 2 mM GTP, 2 mM RF1. Concentrations of translation components were optimized all necessary components for translation except for RF1.

The translation products were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and/or autoradiography after tricine-SDS-PAGE. For MALDI-TOF MS analysis, 1.0–5.0 \( \mu \)l of the translation reaction mixture was incubated at 37°C for 3–30 min (see Supplementary Table S8 for the volume and reaction time in each experiment). After the reaction, the mixture was diluted with the same volume of FLAG-purification buffer [100 mM Tris–HCl (pH 7.6), 300 mM NaCl]. The expressed peptide was immobilized on anti-FLAG M2 agarose beads (Sigma) by incubating at 25°C for 1 h. After washing the beads with 25 \( \mu \)l of wash buffer [50 mM Tris–HCl (pH 7.6), 150 mM NaCl], the immobilized peptides were eluted with 15 \( \mu \)l of 0.2% TFA. Following purification, the peptide was desalted with SPE C-tip (Nikkyo Technos) and eluted with 1 \( \mu \)l of 80% acetonitrile, 0.5% acetic acid solution 50% saturated with the matrix (R)-cyano-4-hydroxycinnamic acid (Bruker Daltonics). MALDI-TOF MS measurements were performed using a ultraflx Xtreme (Bruker Daltonics) under reflect/positive mode and externally calibrated with peptide calibration standard II (Bruker Daltonics) and/or protein calibration standard I (Bruker Daltonics). For the quantification analysis, the translation reaction was performed in the presence of 50 \( \mu \)M [\(^{14}\)C]-Asp instead of 200 \( \mu \)M cold Asp. The translation product was analyzed by 15% tricine-SDS-PAGE and autoradiography using a Typhoon FLA 7000 (GE Healthcare) without FLAG purification. The amount of peptide products was quantified based on the relative band intensity of the peptide product to the sum intensities present in the lane, that is, the sum intensities of unreacted free Asp and peptide products.

**RESULTS AND DISCUSSION**

MeAA-tRNAs lack sufficient EF-Tu affinity

We first quantified the affinities of MeAA-tRNAs to EF-Tu (\( \Delta G \) value) and compared them with those of canonical pAA-tRNAs. The quantification was conducted by means of RNase A protection assay (47) (see methods section for the details). Three canonical pAA-tRNAs (Phe-tRNA\(_{\text{GAA}}\), Tyr-tRNA\(_{\text{GUA}}\) and Ser-tRNA\(_{\text{CGA}}\)) were prepared using flexizymes (25). As expected, all three canonical pAA-tRNAs exhibited similar EF-Tu affinities, ranging from −8.0 to −9.4 kcal/mol (Figure 2A). Although these values were slightly weaker than those previously reported (from −9.5 to −10.5 kcal/mol) (6), this could be attributed to the differences in buffer composition or the presence of the His\( \delta \) tag at the C-terminus of the EF-Tu used in this assay. For quantification of the affinities of MeAA-tRNAs to EF-Tu, we prepared 13 representative MeAA-tRNA\(_{\text{Ame2}}\)\(_{\text{GAC}}\)S [MeG, MeA, MeS, MeT, MeF, MeY, MeV, MeL, MeM, MeD, MeNv (N-methylornithine), MeNl (N-methylnorleucine) and MeYm (N-methyl-p-methoxyphenylalanine)], Figure 2B] by means of the flexizyme technology (25,38,39). Most of the MeAA-tRNA\(_{\text{Ame2}}\)\(_{\text{GAC}}\)S showed undetectably weak affinities to EF-Tu with two exceptions where the affinity of MeG-tRNA\(_{\text{Ame2}}\)\(_{\text{GAC}}\) (−8.4 kcal/mol) and MeS-tRNA\(_{\text{Ame2}}\)\(_{\text{GAC}}\) (−8.4 kcal/mol) are in the affinity range observed for the natural pairs (from −8.0 to −9.4 kcal/mol), while that of MeA-tRNA\(_{\text{Ame2}}\)\(_{\text{GAC}}\) (−7.0 kcal/mol) was measurable but yet out of the range (Figure 2A).

In the cocystal structure of the ternary complex (Escherichia coli EF-Tu-Ecoli Phe-tRNA\(_{\text{Phc}}\)-GTP), the amino group of Phe esterified on the tRNA forms a hydrogen bond with Asn285 of EF-Tu, defining the orientation of Phe in the binding pocket (Figure 1B) (3). This amino group is tightly surrounded by the pocket allowing no space for additional methylation. Thus, the methylation could cause a steric clash with EF-Tu and disrupt the hydrogen bond formation, resulting in the loss of affinity for most
The feasibility of this approach was evaluated using Phe-tRNA$_{GAC}$#1–4 and MeF-tRNA$_{GAC}$#2–4. As expected, the
Figure 3. Reinforcement of EF-Tu affinity of Phe-tRNA and npAA-tRNAs by T-stem engineering. (A) Sequence of the original tRNAAsnE2 (#2) and T-stem variants #1–4. The affinities of tRNA #1–4 were designed to increase as the T-stem number increases from #1 to #4. (B–E) Quantification of the EF-Tu affinities of Phe-tRNAGAC#1–4 (B, C) and MeF-tRNAGAC#2–4 (D, E). Schematic representation of RNase A protection assay, the observed fraction of the ternary complex with the fitting curve to determine the $K_D$ value (B, D), and the calculated $\Delta G$ value (C, E). Asterisk (*) indicates undetectably weak affinity ($\Delta G > -6$ kcal/mol). Error bar indicates the fitting error. (F–J) The EF-Tu affinities of npAA-tRNA#1–4 and MALDI-TOF MS of P1-npAA examined for MeG (F), MeS (G), MeA (H), MeL (I) and AcK (J). Each dagger peak (†) corresponds to a byproduct containing Ile in place of npAA.

affinity of Phe-tRNAGAC#X was enhanced as the T-stem number increased, that is, $\Delta G = -7.0, -8.7, -9.4$ and $-9.7$ kcal/mol for #1–4, respectively (Figure 3B and C). The affinity of MeF-tRNAGAC#2 to EF-Tu was undetectable but the T-stem replacement to #3 and #4 enhanced the affinity to $-7.9$ and $-9.0$ kcal/mol, respectively (Figure 3D and E). A control experiment using uncharged tRNAGAC#1–4 showed that the tRNA itself did not bind to EF-Tu, which is consistent with the notion that EF-Tu selectively binds to aminoacylated tRNAs regardless of the T-stem sequence (Supplementary Figure S3). We also evaluated the EF-Tu affinities for the other twelve distinct MeAAs (MeG, MeS, MeA, and MeL in Figure 3F–I, left panels; MeM, MeT, MeY, MeD, MeV, MeNv, MeNl and MeYm in Supplementary Figure S4) with the series of tRNAs, demonstrating that their reinforcement of EF-Tu affinity can be achieved with all MeAA-tRNAs. Interestingly, in most MeAA cases the affinities measured with T-stem #3 and #4 were stronger than the predicted value based on #2, whereas those of Phe-tRNAs and MeG-tRNAs were close to the predicted value. This suggests that the effect of the affinity reinforcement becomes more pronounced when MeAA-tRNA#2 exhibits undetectably poor affinity. We further evaluated four MeAAs (MeG, MeS, MeA and MeL) and monitored the reprogramming fidelity in expression of P1-MeG, P1-MeS, P1-MeA and P1-MeL using tRNAGAC#1–4 charged with MeG, MeS, MeA, and MeL, respectively (Figure 3F–I, right panels). Consequently, the desired peptide was expressed as the sole product only when the affinity of MeAA-tRNA to EF-Tu was sufficiently reinforced (Figure 3F #2–4, 3G #2–4, 3H #3–4 and 3I #4). In contrast, when the affinity of MeAA-tRNA to EF-Tu was insufficient or undetectable, the expression fidelity of the desired P1-MeAA was poor, giving a peak of P1-Ile (Figure 3F #1, 3G #1, 3H #1–2 and 3I #1–3). It should be noted that MeL was reported as a very poor substrate (39), but P1-MeL was cleanly expressed by using MeL-tRNAGAC#4 (Figure 3I #4). These results clearly show that the reprogramming fidelity can be significantly improved by reinforcing the affinity of MeAA-tRNA to EF-Tu. A similar trend was also observed in expression of P1-AcK, demonstrating the versatility of this strategy in introducing npAAs (Figure 3J).
Expression of N-methyl-peptides by EF-Tu affinity-tuned MeAA-tRNAs

Having the affinity-tuned MeAA-tRNA#1–4 to EF-Tu, we next expressed an N-methyl-peptide containing nine distinct MeAAs using three sets of tRNA: all-#2 set, all-#4 set, and uniform set (Figure 4A). In the all-#2 set, all nine MeAAs were charged onto tRNA#2 with the original tRNAAsnE2 T-stem. In the all-#4 set, all nine MeAAs were charged onto tRNA#4 with the strongest T-stem to simply maximize the EF-Tu affinities. In contrast, the uniform set is a combination of each MeAA and appropriately chosen T-stem with optimal EF-Tu affinities as follows: #2 for MeSa and MeG; #3 for MeA, MeNv, MeYa and MeF; and #4 for MeYm, MeNl and MeV. In this set, the affinities of MeAA-tRNAs to EF-Tu ($\Delta G$) were tuned in the range of $-7.9$ to $-8.8$ kcal/mol, which is comparable to the previously determined $\Delta G$ values of pAA-tRNAs ($-8.0$ to $-9.4$ kcal/mol, the orange bands in Figure 4A). In this range, EF-Tu can bind to all pAA-tRNAs and MeAA-tRNAs with uniform affinities (Figure 4B). We prepared a FIT system where Phe, Leu, Ile, Val, and Ala were omitted and their vacant codons were reprogrammed with nine MeAAs by the addition of MeG-tRNAAGA#X, MeS-tRNAAGG#X, MeA-tRNAAGC#X, MeNv-tRNAAGC#X, MeY-tRNAAGU#X, MeF-tRNAAGG#X, MeYm-tRNAAGA#X, MeNl-tRNAAGC#X and MeV-tRNAAGC#X (Figure 4C) by means of the artificial codon box division methodology (49) to express a linear 32-mer model peptide P2 containing 9 MeAAs (Figure 4D).

The peptide P2 expressed in the FIT system supplemented with a tRNA set (all-#2, all-#4, or uniform set) was analyzed by MALDI-TOF MS (Figure 4E). The exclusive use of the all-#2 set yielded a set of peaks corresponding P2 along with byproducts that have $-28$ and $-14$ m/z compared to P2. Interestingly, the exclusive use of the all-#4 set did not show a detectable peak of P2 at all. The calculated m/z value of P2 is 4019.97, but the monoisotopic m/z value of the observed peak in the all-#4 set was 4021.97 (P2*), indicating that the desired peptide P2 was not synthesized by the all-#4 set (Figure 4E). The P2* peak was also accompanied by various byproducts with $-14$, $+14$ or $+16$ m/z difference relative to the P2* peak. These byproducts could be attributed to misincorporations of a certain amino acid(s); for example, a misincorporation of MeNv instead of MeNl could result in $-14$ m/z difference; a misincorporation of MeNl instead of MeNv could result in $+14$ m/z difference; or a misincorporation of Ser instead of MeG could result in $+16$ m/z difference; and P2* itself, with $+2$ m/z difference from P2 could be derived from the combination of $-14$ and $+16$ m/z. It should be noted that a substantial amount of unidentified peptide byproducts was also observed when using the all-#2 or all-#4 set (Supplementary Figure S5B). In contrast, when the uniform set was tested, the desired peptide P2 was correctly synthesized as a nearly sole product, and the degree of byproducts was drastically suppressed, in-
detection of [14C]-Asp introduced in the C-terminal FLAG P2 by means of tricine-SDS-PAGE with autoradiographic efficiency.

has given the best expression in terms of fidelity and efficiency for MeAA-tRNAs decreasing the fidelity of the multiple MeAA N\-methyl-peptides and (iii) this affinity-tuning strategy is able to improve both the synthetic accuracy and translation efficiency of N-methyl-peptides.

Finally, we designed a macrocyclic peptide, P3, composed of 9 distinct MeAAs and 14 different kinds of pAAs. For macrocyclization, an N-chloroacetyl-L-tyrosine (ClAcY) at the N-terminus and a Cys residue at a downstream (24th) position were also installed and thereby they underwent spontaneously macrocyclization via a thioether bond (44, 50), giving a 24-mer macrocycle (Figure 5A and B). To achieve the desired reprogramming, we used a FIT system that is deficient in Met, Phe, Leu, Ile, Val and Ala, enabling us to install ClAcY (by the addition of ClAcY-tRNA\textsubscript{Im} ) and aforementioned 9 MeAAs by the uniform set of MeAA-tRNAs. As expected, the desired macrocyclic P3 was accurately expressed (Figure 5C) with an expression level of 0.37 μM (1.5 ng/μl).

CONCLUSION

Here, we have reported a new methodology to achieve the ribosomal synthesis of nonstandard peptides containing a rich variety of MeAAs. The inefficient incorporation of MeAAs is attributed to the two major reasons: impaired EF-Tu-mediated accommodation of the MeAA-tRNAs and their slow peptidyl transfer. Indeed, we have quantitatively demonstrated that the binding affinities (ΔG values) of MeAA-tRNA\textsubscript{AnE2} to EF-Tu are generally weaker than those of pAA-tRNAs. \textit{In vitro} translation experiments using MeAA-tRNA\textsubscript{AnE2} with inadequate affinities indicate that the thermodynamic contributions of the EF-Tu affinities play a major role in the translation. Based on this knowledge, we have engineered the T-stem sequence of tRNA to tune the affinity of MeAA-tRNA to EF-Tu. This affinity-tuning strategy dramatically improves the expression of peptides containing multiple MeAAs with high fidelity and expression level, even though some MeAAs are consecutively and/or alternately placed in the single sequence. Consequently, we succeeded in assigning 24 distinct building blocks in the genetic code, where a set of 15 pAAs and 9 MeAAs, or 14 pAAs, 9 MeAAs and ClAcY, and expressing N-methyl-peptides consisting of these amino acids. The present data have exceeded the previous record of the genetic code reprogramming achieved to place 23 building blocks consisting of 20 pAAs and 3 npAAs (49, 51).

Forster et al. kinetically determined the peptidyl-transfer rate where the initiation complex of ribosome and pMet-tRNA was incubated with pre-formed MeF-tRNA-EF-Tu complex, showing that the rate was approximately 8 000-fold slower than the natural pair (Phe-tRNA-EF-Tu) case (21). However, it is hard to generalize this notion to other MeAAs, since the efficiency of MeF incorporation into polypeptides was as high as Phe incorporation in our previous experiment (39). More importantly, when the incorporation of MeAA in a middle of sequence of longer peptides, the observed expression efficiency relies on not only the peptidyl-transfer step but also all steps of elongation. Our work undoubtedly shows that the uniform tuning of MeAA-tRNA affinities to EF-Tu is quite effective to improve their incorporation efficiency, giving the sufficient expression of such exotic peptides in terms of fidelity and yield.
for the practical application to the RaPID selection. Therefore, the most critical determinant for MeAA incorporation to the nascent peptide chain could be a step of delivery of MeAA-tRNA-EF-Tu complex to the A site, or a step of EF-Tu dissociation and MeAA-tRNA accommodation (10), or both steps.

In addition to MeAAs, we have also demonstrated that the incorporation of AcK was enhanced by engineering the T-stem, indicating that the substrate of this affinity-tuning methodology is applicable to other npAAs. In our most recent work aiming at enhancing the incorporation of D-, β- and γ-amino acids into nascent peptide chain, we logically devise a chimeric tRNAPro1E2 based on the T-stem of tRNA#3 (equal to tRNAGluE2) and D-arm of tRNAPro1 (27). In the case of the consecutive incorporation of such exotic amino acids, the EF-P recruitment by the use of tRNAPro1E2 was essential to enhance the efficiency (27–31).

The knowledge established in the tRNA engineering efforts including the present work enables us to further expand the utility of exotic amino acids to the translation chemistry world. Particularly when the knowledge is integrated with the RaPID system, it will allow us to rapid and high-throughput screening of binding peptides to target proteins of interest using a massive library of randomized macrocyclic peptides, yielding therapeutically valuable peptides with improved pharmacological properties (30).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Yuuki Hayashi for providing purified EF-Tu. We also thank Renier Herman Pieter van Neer, Hayden Peacock, Koki Shimbara and Naoya Kawakami for proofreading of the manuscript.

FUNDING

Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for JSPS Fellows [26-9576 to Y.I.]; Grant-in-Aid for Scientific Research (B) [JP18H02080 to T.K.]; Grant-in-Aid for Specially Promoted Research [JP20H05618]; Human Frontier Science Program [RGP0015/2017-302 to H.S.]. Funding for open access charge: Japan Society for the Promotion of Science [JP20H05618].

Conflict of interest statement. None declared.

REFERENCES

1. Schmidt,T.M. and Ramakrishnan,V. (2009) What recent ribosome structures have revealed about the mechanism of translation. Nature, 461, 1234–1242.

2. Pape,T., Wintermeyer,W. and Rodnina,M. (1999) Induced fit in initial selection and proofreading of aminoacyl-tRNA on the ribosome. EMBO J., 18, 3800–3807.

3. Nissen,P., Kjeldgaard,M., Thirup,S., Polekhina,G., Reshetnikova,L., Clark,B.F.C. and Nyborg,J. (1995) Crystal structure of the ternary complex of Phe-tRNA<sup>34</sup>GTP, EF-Tu, and a GTP Analog. Science, 270, 1464–1472.

4. Asahara,H. and Uhlenbeck,O.C. (2002) The tRNA specificity of Thermus thermophilus EF-Tu. Proc. Natl. Acad. Sci. U.S.A., 99, 3490–3494.

5. Sanderson,L.E. and Uhlenbeck,O.C. (2007) Directed mutagenesis identifies amino acid residues involved in elongation factor Tu binding to yeast Phe-tRNA<sup>34</sup>Phe. J. Mol. Biol., 368, 119–130.

6. LaRiviere,F.J., Wolson,A.D. and Uhlenbeck,O.C. (2001) Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. Science, 294, 165–168.

7. Asahara,H. and Uhlenbeck,O.C. (2005) Predicting the binding affinities of misacylated tRNAs for Thermus thermophilus EF-Tu-GTP. Biochemistry, 44, 11254–11261.

8. Dale,T., Sanderson,L.E. and Uhlenbeck,O.C. (2004) The affinity of elongation factor Tu for an aminoacyl-tRNA is modulated by the esterified amino acid. Biochemistry, 43, 6159–6166.

9. Schrader,J.M., Chapman,S.J. and Uhlenbeck,O.C. (2009) Understanding the sequence specificity of tRNA binding to elongation factor Tu using tRNA mutagenesis. J. Mol. Biol., 386, 1255–1264.

10. Schrader,J.M., Chapman,S.J. and Uhlenbeck,O.C. (2011) Tuning the affinity of aminoacyl-tRNA to elongation factor Tu for optimal decoding. Proc. Natl. Acad. Sci. U.S.A., 108, 5215–5220.

11. Uhlenbeck,O.C. and Schrader,J.M. (2018) Evolutionary tuning impacts the design of bacterial tRNAs for the incorporation of unnatural amino acids by ribosomes. Curr. Opin. Chem. Biol., 46, 138–145.

12. Stanzel,M., Schön,A. and Sprinzl,M. (1994) Discrimination against misacylated tRNA by chloroplast elongation factor Tu. Eur. J. Biochem., 219, 435–439.

13. Wang,L. and Schultz,P.G. (2005) Expanding the genetic code. Angew. Chem. Int. Ed., 44, 34–66.

14. Chin,J.W. (2014) Expanding and reprogramming the genetic code of cells and animals. Annu. Rev. Biochem., 83, 379–408.

15. Dumas,A., Lercher,L., Spicer,C.D. and Davis,B.G. (2015) Designing logical codon reassignment – expanding the chemistry in biology. Chem. Sci., 6, 50–69.

16. Hirose,H., Tsiamantas,C., Katoh,T. and Suga,H. (2019) In vitro expression of genetically encoded non-standard peptides consisting of exotic amino acid building blocks. Curr. Opin. Biotechnol., 58, 28–36.

17. Guo,J., Melançon,C.E. III, Lee,H.S., Groff,D. and Schultz,P.G. (2009) Evolution of amber suppressor tRNAs for efficient bacterial production of proteins containing unnatural amino acids. Angew. Chem. Int. Ed., 48, 9148–9151.

18. Doy,I., Ohtsuki,T., Shimizu,Y., Ueda,T. and Sisido,M. (2007) Elongation factor Tu mutants expand amino acid incorporation of protein biosynthesis system. J. Am. Chem. Soc., 129, 1458–1462.

19. Gao,R., Perez,J.G., Carlson,E.D., Nisli,L., Isaacs,F.J., Kelleher,N.L. and Jewett,M.C. (2017) Translation system engineering in Escherichia coli enhances non-canonical amino acid incorporation into proteins. Biotechnol. Bioeng., 114, 1074–1086.

20. Park,H.-S., Hohn,M.J., Umehara,L., Guo,L.-T., Osborne,E.M., Benner,J., Noren,C.J., Rinhart,J. and Söll,D. (2011) Expanding the genetic code of Escherichia coli with phosphoryserine. Science, 333, 1151–1154.

21. Pavlov,M.Y., Watts,R.E., Tan,Z., Cornish,V.W., Ehrenberg,M. and Forster,A.C. (2009) Slow peptide bond formation by proline and other N-alkylamino acids in translation. Proc. Natl. Acad. Sci. U.S.A., 106, 50–54.

22. Liljerum,J., Wang,J., Kwiatkowski,M., Sabari,S. and Forster,A.C. (2019) Kinetics of D-amino acid incorporation in translation. ACS Chem. Biol., 14, 204–213.

23. Zhang,B., Tan,Z., Dickson,L.G., Nalam,M.N.L., Cornish,V.W. and Forster,A.C. (2007) Specificity of translation for N-alkyl amino acids. J. Am. Chem. Soc., 129, 11316–11317.

24. Leon,G.W., Pavlov,M.Y., Kwiatkowski,M., Ehrenberg,M. and Forster,A.C. (2014) A tRNA body with high affinity for EF-Tu hastens ribosomal incorporation of unnatural amino acids. RNA, 20, 632–643.

25. Goto,Y., Katoh,T. and Suga,H. (2011) Flexizymes for genetic code reprogramming. Nat. Protoc., 6, 779–790.
26. Achenbach, J., Jahnz, M., Bethge, L., Paal, K., Jung, M., Schuster, M., Albrecht, R., Jarosh, F., Nierhaus, K. H. and Klussmann, S. (2015) Outwitting EF-Tu and the ribosome: translation with D-amino acids. *Nucleic Acids Res.*, 43, 5687–5698.

27. Katoh, T., Iwane, Y. and Suga, H. (2017) Logical engineering of D-arm and T-stem of tRNA that enhances D-amino acid incorporation. *Nucleic Acids Res.*, 45, 12601–12610.

28. Katoh, T. and Suga, H. (2018) Ribosomal incorporation of consecutive β-amino acids. *J. Am. Chem. Soc.*, 140, 12159–12167.

29. Katoh, T. and Suga, H. (2020) Ribosomal elongation of cyclic γ-amino acids using a reprogrammed genetic code. *J. Am. Chem. Soc.*, 142, 4965–4969.

30. Katoh, T., Sengoku, T., Hirata, K., Ogata, K. and Suga, H. (2020) Ribosomal synthesis and de novo discovery of bioactive foldamer peptides containing cyclic β-amino acids. *Nat. Chem.*, 12, 1081–1088.

31. Katoh, T. and Suga, H. (2020) Ribosomal elongation of aminobenzoic acid derivatives. *J. Am. Chem. Soc.*, 142, 16518–16522.

32. Katoh, T., Iwane, Y. and Suga, H. (2018) tRNA engineering for manipulating genetic code. *RNA Biol.*, 15, 453–460.

33. Conradi, R.A., Hilgers, A.R., Ho, N.F.H. and Burton, P.S. (1992) The influence of peptide structure on transport across Caco-2 Cells. II. Peptide bond modification which results in improved permeability. *Pharm. Res.*, 9, 435–439.

34. Haviv, F., Fitzpatrick, T.D., Swenson, R.E., Nichols, C.J., Mort, N.A., Bush, E.N., Diaz, G., Bannert, G., Nguyen, A., Rhutase, N.S., Nellans, H.N., Hoffman, D.J., Johnson, E.S. and Greer, J. (1993) Effect of N-methyl substitution of the peptide bonds in luteinizing hormone-releasing hormone agonists. *J. Med. Chem.*, 36, 363–369.

35. Chikhale, E.G., Ng, K.Y., Burton, P.S. and Borchardt, R.T. (1994) Hydrogen bonding potential as a determinant of the in vitro and in situ blood–brain barrier permeability of peptides. *Pharm. Res.*, 11, 412–419.

36. Bockus, A.T., Schwochert, J.A., Pye, C.R., Townsend, C.E., Sok, V., Bednarek, M.A. and Lokey, R.S. (2015) Going out on a limb: delineating the effects of β-branching, N-methylation, and side chain size on the passive permeability, solubility, and flexibility of sanguinamide A analogues. *J. Med. Chem.*, 58, 7409–7418.

37. Nielsen, D.S., Shepherd, N.E., Xu, W., Lucke, A.J., Stoermer, M.J. and Fairlie, D.P. (2017) Orally absorbed cyclic peptides. *Chem. Rev.*, 117, 8094–8128.

38. Yamagishi, Y., Shoji, I., Miyagawa, S., Kawakami, T., Katoh, T., Goto, Y. and Suga, H. (2011) Natural product-like macrocyclic N-methyl-peptide inhibitors against a ubiquitin ligase uncovered from a ribosome-expressed de novo library. *Chem. Biol.*, 18, 1562–1570.

39. Kawamura, T., Murakami, H. and Suga, H. (2008) Messenger RNA-programmed incorporation of multiple N-methyl-amino acids into linear and cyclic peptides. *Chem. Biol.*, 15, 32–42.

40. Subtelny, A.O., Hartman, M.C.T. and Szostak, J.W. (2008) Ribosomal synthesis of N-methyl peptides. *J. Am. Chem. Soc.*, 130, 6131–6136.

41. Kao, C., Zheng, M. and Rüdisser, S. (1999) A simple and efficient method to reduce nontemplated nucleotide addition at the 3′ terminus of RNAs transcribed by T7 RNA polymerase. *RNA*, 5, 1268–1272.

42. Yasif'eva, L.A., Ankilova, V.N., Lavrik, O.I. and Moor, N.A. (2002) tRNA discrimination by T. thermophilus phenylalanyl-tRNA synthetase at the binding step. *J. Mol. Recognit.*, 15, 188–196.

43. Murakami, H., Ohta, A., Ashigai, H. and Suga, H. (2006) A highly flexible tRNA acylation method for non-natural polypeptide synthesis. *Nat. Methods*, 3, 357–359.

44. Goto, Y., Ohta, A., Sako, Y., Yamagishi, Y., Murakami, H. and Suga, H. (2008) Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. *ACS Chem. Biol.*, 3, 120–129.

45. Xiao, H., Murakami, H., Suga, H. and Ferre-D’Amare, A.R. (2008) Structural basis of specific tRNA aminoacylation by a small in vitro selected ribozyme. *Nature*, 454, 358–361.

46. Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K. and Ueda, T. (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol.*, 19, 751–755.

47. Pleiss, J.A. and Uhlenbeck, O.C. (2001) Identification of thermodynamically relevant interactions between EF-Tu and backbone elements of tRNA. *J. Mol. Biol.*, 308, 895–905.

48. Schrader, J.M. and Uhlenbeck, O.C. (2011) Is the sequence-specific binding of aminocyl-tRNAs by EF-Tu universal among bacteria? *Nucleic Acids Res.*, 39, 9746–9758.

49. Iwane, Y., Hitomi, A., Murakami, H., Katoh, T., Goto, Y. and Suga, H. (2016) Expanding the amino acid repertoire of ribosomal polypeptide synthesis via the artificial division of codon boxes. *Nat. Chem.*, 8, 317–325.

50. Vinogradov, A.A., Yin, Y. and Suga, H. (2019) Macrocyclic peptides as drug candidates: recent progress and remaining challenges. *J. Am. Chem. Soc.*, 141, 4167–4181.

51. Ohtsuki, T., Manabe, T. and Sisido, M. (2005) Multiple incorporation of non-standard structures. *FEBS Lett.*, 579, 6679–6774.