

Using a Solid-Phase Ribozyme Aminoacylation System to Reprogram the Genetic Code

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Summary

Here, we report a simple and economical tRNA aminoacylation system based upon a resin-immobilized ribozyme, referred to as Flexiresin. This catalytic system features a broad spectrum of activities toward various phenylalanine (Phe) analogs and suppressor tRNAs. Most importantly, it allows users to perform the tRNA aminoacylation reaction and isolate the aminoacylated tRNAs in a few hours. We coupled the Flexiresin system with a high-performance cell-free translation system and demonstrated protein mutagenesis with seven different Phe analogs in parallel. Thus, the technology developed herein provides a new tool that significantly simplifies the procedures for the synthesis of aminoacyl-tRNAs charged with nonnatural amino acids, which makes the nonnatural amino acid mutagenesis of proteins more user accessible.

Introduction

Site-specific mutagenesis of proteins with nonnatural amino acids allows us to utilize unique functional groups that are unavailable from the repertoire of natural amino acids, thereby adding a new dimension to protein research [1, 2]. In the last two decades, this technique has been used for the isolation of various proteins carrying the desired nonnatural amino acids [3]. However, in spite of its tremendous potential toward the engineering and studying of proteins, this technique has not been widely used. This is simply because the method is technically difficult and laborious. Particularly, the synthesis of a suppressor tRNA charged with nonnatural amino acids requires multiple steps, which involve chemical aminoacylation of a dinucleotide (pdCpA), HPLC purification, enzymatic ligation with the tRNA fragment that lacks the 3'-CA, and removal of the *N*-protecting group [4–6].

Schultz and colleagues have recently made remarkable progress in evolving tyrosyl-tRNA synthetase (TyrRS) mutants derived from *Methanococcus jannaschii* (*M. jannaschii*) [7, 8]. They have developed a genetic selection method where active TyrRS mutants can be selected based on the charging activity of phenylalanine (Phe) analogs onto a *M. jannaschii* tRNA^{Tyr}_{CUA}. This tRNA is inert against endogenous *Escherichia coli* aminoacyl-

tRNA synthetases (*E. coli* ARSs), and thus called orthogonal tRNA (otRNA). They have so far succeeded in evolving several sets of TyrRS mutants capable of charging Phe analogs onto this particular otRNA (generally each TyrRS mutant is designated to one Phe analog) and expressing proteins containing Phe analogs in *E. coli* under the conditions where natural amino acid concentrations were controlled [7, 9–12].

The above demonstration represents a powerful strategy to evolve a naturally occurring ARS. However, a drawback of this method is that since the individual mutant is generally specific to its Phe analog, additional evolution is required to alter the ARS specificity when a new kind of analog is used. This would not be a routine process for many investigators because it requires multistep experiments, such as preparation of the initial libraries, optimization of the selection conditions, and characterization of the mutants. Moreover, the above orthogonal ARS/tRNA pair selected in *E. coli* must be reevolved in order to be applied to other organisms. This is in contrast to the aforementioned cell-free synthetic method, which is more flexible toward various nonnatural amino acids and suppressor tRNAs that are orthogonal in prokaryotic [3] and eukaryotic translation systems [13].

Here, we report a novel and highly practical tRNA aminoacylation system based upon a resin-immobilized ribozyme, facilitating the step for the synthesis of nonnatural aminoacyl-tRNAs (Figures 1A and 1B). The method is simple; a user-specified tRNA and a Phe analog are reacted on this *reusable* ribozyme resin, and the eluent containing the desired aminoacyl-tRNA can be used in a cell-free transcription-translation coupling system (Figure 1C). In support of this claim, we have demonstrated the incorporation of seven Phe analogs with a variety of *p*-substitutions into green fluorescent protein (GFP) at one or two sites specifically. It should be noted that the entire process, including tRNA charging, in vitro translation, and purification of protein, can be done in one day. Thus, the ribozyme aminoacylation system in combination with a highly efficient cell-free translation system advances the method of the protein nonnatural amino acid mutagenesis to a more user-accessible technology.

Results and Discussion

Resin-Immobilized Ribozyme

The ARS ribozyme used for the development of the resin-immobilized form is a *de novo* catalyst that was originally selected from a pool of random RNA sequences [14]. The first generation ribozyme was further optimized by a combination of in vitro evolution and systematic engineering, affording a 45 nucleotide (nt) ribozyme [15]. This ribozyme, called Flexizyme, displays a broad spectrum of activity toward various tRNAs and Phe analogs, the properties of which would be suitable for nonnatural amino acid mutagenesis.

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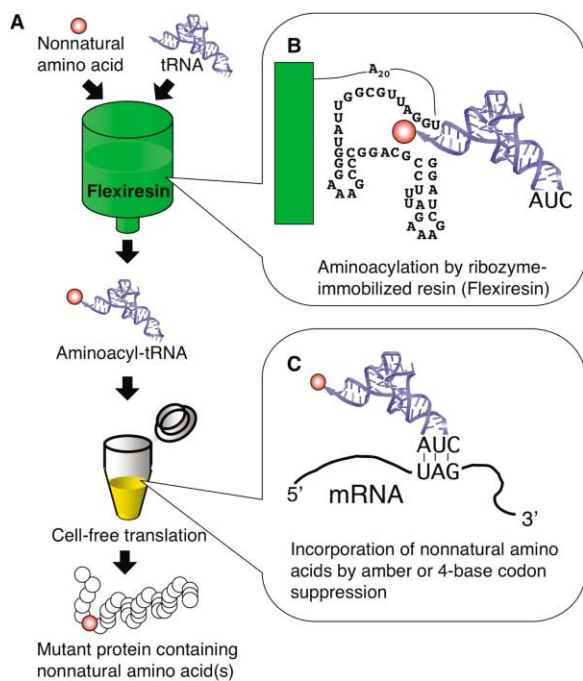


Figure 1. Overview of Flexiresin-Facilitating Nonnatural Amino Acid Mutagenesis

(A) The process of nonnatural amino acid mutagenesis of protein using Flexiresin. Flexiresin is capable of charging various Phe analogs onto various tRNAs, and therefore, this method allows users to utilize desired pairs of Phe analog and tRNA.

(B) By simply mixing a Phe analog and a tRNA that are chosen by users, the tRNA is charged with the nonnatural amino acid on the solid phase of Flexiresin in 2 hr.

(C) The aminoacyl-tRNA product is added to a high-performance cell-free translation system coupled with transcription in the presence of a mutant protein DNA template. The mutant gene contains an amber codon at a specific position. An amber suppressor tRNA suppresses the amber codon and incorporates a Phe analog. Alternatively, a 4-base codon and frame-shift suppressor tRNA pair can be used, and a Phe analog is incorporated by the “programmed frame-shift” suppression.

In order to make the Flexizyme aminoacylation system more practical, we have carried out 3'-end specific immobilization of the ribozyme onto a resin [16]. Flexizyme was engineered to bear an additional 20 adenosines at the 3'-end, and the cis-diol of this terminal adenosine was converted to the 3'-dialdehyde by periodate oxidation. This construct was then coupled to a hydrazine resin, followed by mild reduction of the hydrazide bond, yielding an irreversible covalent bond. The preparation process generally takes a few hours, giving 80%–90% immobilization efficiency. The resin-immobilized Flexizyme, referred to as Flexiresin, is stable and can be stored for months without any loss of activity.

Aminoacylation of Suppressor tRNA^{Asn_{CUA}}

Flexiresin was tested for aminoacylation activity onto an engineered suppressor tRNA, derived from *E. coli* tRNA^{Asn_{CUA}} [17]. This suppressor tRNA was engineered to have a G73A mutation, which was found effective for enhancing its inertness against endogenous ARSs in an *E. coli* cell-free translation system (vide infra). Flexiresin

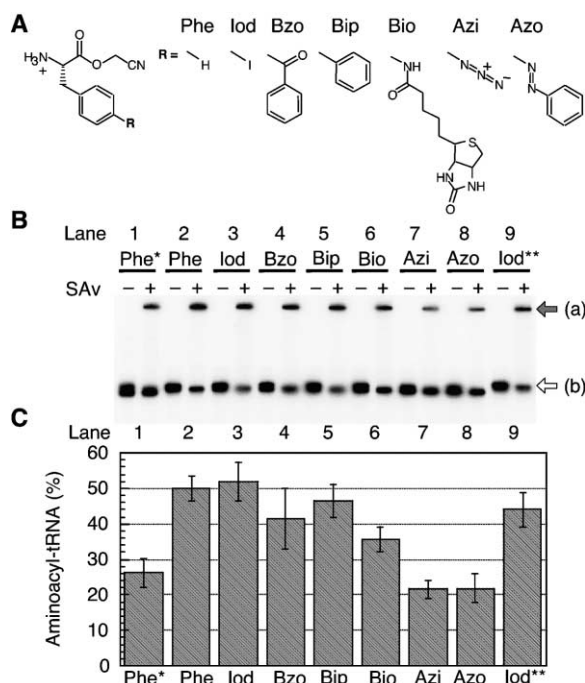


Figure 2. Aminoacylation of Engineered Suppressor tRNAs with Phe and Phe Analogs by Flexiresin

(A) Cyanomethyl ester-activated Phe analogs used in this study. These amino acids have various groups at the *p*-position of phenyl ring.

(B) Aminoacylation analysis by streptavidin-dependent gel-shift assay. Amino acid-specific biotinylation was done after an aminoacylation reaction to detect aminoacyl-tRNA.

(C) Aminoacylation efficiency. The middle of the error bar represents the mean score from three different trials. The error bar represents a standard deviation of all trials. Abbreviations: a, Aminoacyl-tRNA-streptavidin complex; b, unaminoacylated tRNA; *, aminoacyl-tRNA containing dC75, which was prepared by the chemical aminoacylation method; **, tRNA that has a ACCC 4-base anticodon instead of CUA anticodon; Phe, phenylalanine; lod, *p*-iodophenylalanine; Bzo, *p*-benzoylphenylalanine; Bip, *p*-biphenylalanine; Bio, *p*-biotinyl-aminophenylalanine; Azi, *p*-azidophenylalanine; Azo, *p*-phenylazophenylalanine; SAV, streptavidin.

was incubated with tRNA^{Asn_{CUA}} and Phe cyanomethyl ester (Phe-CME) in the presence of Mg²⁺ for 2 hours. Following removal of the supernatant, the resin was washed with buffer containing no Mg²⁺ to elute the desired Phe-tRNA^{Asn_{CUA}}. After biotinylation of the α -amino group of Phe, the product was analyzed using a streptavidin-dependent gel-shift assay, revealing a yield of approximately 50% of Phe-tRNA^{Asn_{CUA}} (Figures 2B and 2C, lane 2). In comparison, the chemoenzymatic synthesis of Phe-tRNA^{Asn_{CUA}} containing dC₇₅ gave approximately a 30% yield in our laboratory (Figures 2B and 2C, lane 1; although this value could vary from 30% to 60% depending on the purity of pdCpA-Phe). Most significantly, neither HPLC purification nor multistep procedures are involved in the aminoacylation of tRNA when using Flexiresin, and hence, the time required for the synthesis of Phe-tRNA^{Asn_{CUA}} is significantly shortened from days to a few hours.

We extended the aminoacylation testing further with six different Phe analogs that have a variety of *p*-substi-

tutions ranging from small to large groups (Figure 2A). Remarkably, Flexiresin is able to charge all these Phe analogs with yields ranging from 20% to 50% (Figures 2B and 2C, lanes 2–8). This result clearly indicates that the flexible nature of Flexizyme is maintained in Flexiresin, but there are two technical advantages of using Flexiresin over Flexizyme. First, Flexiresin is reusable. A simple wash with water regenerates Flexiresin for the next use in minutes. Indeed, we have used it at least ten times and have no reason to believe that it could not be used more times. Second, the desired aminoacyl-tRNA is readily isolated. Upon the addition of tRNA to Flexiresin, tRNA is immobilized over 90%. Thus, the unreacted amino acid substrate and undesired side products can be readily removed by simply taking the supernatant after reaction. Moreover, the aminoacyl-tRNA is separated from the catalyst by the simple elution procedure. This convenient purification procedure of aminoacyl-tRNA is advantageous when the application to cell-free translation or other applications were considered, because any unnecessary contaminants in the reaction may reduce the efficiency of the translation. These two features unequivocally make this technology simple, practical, and economical, particularly when scale up is required.

Nonnatural Amino Acid Mutagenesis

To demonstrate the utility of Flexiresin toward protein mutagenesis, we chose a high performance *E. coli* in vitro transcription-translation coupling system available from Roche, called rapid translation system (RTS). GFP was used as a model protein for expression with nonnatural amino acids, since formation of the full-length protein can be readily detected by fluorescence [18]. We selected the Tyr151 residue for a site-specific mutation, which is located on the surface of GFP and therefore the mutation should not interfere with fluorescent activity (Figure 3) [19]. As expected, the 151 amber mutation on the *gfp* expression plasmid halts elongation, thus producing a truncated protein (Figure 4A, lane 2) that has no fluorescence (Figure 4B, lane 2). The addition of tRNA^{Asn_{CUA}} alone does not suppress the amber mutation (Figure 4A, lane 3), confirming the inertness of this tRNA against endogenous ARSs in RTS. As a positive control, we prepared Phe-tRNA^{Asn_{CUA}}, containing dC at position 75, by means of the traditional chemoenzymatic method, and used it to confirm the suppression activity of this tRNA (Figure 4A, lane 4). We then prepared tRNA^{Asn_{CUA}} charged with Phe or its analogs by using Flexiresin and tested the suppression of the amber codon in parallel (Figure 4A, lanes 5–11). In all cases, the amber mutation was effectively suppressed, yielding the full-length proteins. The suppression efficiency observed was in most cases over 30% and as high as 80% (Figure 4C).

To assess the expression efficiency, the translation was scaled up to a semipreparative level where a higher concentration of methionine (Met) was used compared with the analytical scale (2 mM instead of 0.05 mM, according to the manufacturer's protocol), and the full-length protein was purified via histidine tag. In a 110 μ l scale translation reaction, 19.5 μ g of the wild-type GFP was isolated, while 8.4 μ g of the Tyr151Ido was isolated.

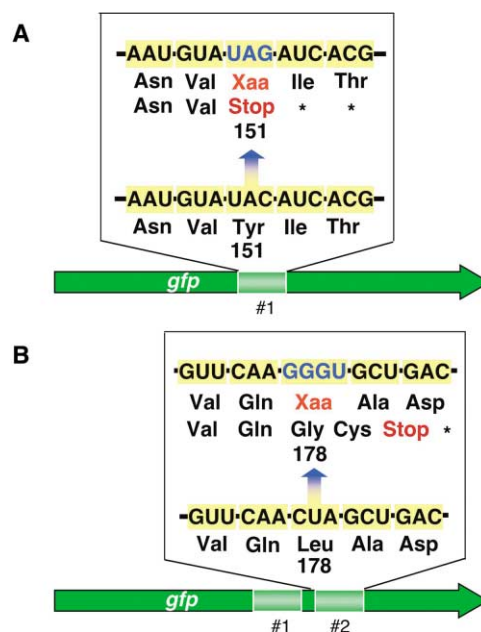


Figure 3. Mutant Genes Used in This Study

(A) Amber (TAG) codon was introduced at position 151 (#1) for incorporation of Phe analogs. Asterisks indicate the residues that are not incorporated into protein.

(B) Amber and a 4-base (GGGT) codon were introduced at 151 (#1) and 178 (#2) positions, respectively, for the incorporation of two Phe analogs. The amino acids at 151 and 178 are present on the surface of GFP, which should not perturb the protein folding.

These values translate to an expression efficiency of 177 μ g/mL and 76 μ g/mL, respectively. Interestingly, the difference in the efficiency between the wild-type and mutant proteins in the semipreparative scale was greater than that observed in the analytical scale (Figure 4A, the ratio of full-length protein in lane 6 to lane 1 was approximately 9:10). We attributed this to the higher concentration of Met, which enhanced the expression level of the wild-type more than that of the mutant. We speculated that the addition of Met could result in accelerating the initiation step. Perhaps the expression of the wild-type protein was effectively enhanced because initiation could be the rate-limiting step in this case. On the other hand, in the case of expression of the mutant proteins, other steps related to the suppression event, such as amber suppression or peptidyl-transfer of the nonnatural amino acid, might be the rate-limiting step. Further investigations are necessary to draw a more definitive conclusion regarding this observed difference.

The isolated GFPs were analyzed to confirm the incorporation of Tyr and Ido into position 151. The protein was digested with trypsin, and the resulting fragments were analyzed by LC-MS. We detected the expected peptide fragment containing Tyr (observed mass = 972.6 *m/z*, calculated mass = 972.5 *m/z*) for the wild-type fragment and that containing Ido (observed mass = 1027.9 *m/z*, calculated mass = 1027.4 *m/z*) for the mutant fragment (see Supplemental Data available online at <http://www.chembiol.com/cgi/content/full/10/11/1077/DC1>). This result confirmed the incorporation of the Phe analog at the expected position.

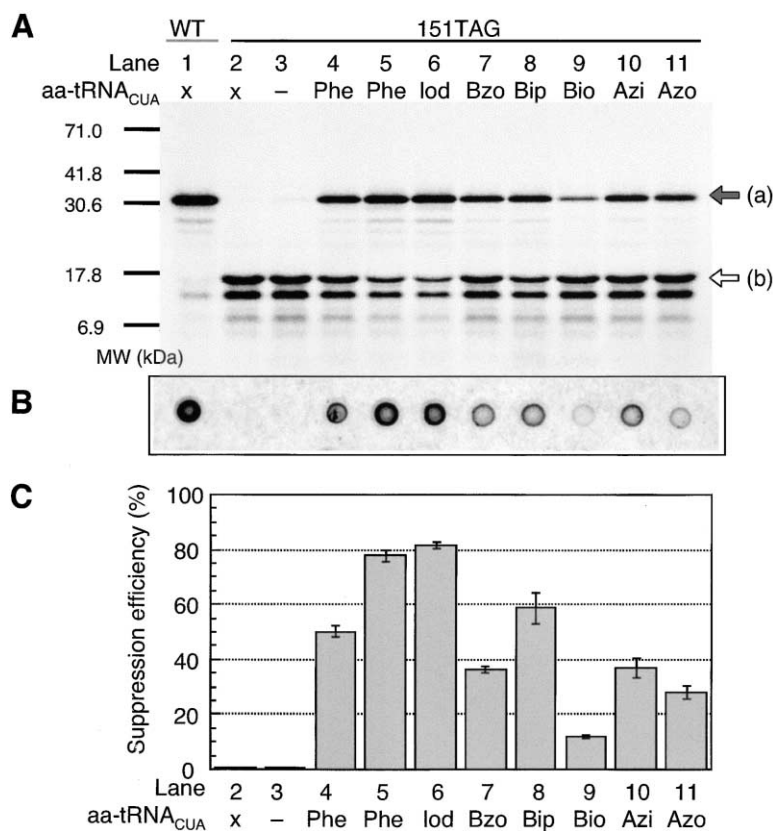


Figure 4. Nonnatural Amino Acid Mutagenesis of GFP at 151

(A) SDS-PAGE analysis of translation reaction. Lane 1, wild-type; lane 2, in the absence of suppressor tRNA^{Asn}_{CUA}; lane 3, in the presence of suppressor tRNA^{Asn}_{CUA}; lane 4, in the presence of chemically synthesized Phe-tRNA^{Asn}_{CUA} containing dC75; lanes 5–11, in the presence of aminoacyl-tRNA^{Asn}_{CUA} prepared by the Flexizyme-resin. Abbreviations: aa-tRNA, aminoacyl-tRNA^{Asn}_{CUA}; x, no tRNA^{Asn}_{CUA}; -, no aa-tRNA^{Asn}_{CUA}; wt, wild-type; (a), full-length protein; (b), truncated peptide. The band below (b) is an unknown truncated peptide.

(B) Fluorescence activity of each sample in (A), analyzed by Molecular Imager FX.

(C) Suppression efficiency. The middle of the error bar represents the mean score from three different experiments. The error bar represents a standard deviation of all trials. The suppression efficiency was calculated based on the radioactivity of bands (a) and (b), fitted to $(a)/[(a)+(b) \times 5/3]$. Intensity of (b) was corrected in order to reflect the number of Met residues, based on the consideration that the full-length and truncated proteins contain 5 and 3 Met, respectively. We excluded the shortest peptide fragment (the fastest migrating band) from the calculation of suppression efficiencies, since this truncation might occur due to an abnormal termination, such as a drop-off of peptidyl-tRNAs from the ribosome [39–41]. It should be noted that this termination was also observed in the wild-type expression (Figure 4A, lane 1). Therefore, in the case of mutant expression, translation might pause at the amber codon, resulting in an increase in the chance of a drop-off event.

Posttranslational Modification

Although we have shown in Figure 4 that most Phe analogs were incorporated into GFP efficiently, the incorporation of Bio (lane 9) was modest compared with the other Phe analogs. This is most likely an intrinsic limitation of the translational machinery against nonnatural amino acids with large side chains. To avoid this problem, a Phe analog bearing an electrophile, such as a ketone, can be incorporated into proteins and then a posttranslational modification reaction can be performed using hydrazine-tagged molecules [20]. The efficiency of Flexiresin-catalyzing tRNA aminoacylation with *p*-aceto-Phe (AcPhe) was $45.0\% \pm 1.2\%$ determined by streptavidin-dependent gel-shift assay, and this nonnatural amino acid was incorporated into the position 151 with $28.9\% \pm 0.9\%$ suppression efficiency (data not shown). This mutant protein was then treated with biotin-hydrazide and the 151 keto residue was selectively tagged with the biotin molecule. As expected, the gel mobility of the biotin-modified protein was retarded in the presence of streptavidin (Figure 5A) and pulled down by streptavidin-agarose (Figure 5B), whereas the wild-type was not. Thus, site-specific posttranslational modification can be performed with a variety of hydrazide molecules.

Double Site-Specific Mutagenesis with Two Nonnatural Amino Acids

Like Flexizyme, Flexiresin does not recognize the anticodon sequence of tRNA (Figures 1B and 1C, lane 9). This feature allows us to create a second set of Phe analogs and suppressor tRNAs with an anticodon other than amber. Programmed frame shift or the so-called “4-base codon” is an alternative approach to expand the genetic code [21]. When the programmed frame-shift mutation is introduced into mRNA, termination of the translation reaction occurs after the 4-base codon because it remains in the “zero reading frame” and encounters a stop codon therein (see Figure 3B). By reading through the 4-base codon with the frame-shift suppressor tRNA bearing the corresponding 4-base anticodon, translation can continue in the “+1” frame and hence the full-length protein is produced. It has been shown that 4-base codons can be designed based on less abundant codons in *E. coli*, such as Arg CGG, Arg AGG, or Gly GGG; hence, the 4-base codon consists of CCGN, AGGN, or GGGN [22, 23]. We prepared pairs of 4-base codons and the corresponding frame-shift suppressor tRNA mutants and screened appropriate combinations for RTS activity using Bzo as the nonnatural amino acid. We found that an orthogonal *E. coli* tRNA^{Asn}_{ACCC} sup-

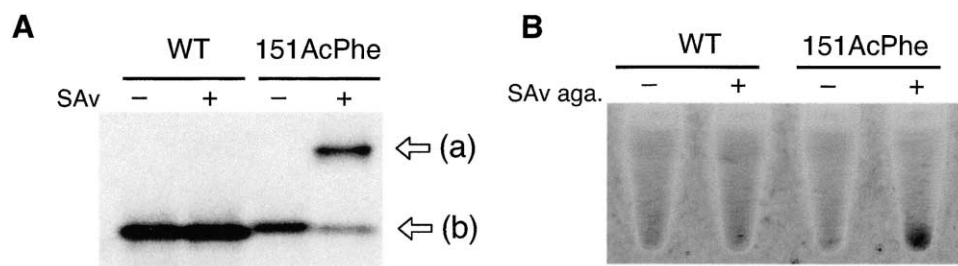


Figure 5. Posttranslation Modification of AcPhe at Position 151

(A) Streptavidin-dependent gel-shift assay for the wild-type and biotinylated 151AcPhe mutant GFPs. Approximately 80% of the mutant GFP band was retarded by the addition of streptavidin, whereas no shift was observed for wild-type. Abbreviations: SAv, streptavidin; wt, wild-type.

(B) Streptavidin-agarose capture of the mutant GFP. Streptavidin-agarose was successfully used to capture the biotinylated mutant GFP and concentrated on the resin, whereas no capture was observed for wild-type. Abbreviation: SAv-aga, streptavidin-agarose.

presses the corresponding frame-shift mutation (GGGU) most efficiently in RTS (H.M. and H.S., unpublished data).

By combining the amber and the programmed frame-shift codons [24], we attempted to incorporate two Phe analogs (Iod and Bip, see Figure 2A) into GFP at two specific sites (Figure 3B). We chose positions 151 and 178 to have the UAG and GGGU mutations, respectively. Two corresponding suppressor tRNAs, $tRNA^{Asn}_{CUA}$ and $tRNA^{Asn}_{ACCC}$, were charged with Bip and Iod via Flexiresin (Figure 2C, lanes 5 and 9, respectively) and used to suppress these mutations. As expected, the full-length GFP could be translated only in the presence of both suppressor tRNAs (Figures 6A–6C, lane 6), whereas the expected truncated proteins were observed in the presence of either of the charged tRNAs, as expected (lanes 2–5). These results support the incorporation of two nonnatural amino acids into the designated positions of the mutant GFP.

Conclusion and Outlook

We have demonstrated that the combination of a resin-immobilized form of an ARS ribozyme, called Flexiresin, and a high-performance cell-free translation system facilitates the mutagenesis of proteins with nonnatural amino acids. Owing to the flexible feature of Flexiresin toward different aromatic amino acid and tRNA substrates, this single catalytic system can be applied to a variety of user-specified combinations. The method is simple and practical in terms of the preparation of the Flexiresin as well as the procedures for the synthesis and isolation of aminoacyl-tRNAs. Moreover, the durability and recyclability of Flexiresin make this technology economical. Most importantly, the methods used throughout these studies, including the cell-free translation, are not specialized, and therefore, nonnatural amino acid mutagenesis becomes a user-accessible technology.

The flexibility of Flexiresin is perhaps even more significant when other applications are considered. For instance, the combinations of amber and 4-base codons will allow us to readily reprogram the genetic code with more than two nonnatural amino acids [24–26]. Site-specific multiple mutations with unique nonnatural amino acids, such as fluorophores or spin labels, provide a new

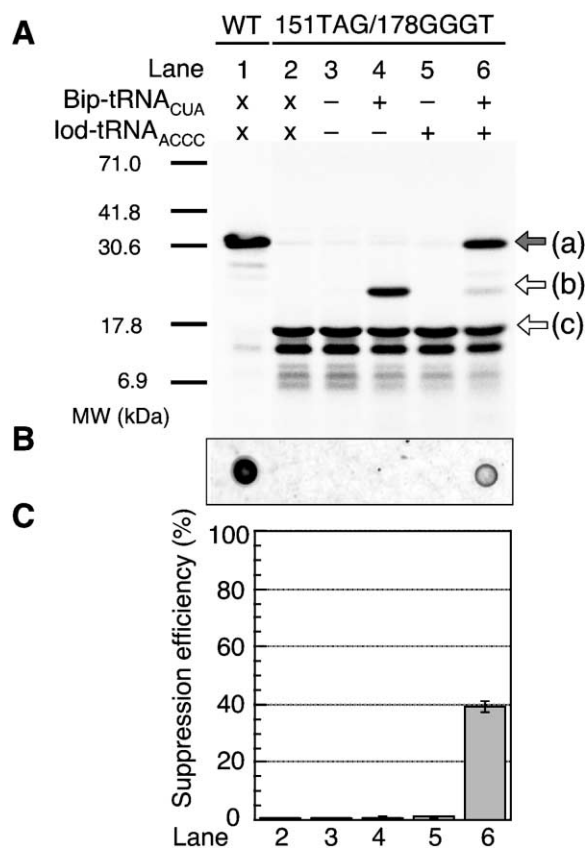


Figure 6. Nonnatural Amino Acid Mutagenesis of GFP at 151/178

(A) SDS-PAGE analysis of the translation reaction. Abbreviations: x, no tRNA; -, no aa-tRNA; +, aa-tRNA; wt, wild-type; (a), full-length protein; (b), truncated peptide at position 180 (failed frame shift); (c), truncated peptide at position 151 (amber stop codon).

(B) Fluorescent activity of each sample in (A), analyzed by Molecular Imager FX.

(C) Suppression efficiency. The suppression efficiency was calculated based the radioisotope of the bands of (a), (b), and (c), fitted to $(a)/[(a)+(b) \times 5/3 + (c) \times 5/3]$. The middle of the error bar represents the mean score from three different trials. The error bar represents a standard deviation of all trials.

tool for studying protein dynamics [24, 27–29]. Flexiresin can be used for the synthesis of nonnatural aminoacyl-tRNAs that are orthogonal in other translation systems, e.g., rabbit reticulocyte lysate and wheat germ extract [30], both of which are suitable for translating mRNAs or cDNAs derived from eukaryotic cells. For example, an amber suppressor tRNA derived from *E. coli* tRNA^{Tyr} is known to be orthogonal in wheat germ translation system [13], and hence, the Flexiresin method can be readily applied to the preparation of the corresponding aminoacyl-tRNAs. Similarly, Flexiresin allows users to synthesize suitable aminoacyl-tRNAs that can be imported into COS1 cells [31] or *Xenopus* oocyte [32], thus expressing proteins containing nonnatural amino acids in vivo. Thus, this new enabling technology based on Flexiresin allows for nonnatural amino acid mutagenesis to become more accessible for many researchers.

Yet we still need more investigations in order to adapt this technology to a variety of applications. Flexiresin is able to charge a fairly wide range of aromatic amino acids, including those not shown in this study, such as *N*-alkyl-Phe and tryptophan analogs onto tRNAs (D.K., H.M., and H.S., unpublished data), but the full scope of Phe analogs has not been fully investigated. On the other hand, it is clear that Flexiresin is unable to charge nonnatural amino acids possessing aliphatic side chains [14, 33]. Although our previous studies on Flexizyme and its parental ribozyme have revealed the potential bases involved in Phe binding [33], the exact mode of the substrate recognition is unknown. Perhaps further structural characterizations of the ribozyme, e.g., by means of X-ray crystallography, would help in understanding such details. However, even without a high-resolution structure, our accumulated knowledge of the critical residues in Flexizyme [15, 33–35] would facilitate the design of doped RNA pools such that rapid in vitro evolution could be carried out. These studies are currently underway in our laboratory.

Significance

The resin-immobilized ribozyme, called Flexiresin, is able to charge a wide range of Phe analogs onto engineered amber and frame-shift suppressor tRNAs. Flexiresin can be readily prepared and stored at 4°C, and most importantly, it is recyclable. This technology was coupled with a high-performance cell-free translation system, and Phe analogs were incorporated into a model protein at one or two specific sites. Thus, the solid phase ribozyme aminoacylation system would enable many researchers to perform protein mutagenesis using nonnatural amino acids with greater ease.

Experimental Procedures

Preparation of Ribozymes and tRNAs

The following nucleotides were chemically synthesized and purified by 6% denaturing polyacrylamide gel electrophoresis (PAGE): Fx (5'-ACCTAACGCC AATACCCTTT CGGGCCTGCG GAAATCTTTC GATCC-3'), P5-1 (5'-ACGCATATGT AATACGACTC ACTATAGGAT CGAAAGATT CCGC-3'; T7 promoter sequence is italicized), P5-2 (5'-GGTAACACGC ATATGTAATA CGACTC-3'), P3-1 (5'-T₂₀ACC TAACGCC AATACCCTTT-3'), P3-2 (5'-T₂₀ACCTAACGCC-3'), tR (5'-TGGTGCCTCT GACTGGACTC GAACCACTGA CATACGGATT XXX

AGTCCCG CGTTCTACCG ACTGAACTAC AGAGGC-3', XXX = TAG or GGGT), P5-3 (5'-ACGCATATGT AATACGACTC ACTATAGCCT CTGTAGTTCAG TCGGT-3'), and P3-3 (5'-TGGTGCCTCT GACTG GACTC-3').

The corresponding 5'- and 3'-primers (P5-1 and P3-1) were used to amplify the template DNA (Fx) coding the Flexizyme sequence, and the resulting dsDNA was further amplified using the shorter primers (P5-2 and P3-2). The corresponding 5'- and 3'-primers (P5-3 and P3-3) were used to amplify the template DNA (tR) coding the engineered tRNA^{Asn}, and the resulting dsDNA was further amplified using the shorter primers (P5-2 and P3-3). The Fx dsDNA or tR dsDNA was in vitro transcribed in the presence of 7.5 mM GMP, 3.75 mM each NTPs, and the RNA transcript was purified by 6% denaturing PAGE. For the aminoacylation assay, body-radiolabeled tRNA was synthesized using the same protocol except for the presence of [α -³²P]GTP in the transcription reaction.

Preparation of Resin-Immobilized Flexizyme

Freshly prepared 0.1 M NaIO₄ (400 μ L) was added to 28.1 μ M Flexizyme (1 mL), and the mixture was incubated at 0°C for 20 min. The 3'-dialdehyde RNA was precipitated with 14 ml of 2% lithium perchlorate in acetone followed by a 1 ml wash with acetone. The pellet was dissolved in 1.4 ml of 0.1 M sodium acetate (pH 5.) and then mixed with 0.7 ml adipic acid dihydrazide-agarose that was prewashed with DEPC-treated water. The reaction solution was mixed at room temperature for 3 hr. The resulting imine moiety of the RNA-resin was reduced by adding 1 M sodium cyanoborohydride (300 μ L) and then was incubated at room temperature for 30 min. The agarose was washed with 1.4 ml of W1 (consisting of 0.1 M sodium acetate buffer [pH 5.0] containing 300 mM NaCl, 7.5 M urea, and 0.1% sodium dodecyl sulfate) and then suspended in 2.1 ml of W1. The immobilization efficiency was estimated by the amount of the recovered ribozyme in the flow through based on absorbance at UV₂₆₀. The Flexizyme-resin (Flexiresin) can then be stored in 4°C. Before the aminoacylation reaction, Flexiresin is washed with 4 volumes of DEPC-treated water three times and then suspended in three-resin volumes of DEPC-treated water.

Preparation of Amino Acid Substrates

The same procedure as previously described [14] was used to synthesize cyanomethyl ester of Phe (phenylalanine), Bzo (*p*-benzoyl-phenylalanine), and Azo (*p*-phenylazophenylalanine), Iod (*p*-iodophenylalanine), Bip (*p*-biphenylalanine), Azi (*p*-azidophenylalanine), and AcPhe (*p*-acetylphenylalanine) from the corresponding *N*-Boc amino acids. Bio (*p*-biotinyl-aminophenylalanine) was synthesized from *N*-Boc-*p*-aminophenylalanine and biotin-NHS, followed by the same procedure as above.

Aminoacylation of tRNA on Flexiresin

The following conditions were generally used for analytical purposes: 5 μ l Flexiresin suspended in DEPC-treated water (the resin volume is 1.2 μ l corresponding to 7.5–10 μ M ribozyme concentration) was taken to a microtube, and the supernatant was removed to leave the resin. Folded tRNA (2.25 μ l of 12 μ M) in EKM buffer [pH 7.0, 50 mM N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid) (EPPS), 12.5 mM KCl, 1.2 M MgCl₂] was added to the resin on ice, and then added to this mixture was 0.25 μ l of 50 or 100 mM amino acid substrate followed by 0.1 μ l of 0.25 M KOH (to adjust pH to 7.0–7.2). After 2 hr incubation on ice, the supernatant was removed, and the resin was washed with 5 μ l of EK buffer (pH 7.5) containing 10 mM EDTA at room temperature three times to elute the aminoacyl-tRNA product. After addition of 0.75 μ l of 3.0 M potassium acetate (approximately 0.15 M final concentration), the aminoacyl-tRNA was precipitated by the addition of 30 μ l of ice-cold ethanol. After 5 min on dry ice, the tube was centrifuged, the pellet was washed with 70% ethanol, and dried under vacuum.

For analysis of aminoacylation efficiency, selective biotinylation of the α -amino group of aminoacyl-tRNA was carried out as follows [14, 36]. The pellet was dissolved in 2.5 μ l of EPPS (0.1 M; pH 5.9) containing 20 mM biotin-3-sulfo-*N*-hydroxysuccinimide ester, and after cooling on ice, the reaction was initiated by the addition of 0.86 μ l of EPPS-KOH (0.3 M; pH 9.1, which brought the pH to 8.0). After 1 hr, the reaction was terminated by ethanol precipitation, and

the pellet was washed with 70% ethanol and dissolved into 10 μ l of water. A 1 μ l portion of the RNA solution was mixed with 4 μ l of the loading buffer (0.62 mg/mL streptavidin, 50 mM EDTA, 33 mM piperazine-*N,N*-bis-[2-ethanesulfonic acid] [pH 6.1], 6 M urea), heated for 30 s at 95°C, and then cooled to 25°C. This sample was analyzed by 8% denaturing PAGE, performed in a cold room in order to keep the gel temperature below 20°C. Under these conditions, the streptavidin-biotin complex is stable to retard the aminoacyl-tRNA band, but the RNA structure is mostly denatured. The radioisotope of each band was quantified using the Quantity One program equipped with Molecular Imager FX (Bio-Rad).

For a semipreparative scale, the reaction volume described above was scaled up 20 or 200 times depending upon the needs. For example, a total of 450 μ l tRNA aminoacylation reaction volume (approximately 150 μ l resin was involved) was generally needed for 100 μ l of translation. A disposable spin column (Bio-Rad) was used for this scale instead of the conventional microtube. After the aminoacylation reaction, the resulting aminoacyl-tRNA was eluted from the spin column and ethanol precipitated. The aminoacyl-tRNAs could be stored at -80°C for one week. Regeneration of Flexiresin could be carried out by washing the resin with 4 volumes of DEPC-treated water 4–5 times. Flexiresin could also be stored in solution W1 and could be reused multiple times (generally more than 10 times) without any deterioration of activity.

Plasmid Construction

The GFP_{UV}-coding region was obtained from pGFP_{UV} (Clontech, Palo Alto, CA) and His-Tag sequence was added by PCR using 5'-CATATGGCTA GCAAAGGAGA AGAACTTTTC ACTGG-3' and 5'-ATACTCAAGC TTAGTGTTGG TGGTGGTGGT GTTTGTAGAG CTCATCCATGC-3' as primers. The PCR product was cloned into NheI and HindIII site of pGEMEX-1 (Promega). Mutation of G72T, C192A, T193A, G240T, G312T, and G684T were introduced using standard mutagenesis protocols. The mutations at C192A and T193A were introduced to enhance fluorescence at 488 nm [37], and those at G72T, G240T, G312T, and G684T were done to remove minor codons (CGG and GGG).

Cell-free Translation

Batch translation (2.5 μ l) was done using rapid translation system (RTS-100, Roche) in the presence of ³⁵S-Met. The reaction was prepared according to the manufacturer's protocol with minor adjustments to the following conditions. Half the amount of the amino acid mix was used instead of the manufacturer's suggested amount, and 30 μ M (final concentration) suppressor tRNA charged with Phe or Phe analogs was added. Each aminoacyl-tRNA was synthesized with the protocol described in solid-phase aminoacylation. As a control, Phe-tRNA containing pC75 was prepared according to the literature procedure [38]. After 1 hr incubation at 30°C, the translation results were analyzed by 15% SDS-PAGE. Fluorescent activity of GFP was analyzed by Molecular Imager FX equipped with an external 488 nm laser (Bio-Rad). Prior to this analysis, 2 μ l of 0.1 M Tris-HCl (pH 8.8) was added to 1 μ l of the translation mixture and kept at 4°C overnight in order to form the active fluorescent species.

For mass spectroscopic analysis, the wild-type and mutant (Tyr151Iod) proteins were translated on a 110 μ l scale under manufacturer's conditions containing 2 mM Met. The protein was then purified using TALON (Clontech), eluting with an imidazole buffer (250 mM imidazole [pH 8], 150 mM NaCl, 0.5% polyethylene glycol 8000). Purity of the protein was confirmed by 15% SDS-PAGE analysis followed by staining using GelCode Blue Stain Reagent (Pierce). The proteins were digested with modified Trypsin (Promega) and analyzed by LC-MS (Thermo Finnigan). The protein concentration was also determined with a Micro BCA protein assay reagent using BSA as the standard (Pierce).

Posttranslational Modification

Mutant GFP (Tyr151AcPhe) and wild-type GFP labeled with ³⁵S-Met were translated in a 30 μ l scale reaction and purified using TALON as described above. Sodium acetate (1 μ l of 3 M; pH 5.0) was added to 10 μ l of purified protein and pH was adjusted to 5.0 by adding 1 μ l of 2 M HCl. Biotin-LC-hydrazide (3 μ l of 0.1 M; Pierce) in dimethyl sulfoxide was added to the above protein solution. After 24 hr incu-

bation, 0.75 μ l of 1 M NaCNBH₃ was added and the reaction mixture was incubated for another 24 hr to reduce the imine bond. To remove the excess amount of biotin-LC-hydrazide, the solution was first diluted with 200 μ l of 10 mM Tris-HCl (pH 8.8) and centrifuged to remove any insoluble reagents. The solution was then applied to BSA-precoated Microcon YM-10 (Millipore) and spun to remove any unreacted biotin. The solution that did not pass through the filter was diluted with 500 μ l of 10 mM Tris-HCl (pH 8.8). After six more dilutions and subsequent spinning, the solution was collected. The collected proteins were analyzed by streptavidin gel-shift assay using 10% native PAGE. For the streptavidin-agarose capturing experiment, 5 μ l of streptavidin-agarose (Pierce) was added to the protein and incubated for 10 min. The resulting mixture was then diluted with 200 μ l of 0.1 M Tris-HCl (pH 8.8), and the fluorescence of the protein was analyzed using Molecular Imager FX (Bio-Rad).

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