Advances in *in vitro* genetic code reprogramming in 2014–2017

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Abstract

To date, various genetic code manipulation methods have been developed to introduce non-proteinogenic amino acids into peptides by translation. However, the number of amino acids that can be used simultaneously remains limited even using these methods. Additionally, the scope of amino acid substrates that are compatible with ribosomal translation systems is also limited. For example, difficult substrates such as D-amino acids and β-amino acids are much less efficiently incorporated into peptides than L-α-amino acids. Here, we focus on three recently developed methodologies that address these issues: (i) artificial division of codon boxes to increase the number of available amino acids, (ii) orthogonal ribosomal translation systems to 'duplicate' the codon table and (iii) development of novel artificial tRNAs that enhance incorporation of difficult amino acid substrates.

Key words: genetic code; tRNA; ribosome; non-proteinogenic amino acid; reprogramming

Introduction

In standard ribosomal translation, only the 20 canonical proteinogenic amino acids (PAAs) are utilized for peptide/protein synthesis. However, various artificial methodologies to expand or reprogram the genetic code in order to introduce non-proteinogenic amino acids (nPAAs) have been developed over the last half-century (1–3). The canonical genetic code consists of 64 codons, out of which 61 are used as sense codons for designating the 20 PAAs, and the remaining three are used as stop codons. In contrast, in expanded or reprogrammed genetic codes nPAAs are assigned to at least one of the sense or stop codons, or to extra codons (e.g. four base codons) artificially prepared beyond the 64 canonical codons.

For instance, non-sense codon-based methods expand the genetic code by utilizing one or two of the three stop codons to assign nPAAs in place of the translation termination signal (4–6). Alternatively, the programmed frame shift method, which is also referred to as the four-base codon method, utilizes one or more four-base codons consisting of a rare triplet codon with

an adjacent fourth nucleotide to designate nPAAs (7). Similarly, the non-standard base method creates extra codons for the assignment of nPAAs beyond the 64 canonical ones by introducing artificial nucleotide pairs, such as an isoG/isoC pair, in addition to the natural A/U and G/C pairs (8,9); theoretically, increasing the number of codons from 64 ($=4^3$) to 216 ($=6^3$). These techniques can be considered to be genetic code expansion techniques, since the resulting polypeptides may contain all of the 20 PAAs in addition to any nPAAs. In contrast, genetic code reprogramming methods utilize sense codons for assigning nPAAs, in which PAAs designated at the sense codon(s) to be reprogrammed are sacrificed (10).

By using these methods, various nPAAs including L-α-amino acids with non-natural side chains, N-methyl amino acids, D-amino acids and β-amino acids have been successfully introduced into peptides (11–16). These amino acids are often found in natural polypeptides made through non-ribosomal peptide synthesis and have biologically important functions related to catalytic activity, membrane permeability, structural rigidity and peptidase resistance. For this reason, methods that enable

incorporation of these substrates have attracted substantial attention recently.

However, these techniques still have notable limitations. A principal issue is the limited number of codons available for nPAAs in the codon table. For example, in the non-sense codon method, only one or two stop codons can be used to designate additional nPAAs because at least one stop codon must be left unassigned in order to be recognized by a release factor(s) for translation termination [17–19]. Thus, only 22 amino acids (=20 PAAs + 2 nPAAs) at a maximum can be introduced into a peptide at once. Moreover, competition of the release factor(s) with nPAA incorporation often causes undesired translation termination. Similarly, in the programmed frame shift method, the number of available rare codons is limited. Competition of PAA-tRNA that designates the rare codon against the nPAA-tRNA is also non-negligible. In genetic code reprogramming, as sense codons are used for introducing nPAAs, the PAAs which are endogenously assigned at the corresponding codons must be removed from the codon table. Thus, the number of available amino acids cannot normally be increased above 20. From this point of view, the use of non-standard bases appears favorable since it enables expansion of the codon table to 216 codons. However, this approach suffers from low fidelity of decoding in translation as well as insufficient orthogonality of artificial base pairs to the natural bases in transcription and replication [20]. Therefore, development of improved base pairs without these defects is required.

Another significant issue is the limited range of amino acids that are compatible with ribosomal translation. Although diverse amino acid substrates have been tested to date, not all could be efficiently introduced into peptides, and incorporation efficiency differs depending on the specific structure of each nPAA. For instance, L-α-amino acids with small side chains are relatively easily incorporated, but ones with bulkier side chains are generally more difficult. D-Amino acids, β-amino acids and N-methyl-amino acids also tend to be very difficult to be translated [15,16,21,22]. In addition, consecutive incorporation of these more challenging amino acids is far more difficult than single incorporation. This is especially true of β-amino acids, for which consecutive incorporation has not yet been reported. Therefore, improvement of nPAA incorporation methods so as to broaden the amino acid substrate scope is required.

In this review, we focus on three recently developed methodologies to address these issues: (i) artificial division of codon boxes and (ii) codon table duplication by orthogonal tRNA/ribosome pairs are novel methods for manipulating codon tables so as to increase the number of available codons for nPAAs and (iii) use of engineered tRNAs with high EF-P/EF-Tu binding affinity, a new approach to improve the incorporation efficiency of intractable amino acid substrates.

Artificial division of codon boxes to increase the number of available codons

As described above, genetic code reprogramming involves the translation of polypeptides containing nPAAs, in which the nPAAs of interest are assigned to one (or more) of the 61 sense codons. Since, under normal translation conditions, all of the sense codons are utilized by the 20 PAAs in the canonical codon table, the codons targeted for reprogramming to nPAAs need to be 'vacated' by removal of the relevant PAA from the reaction. Thus, the number of available PAAs is reduced to <20, although

the total number of amino acids (i.e. the sum of PAAs and nPAAs) that can be translated remains 20.

In order to overcome this limitation, Iwane *et al.* recently developed a method to artificially divide a codon 'box' (i.e. four codons which share identical 5′ and central nucleobases, differing only at the 3′ base, and which encode a single amino acid) and assign two amino acids to the divided codons [23]. Since the 20 PAAs are redundantly assigned to the 61 sense codons, if the degenerate codons can be assigned to multiple amino acids, the number of available amino acids will increase. However, in natural translation systems, a single aminoacyl-tRNA can, in some cases, decode multiple codons since 'wobble' base pairs, in addition to standard Watson–Crick base pairs are tolerated at the third base of codons [24]. Therefore, such codon boxes decoded by a single aminoacyl-tRNA cannot be easily divided into two (Figure 1A). For example, in *Escherichia coli* translation, the Val GUN codon box is decoded by two aminoacyl-tRNAs bearing GAC or cmo⁵UAC anticodons, with the former decoding GUU and GUC codons, and the latter decoding all of the four GUN codons, complicating Val GUN codon box division.

To overcome this issue, an *E. coli* cell-free translation system lacking tRNA was reconstituted with 32 *in vitro*-transcribed tRNAs with SNN anticodons (S = G or C) to decode the 20 PAAs. Using this system, NNY (Y = U or C) and NNG codons are independently decoded by the tRNAs with GNN and CNN anticodons, respectively, whereas NNA codons are rendered unavailable because no corresponding tRNA is present in the system. Then, three redundant NNY codons were reprogrammed using pre-charged non-proteinogenic aminoacyl-tRNA$_{GNN}$, with NNG codons used for PAA incorporation. For example, at the Val GUN codon box, citrulline (Cit) and Val were introduced at the GUY and GUG codons, respectively (Figure 1B). Similarly, the Arg CGN and Gly GGN codon boxes were also divided to introduce 4-iodophenylalanine (^Iodo^F) at the CGY codon and N-ε-acetyllysine (^Ac^K) at the GGY codon without sacrificing Arg and Gly translation. Consequently, this approach succeeded in increasing the number of available amino acids to 23 (3 nPAAs and 20 PAAs), and a 32-mer peptide with 23 different amino acids could be translated using this system (Figure 1C, peptide 1). As 11 vacant codons can theoretically be created through this approach, up to 31 different amino acids (11 nPAAs and 20 PAAs) could be simultaneously assigned in one codon table.
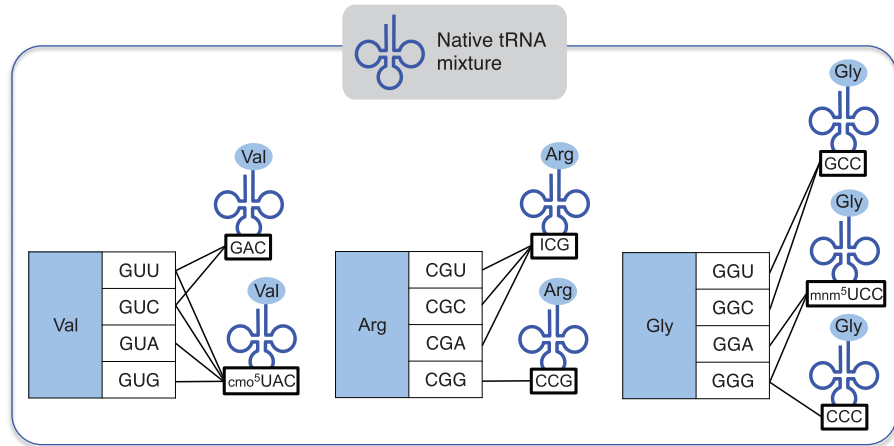
As another example, translation of a macrocyclic N-methyl-peptide CM$_{11}$-1, an E6AP inhibitor [25], was also demonstrated with N-methylphenylalanine (^Me^F), N-methylserine (^Me^S) and N-methylglycine (^Me^G) assigned to the GUY, CGY and GGY codons by codon box division (Figure 1D, E, peptide 2). For macrocyclization of this peptide, the initiator fMet at the AUG codon was also reprogrammed to N-chloroacetyl-D-tryptophan (ClAc^d^W), the chloroacetyl group of which spontaneously reacts with the thiol moiety of the downstream Cys to form a non-reducible thioether bond.

Codon table duplication by means of an orthogonal tRNA/ribosome pair

Codon table duplication is another approach to expand the genetic code through the use of an orthogonal tRNA/ribosome pair that does not interact with the wild-type tRNA/ribosome pair. In a translation system in which both the orthogonal and the wild-type tRNA/ribosome pairs coexist, two duplicated codon tables are independently decoded by the two pairs (Figure 2). In the
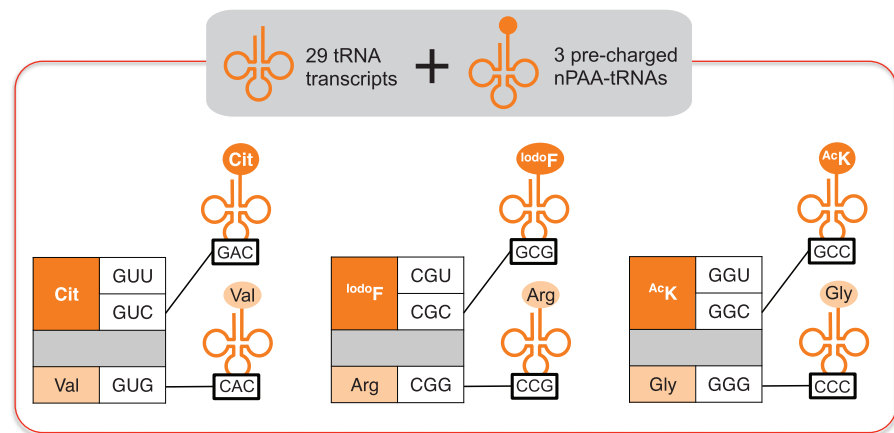
A Canonical codon table

B Artificially divided codon table

(stop codon = UAA)

C

mRNA1: AUG AAG AAG AAG CAG **CGG** UCG AUG **GGC** CUC CCG **CGC** UGG ACC GCC GAG AAC **GUC** CAC AUC **GGG** GUG UUC UGC (Flag) UAA

peptide1: fMet Lys Lys Lys Gln Arg Ser Met AcK Leu Pro IodoF Trp Thr Ala Glu Asn Cit His Ile Gly Val Phe Cys Flag Stop
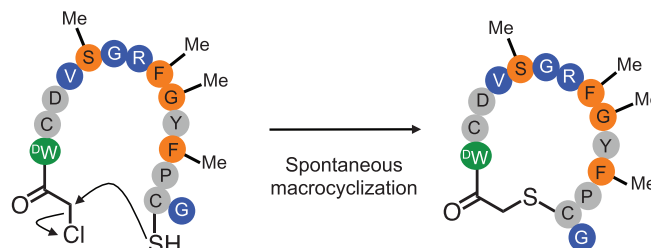
(Flag = Asp Tyr Lys Asp Asp Asp Asp Lys)

D Codon box division
+
Initiation suppression

(stop codon = UAA)

E

mRNA2: **AUG** UGC GAC **GUG CGC GGG CGG GUC GGC** UAC **GUC** CCG UGC **GGG** UAA

peptide2: ClAcDW Cys Asp Val MeS Gly Arg MeF MeG Tyr MeF Pro Cys Gly Stop
(CM11-1)

Figure 1. Overview of artificial division of codon boxes. (**A**) The canonical codon table (left) and natural *E. coli* tRNAs that decode Val GUN, Arg CGN and Gly GGN codon boxes (right). cmo⁵U: 5-oxyacetyl uridine, I: Inosine, mnm⁵U: 5-methylaminomethyl uridine. (**B**) An artificially divided codon table containing 23 different amino acids (left) and *in vitro*-transcribed tRNAs that decode GUN, CGN and GGN codon boxes (right). The GUN codon box is divided into GUY and GUG codons which are assigned to citrulline (Cit) and Val, respectively. Similarly, the CGN codon box is divided to CGY (4-iodophenylalanine, IodoF) and CGG (Arg) codons and the GGC codon box into GGY (N-ε-acetyllysine, AcK) and GGG (Gly) codons. Cit, IodoF and AcK are pre-charged onto the relevant tRNAs by means of flexizymes, whereas Val, Arg and Gly are charged by aminoacyl-tRNA synthetases *de novo*. (**C**) Sequence of mRNA (mRNA1) and translated peptide (peptide 1) using the artificially divided codon table shown in (B). Peptide 1 contains 23 different amino acids including Cit, IodoF and AcK assigned at the GUC, CGC and GGC codons, respectively. (**D**) An artificially divided codon table combined with initiation suppression. The GUN, CGN and GGN codon boxes are divided and N-methylphenylalanine (MeF), N-methylserine (MeS) and N-methylglycine (MeG) are assigned at the GUY, CGY and GGY codons, respectively. The initiator AUG codon is suppressed by chloroacetyl-D-tryptophan (ClAcDW). (**E**) Sequence of mRNA (mRNAs) and translated peptide (peptide 2) using the codon table shown in (D). The N-terminal chloroacetyl group reacts with the thiol of the downstream Cys to form a thioether bond, resulting in a macrocyclic peptide.
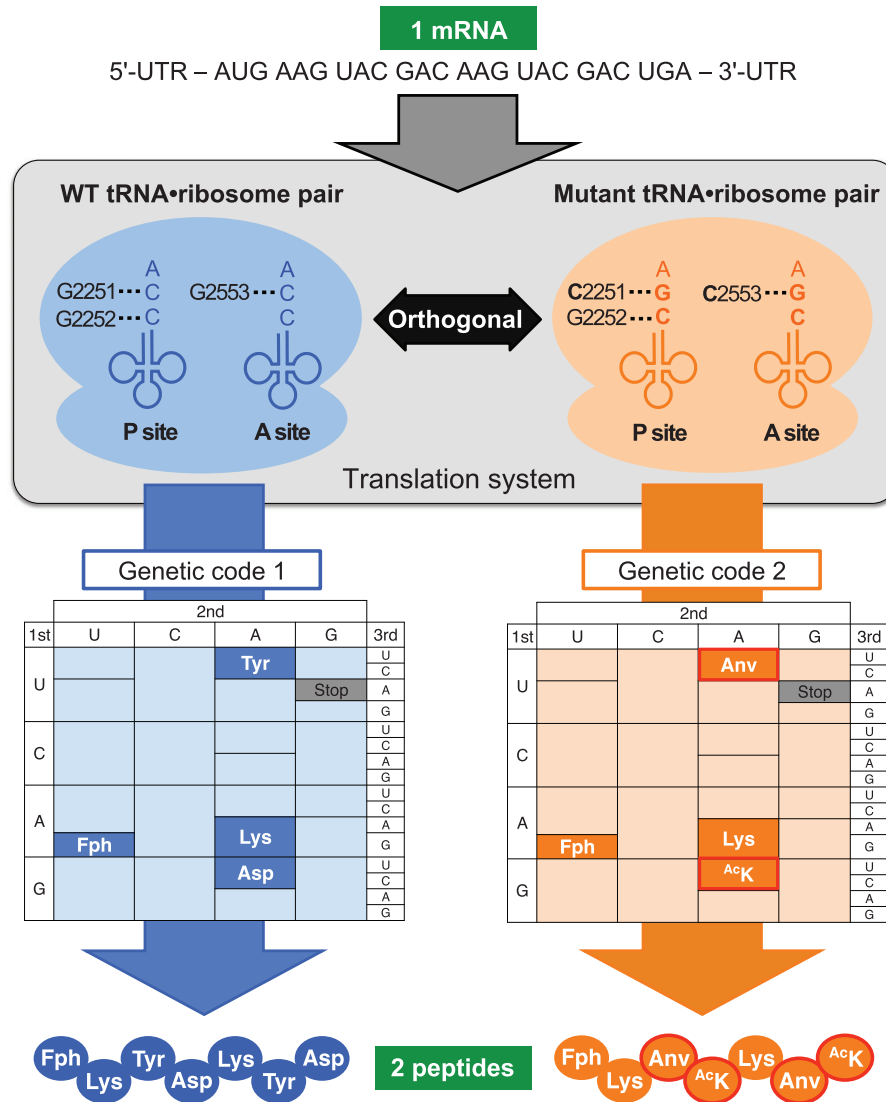
Figure 2. Overview of codon box duplication by means of an orthogonal tRNA/ribosome pair. The tRNA with a C75G mutation can be recognized by the 23S rRNA with G2251C/G2253C mutations, and does not cross-react with the wild-type (WT) ribosome. Similarly, WT tRNA can be recognized only by the WT ribosome, and is not utilized by the mutant ribosome. Genetic code 1 is exclusively utilized by the WT tRNA/ribosome pair, whereas genetic code 2 is exclusively utilized by the mutant tRNA/ribosome pair. Genetic code 2 is reprogrammed to include azidonorvaline (Anv) and ^{Ac}K at UAY and GAY codons, respectively. *N*-(5-FAM)-L-phenylalanine (Fph) is introduced at the initiator AUG codon of both codon tables. By adding a single mRNA, two distinct peptides can be simultaneously synthesized according to the two genetic codes.

wild-type tRNA/ribosome pair, the Watson–Crick base pairs between C74 and C75 of the tRNA and G2251 and G2252 at the P site of 23S rRNA as well as with G2553 at the A site are requisite for catalyzing the peptidyl transfer reaction (26–28). However, introduction of compensatory mutations to keep base-pair formation at these positions, e.g. a paired tRNA with a C75G mutation and a 23S rRNA with G2251C and G2553C mutations, is tolerated to retain peptidyl transfer activity (29). Moreover, the C75G tRNA only reacts with the G2251C/G2553C ribosome without cross-reaction with the wild-type tRNA/ribosome pair. Conversely, the wild-type does not cross-react with the mutant tRNA/ribosome pair.

Importantly, the duplicated codon tables constructed in this way can be combined with other genetic code manipulation methods such as genetic code reprogramming. For instance, while the wild-type codon table retains PAAs decoded by wild-type aminoacyl-tRNAs charged by aminoacyl-tRNA synthetases, the orthogonal codon table can be reprogrammed to have

nPAAs decoded by orthogonal nPAA-tRNAs pre-charged using flexizyme aminoacylating ribozymes (30,31). Whilst the original flexizymes recognize the conserved 3′-terminal NCCA sequence of tRNAs, this sequence is mutated in the tRNA for orthogonal translation. However, mutant flexizymes with compensatory mutations can also aminoacylate these mutant tRNAs. For example, translation of azidonorvaline (Anv), Lys and ^{Ac}K at the UAC, AAG and GAC codons, respectively, of the orthogonal codon table has been demonstrated (Figure 2, Genetic code 2), whilst Tyr, Lys and Asp were retained in the wild-type codon table for the same reaction (Figure 2, Genetic code 1). In addition, in these experiments, *N*-(5-FAM)-L-phenylalanine (Fph) was introduced at the initiator AUG codon of both codon tables for fluorescent labeling of the resulting peptides. By adding a single mRNA to the translation system, the two tRNA/ribosome pairs successfully produced two distinct peptides according to the two independent codon tables without any hybrid products derived from codon cross-reading. Thus, the two codon tables
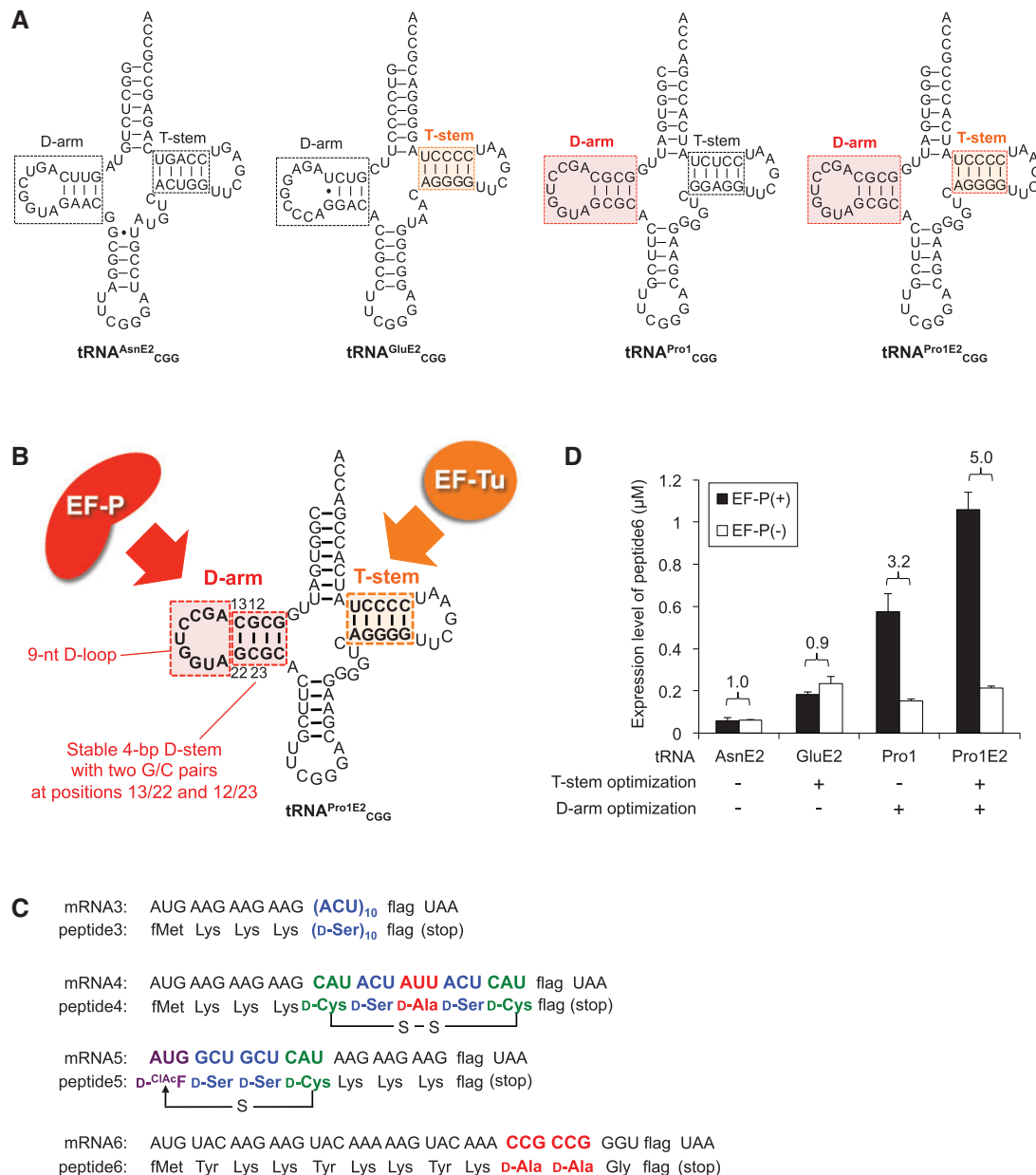
Figure 3. Improvement of nPAA incorporation efficiency using engineered tRNAs with higher EF-P/EF-Tu binding affinity. (**A**) Examples of tRNAs used for nPAA incorporation. tRNA^{AsnE2} is unoptimized for EF-P/EF-Tu binding. tRNA^{GluE2} has a T-stem structure with higher EF-Tu affinity. tRNA^{Pro1} has a D-arm motif that can be recognized by EF-P. tRNA^{Pro1E2} is a chimeric tRNA based on tRNA^{Pro1} in which the T-stem of tRNA^{GluE2} is inserted to provide both the D-arm and T-stem binding motifs. (**B**) Binding motifs of EF-P and EF-Tu in tRNA. EF-P recognizes a specific D-arm motif consisting of a 9-nt D-loop closed by a stable 4-bp D-stem with two G/C base pairs at positions 13/22 and 12/23. EF-Tu recognizes the T-stem region of tRNA, and the binding affinity between EF-Tu and tRNA differs depending on the structure of the T-stem. (**C**) Sequences of mRNAs (mRNAs 3–6) and translated peptides (peptides 3–6). (**D**) Expression level of peptide 6 in which two consecutive D-Ala residues were introduced by the four different tRNAs shown in (B). Black bars indicate the results of EF-P(+) translation and white bars EF-P(−) translation. Numbers above the bars show relative translation yield calculated as the ratio of EF-P(+) to EF-P(−). Error bars, s.d. ($n = 3$).

could be independently decoded by the orthogonal and the wild-type machineries and functioned in parallel.

Improvement of efficiency of nPAA incorporation by means of engineered tRNAs

Although various nPAAs have been successfully introduced into peptides by the aforementioned methodologies, translation of some amino acids, such as D-amino acids, β-amino acids and N-methyl amino acids, remains challenging. The incompatibility

of these substrates with the ribosomal translation system can be mainly attributed to the following causes: (i) inefficient accommodation of nPAA-tRNA at the ribosomal A site, (ii) mistranslocation of nPAA-tRNAs occurring prior to peptidyl transfer and (iii) slow peptidyl transfer between the nPAAs.

During accommodation, EF-Tu introduces aminoacyl-tRNAs to the ribosome by recognizing their T-stem and amino acid moieties (32,33). However, if the interaction between EF-Tu and the nPAA is too weak, EF-Tu cannot efficiently load the nPAA-tRNA on to the ribosome. To overcome this, we developed a new tRNA named tRNA^{GluE2}, the T-stem of which has higher

binding affinity to EF-Tu than previously used tRNAs such as tRNA^{AsnE2} (Figure 3A and B) (29,34). On the other hand, EF-Tu mutants that have higher binding affinity to nPAAs have been also developed, and increasing the concentration of wild-type EF-Tu in the translation system can also be effective for incorporation of difficult nPAAs. Doi *et al.* (35) developed EF-Tu mutants with enlarged binding pockets for bulky nPAAs, e.g. ʟ-1-pyrenylalanine, ʟ-2-pyrenylalanine and ᴅʟ-2-anthraquinonylalanine, whose incorporation was almost impossible by wild-type EF-Tu. Park *et al.* (36) also devised a new-engineered EF-Tu named EF-Sep that efficiently accommodates phosphoseryl-tRNA (Sep-tRNA). As the binding pocket of the wild-type EF-Tu is negatively charged, tRNAs with negatively charged amino acids, including Sep, are less efficiently accommodated. By substituting the amino acid residues around the binding pocket with relatively positive amino acids, they could successfully improve the binding affinity of EF-Tu to Sep-tRNA and thereby increase the expression level of peptides containing Sep.

A second cause of translational incompatibility is drop-off of peptidyl-tRNA from the P site caused by EF-G (37). We have shown that efficiency of consecutive incorporation of ᴅ-amino acids could be improved by reducing EF-G concentration from 0.3 to 0.1 µM to suppress this mis-translocation event (22). By combining the tRNA^{GluE2} and the translation system with optimized EF-G and EF-Tu concentrations, incorporation of 10-consecutive ᴅ-Ser residues could be accomplished (Figure 3C, peptide 3) as well as synthesis of macrocyclic peptides with four or five consecutive ᴅ-amino acids consisting of mixtures of ᴅ-Phe, ᴅ-Ser, ᴅ-Ala or ᴅ-Cys closed by either a disulfide or a thioether bond (Figure 3C, peptides 4 and 5, respectively).

The third cause of translational incompatibility is slow peptidyl transfer between some nPAAs. To accelerate the peptidyl transfer rate between consecutive ᴅ-amino acids, we took advantage of EF-P, a translation factor that accelerates peptidyl transfer between two consecutive ʟ-Pro residues in natural translation systems (38,39). As the peptide bond formation between ʟ-Pro is the slowest among the 20 PAAs and thus causes ribosomal stalling, EF-P is an indispensable factor for translation of peptides containing consecutive ʟ-Pro residues (40). EF-P recognizes the specific D-arm structure of P-site peptidyl-prolyl-tRNA^{Pro}, specifically, the 9-nt D-loop closed by the 4-bp stable D-stem with two G/C pairs at positions 12/23 and 13/22. This motif is shared among the three tRNA^{Pro} isoacceptors in *E. coli* (Figure 3B) (41). We demonstrated incorporation of two consecutive ᴅ-Ala residues into peptide 6 (Figure 3C) by using tRNA^{Pro1}, one of the tRNA^{Pro} isoacceptors (Figure 3A), as a carrier of ᴅ-Ala, and observed 3.2-fold improvement of peptide expression efficiency on addition of EF-P to the translation system. tRNA^{GluE2}, which does not have the conserved D-arm motif, did not demonstrate EF-P-dependent enhancement in this context, indicating the importance of the conserved D-arm motif (42).

As the T-stem and the D-arm are important recognition motifs for binding of EF-Tu and EF-P, respectively, an artificially designed tRNA containing both motifs would be expected to be efficiently recognized by both of EF-Tu and EF-P and achieve efficient accommodation and peptidyl transfer. Such a tRNA, tRNA^{Pro1E2}, including both the T-stem and D-arm motifs, was designed and used for consecutive incorporation of ᴅ-Ala into peptide 6 (Figure 3A–D). tRNA^{Pro1E2} exhibited comparable translation yield to tRNA^{GluE2} even without EF-P due to its higher EF-Tu binding affinity, and in the presence of EF-P a further 5-fold improvement in translation yield was observed (Figure 3D), demonstrating the potency of implanting the T-stem and D-arm motifs into a single tRNA (42).

Conclusions and perspective

In this review, we have summarized three methodologies that increase the number of available codons for translation of nPAAs or broaden the substrate scope of translation by facilitating incorporation of challenging nPAAs. Translation of various nPAAs into peptides by ribosomal synthesis is attractive due to a wide variety of special characteristics that nPAAs can impart. For instance, *N*-methylation increases the hydrophobicity of peptides and thus can lead to higher membrane permeability, and macrocyclic scaffolds made by introduction of stapling amino acids increase both rigidity and stability (43–48). Further, since nPAAs are not efficiently recognized by peptidases, peptides consisting of nPAAs generally have better peptidase resistance. Such properties (membrane permeability, rigidity and stability) are desirable in a therapeutic context and have been leveraged for the development of novel peptidic drugs.

However, whilst the techniques described above allow the translation of diverse nPAAs, the translation of some amino acids remains challenging. Generally, charged ᴅ-amino acids, β-amino acids and *N*-methyl-amino acids, such as ᴅ-Asp, ᴅ-Glu, ᴅ-Lys, β-Glu and *N*-methyl-Glu, are relatively more difficult to incorporate (15,16). Consecutive incorporation of β-amino acids and even single incorporation of γ- or longer backbone amino acids is even more challenging, and has not, to the best of our knowledge, been reported. Therefore, further improvement of these methodologies is required to broaden substrate amino acid scope.

One of the advantages of ribosomally synthesizing peptides with nPAAs, as compared to organic synthesis, is that such peptides can be applied to screening methods such as mRNA display (49,50) and ribosome display (51) for the discovery of bioactive peptides from random peptide libraries. Whilst the details of such techniques is beyond the scope of this review, we have previously shown that the combination of mRNA display and genetic code reprogramming, so called RaPID (Random non-standard Peptides Integrated Discovery), enables the discovery of peptide ligands containing various *N*-methyl (^{Me}Phe, ^{Me}Ser, ^{Me}Gly and ^{Me}Ala) and side-chain modified amino acids against various target proteins (52,53). We envisage that by expanding translation beyond 20 amino acids per reaction, and by allowing the translation of various challenging nPAAs, the methodologies that we have discussed here will lead to the construction of peptide libraries with higher diversity and in broader chemical space, thereby facilitating the discovery of peptide ligands with even higher affinity, rigidity and stability than is currently achieved.

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