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Replacing amino acids in translation: Expanding chemical diversity with non-natural variants

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ABSTRACT

Here, we describe a strategy for synthesis of peptides with multiple unnatural amino acids (UAAs) using in vitro translation. Our method involves removing a natural amino acid and replacing it with an UAA variant in a reconstituted translation system. Whereas other systems require engineered components or chemical synthesis to charge UAAs onto tRNAs prior to translation, our strategy utilizes the wild-type machinery and charging occurs concomitant with translation. The design of the system allows for easy quantification of the UAA's incorporation efficiency and fidelity.

1. Introduction: incorporation of unnatural amino acids in translation

The ability to incorporate UAAs into proteins or peptides via the translational machinery offers many benefits [1]. For example, the ability to directly incorporate unnatural amino acids affords direct and simple access to post-translationally modified proteins [2–6]. Similarly, the ability to site-specifically label proteins with unique functional groups not found in the standard proteinogenic AAs has enabled new means to control protein function inside cells [7,8]. For peptides, the incorporation of UAAs can lead to enhanced stability and permeability, problems that have traditionally hindered the development of peptides as therapeutics [9]. For instance, peptides that contain even a single *N*-methyl amino acid can show enhanced bioavailability and protease stability [10,11]. A chief reason to pursue *translational* incorporation of UAAs into peptides is that it in principle allows the synthesis of extremely diverse (>10^{13}-member) drug-like peptide libraries using techniques like mRNA display [12,13]. This ability to create these libraries hinges on the development of methods to deliver UAAs into the translation apparatus.

The first step for the introduction of UAAs using in vitro translation is their ligation onto tRNAs. There are several strategies to achieve formation of non-natural aminoacyl-tRNAs. The original approach involved chemical attachment of the UAA onto a dinucleotide followed by enzymatic ligation onto a truncated tRNA [14,15]. These chemically charged tRNAs could then be used with in vitro translation reactions [16,17]. This strategy has now been extended to a whole family of orthogonal aminoacyl-tRNA synthetase/suppressor tRNA pairs, that can be used to incorporate UAAs site specifically in vivo [18,19]. An alternative strategy involves charging of a proteinogenic amino acid onto a tRNA, followed by converting it into an UAA while attached to the tRNA. For example, reductive amination can convert a proteinogenic aminoacyl-tRNA into its N-methylated form. This approach can be used to synthesize peptides containing N-methylated backbones [20,21]. Finally, Suga, using an RNA catalyst, has developed a means to charge virtually any UAA ester onto tRNAs. In their method, an artificial, flexible ribozyme noted as flexizyme recognizes the 3′ end of the tRNA in conjunction with benzylic esters of amino acids and charges the amino acid to the tRNA [22]. This general strategy allows for the incorporation of many UAAs into peptides [23–26].

Surprisingly, the wild-type aminoacyl-tRNA synthetases are able to charge a wide variety of unnatural amino acids onto tRNAs [27,28]. This ability suggests that such laborious engineering approaches may not be necessary because incorporation of an UAA would only require adding it to an in vitro translation reaction in place of its natural amino acid counterpart. While conceptually simple, standard cell extracts are highly contaminated with natural AAs, precluding this strategy. The reconstituted PURE (protein synthesis using recombinant elements) translation system [29] solves this problem because the natural amino acids can be withheld from translation. Thus the PURE system components can be tailored such that UAAs are substituted for natural amino acids. In this article, we show how to optimize PURE translation for the aminoacyl-tRNA synthetase-directed incorporation of UAAs (Scheme 1).

Abbreviations: AARS, aminoacyl-tRNA synthetase(s); PURE, protein synthesis using recombinant elements; UAA, unnatural amino acid(s).

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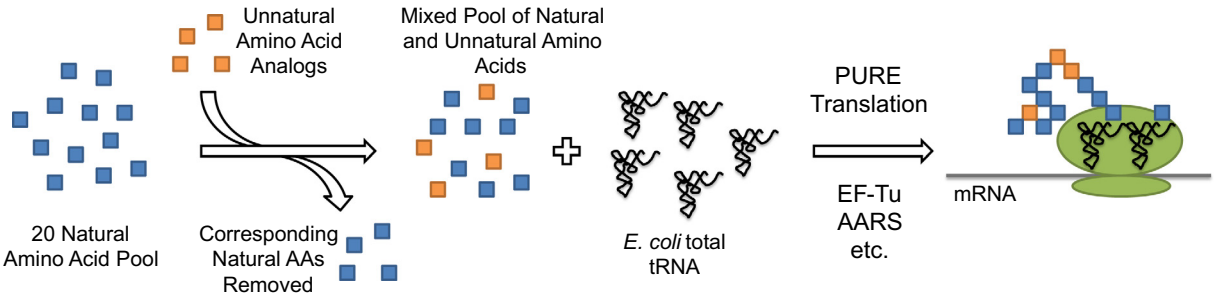

Scheme 1. Strategy for introducing UAAs into translation products. Using the PURE translation system, natural amino acids can be selectively removed from translation allowing analogous UAAs to be incorporated in their place.

2. Materials and methods

2.1. General reagents

Putrescine, spermidine, potassium chloride (KCl), ammonium chloride, magnesium acetate tetrahydrate ($Mg(OAc)_2$), calcium chloride ($CaCl_2$), potassium hydroxide (KOH), nucleoside 5′-diphosphate kinase from bovine liver, D,L-dithiothreitol (DTT), myokinase from rabbit muscle, adenosine 5′-triphosphate disodium salt, guanosine 5′-triphosphate sodium salt hydrate, ANTI-FLAG M2-Agarose from mouse, trifluoroacetic acid spectrophotometric grade and α-cyano-4-hydroxy-cinnamic acid were purchased from Sigma–Aldrich. Potassium acetate (KOAc), water Optima LC/MS grade and acetonitrile Optima LC/MS grade were purchased from Fisher. Potassium phosphate dibasic (K_2HPO_4) was purchased from Caledon. Creatine kinase and *Escherichia coli* total tRNA were purchased from Roche Applied Science. Creatine phosphate potassium salt was purchased from Merck/EMD. (6R,S)-5,10-formyl-*5,6,7,8*-tetrahydrofolic acid (methyl tertahydrofolate) was purchased from Schircks Laboratory. All natural L-amino acids were purchased from Fluka in their highest purity form. ^{35}S-Met (Specific Activity: >1000 Ci (37.0 TBq)/mmol) was purchased from Perkin-Elmer. 1-Aminocyclopentanoic acid was purchased from Chem-Impex International. Zip Tip C-18 columns were purchased from Millipore. Ni-NTA agarose was purchased from Qiagen.

2.2. Instrumentation

MALDI-MS experiments were performed on a Micromass MALDI-R MALDI-TOF Mass Spectrometer.

2.3. Preparation of mRNAs for translation

mRNAs were prepared using T7 in vitro transcription according to described protocols [27]. The DNA templates were created using two different methods. The first method involved ligating synthetic DNA duplexes with sticky ends into a pET12b vector, followed by PCR using the primers complementary to the vector encoded T7 promoter and terminator. Alternatively, a single synthetic oligonucleotide complementary to the T7 promoter, Epsilon enhancer, Shine-Dalgarno, and coding region was used as a template for runoff T7-mediated in vitro transcription.

2.4. Preparation of amino acid stocks for translation

Each UAA was dissolved to a final concentration of 10 mM, the pH was adjusted to 7.0–7.5 with 1 M KOH, filtered through 0.22 μm syringe filter, and stored at −20 °C.

2.5. In vitro translation

Each translation reaction was carried out as previously described [27]. Each reaction (50 μL) contained putresciene (8 mM), spermidine (1 mM), potassium phosphate (5 mM), potassium chloride (95 mM), ammonium chloride (5 mM), magnesium acetate (5 mM), calcium chloride (0.5 mM), dithiothreitol (1 mM), inorganic pyrophosphatase (1 μg/mL), creatine kinase (4 μg/mL), nucleotide diphosphate kinase (1.1 μg/mL), (6R,S)-5,10-formyl-*5,6,7,8*-tetrahydrofolic acid (30 μM), myokinase (3 μg/mL), creatine phosphate (20 mM), ATP (2 mM), GTP (2 mM), *E. coli* total tRNA (2.4 mg/mL), IF-1 (1 μg), IF-2 (2 μg), IF-3 (0.75 μg), EF-G (1 μg), EF-TS (1 μg), EF-Tu (2.24 μg), RF-1 (0.5 μg), RR-F (0.5 μg), RF-3 (0.5 μg), ribosomes (0.5 μM), ^{35}S-Methionine (0.4 μM), methionine (10 μM), 19 AA (200 μM), MetRS (0.1 μM), LeuRS (0.3 μM), GluRS (0.6 μM), ProRS (0.2 μM), GlnRS (1.0 μM), HisRS (1.0 μM), PheRS A294G (2.5 μM), TrpRS (1.5 μM), SerRS (0.2 μM), IleRS, (0.2 μM) ThrRS (0.4 μM), AsnRS (0.6 μM), AspRS (0.6 μM), TyrRS (0.5 μM), LysRS (0.5 μM), ArgRS (0.4 μM), ValRS (0.2 μM), AlaRS (0.2 μM), CysRS (0.5 μM), GlyRS (0.6 μM), MTF (0.2uM) and mRNA template (1.14 μM). The translations were initiated by addition of the appropriate mRNAs (described in the Section 3.2 of the text). For initial testing of UAAs, 19 natural amino acids were included (200 μM each) with only one UAA. After incubation of the translation for 1 h at 37 °C, the reactions were quenched with 150 μL PBS (if using FLAG tag) or 150 μL TBS with 5 mM BME (if using His-tag). Forty microliters of Ni-NTA resin or 10 μL ANTI-FLAG M2 agarose was added to a 500 μL centrifugal filter along with the quenched translation reaction, and the mixture was tumbled at room temperature. After 1 h, the resin was washed three times with 500 μL TBS and eluted with 1% trifluoroacetic acid (TFA) (50 μL). For reactions labeled with ^{35}S-Met, the yield was determined by scintillation counting of half (25 μL) of the elution. To examine the fidelity of the UAA incorporation, the non-radiolabeled reactions were purified and concentrated by Zip-Tip C_{18} chromatography. The Zip-tips were first wetted with acetonitrile, followed by 1:1 acetonitrile, then with 0.1% TFA. Then the peptide was loaded onto the tip by pipetting up and down 15 times in the peptide solution. The tip was washed three times with 0.1% TFA, and then eluted with 5 μl CHCA matrix (α-cyano-4-hydroxycinnamic acid in 1:1 MeCN:0.1% TFA). An aliquot (1 μL) of the resulting suspension was spotted on a MALDI plate and analyzed.

2.6. tRNA deacylation

E. coli total tRNA (100 mg/mL) dissolved in 1 M Tris–HCl (pH 8.8) and was incubated at 37 °C for 2 h, followed by dialysis overnight against 50 mM Tris–HCl (pH 8.8). The tRNA was precipitated by first adding 0.1 volume of a solution of KOAc (3.0 M, pH 5.5) and 3 volumes of ethanol. The pellet was washed twice with 70% ethanol, and allowed to air-dry at room temperature. The tRNA was resuspended

in ddH_2O and the concentration was adjusted to 100 mg/mL (1.6 A_{260}/μL). The tRNA was aliquoted and stored at −80 °C.

3. Theory

3.1. Non-natural amino acid selection

The basis of our method is removal of one or more natural amino acids from translation and substitution of UAA analogs that are charged by the natural aminoacyl-tRNA synthetases (AARS) onto the corresponding tRNA (Scheme 1). We have previously developed an assay to discover unnatural amino acids that are substrates for AARS. Over 92 UAAs can be successfully charged [28]. Our current work has generally chosen UAAs from this list for incorporation into our translation products; however the synthetase screening assay can be used to expand the list of UAAs that can be used. After selecting the group of UAAs to incorporate, the next step is to optimize each UAA individually in translation.

3.2. mRNA template design

PURE translations are conducted with mRNA templates specifically designed for this purpose. The non-coding portions of the mRNA include a T7 promoter, epsilon enhancer, Shine Dalgarno sequence, and a spacer sequence, which is followed by the coding region containing one of five amino acid sequences, and a C-terminal epitope tag. His and FLAG tags are used for this purpose as they contain few amino acids, permitting maximum substitution with UAAs. Our standard coding templates are shown in Table 1. The five templates are designed to test UAAs for each of the 20 natural AAs, four for each template. The templates have also been designed so that there is no overlap of amino acids in the coding region and the C-terminal epitope tag. When creating new templates, several things must be considered. First, the FLAG tag has some advantages over His tags. Ni-NTA resin will capture both the peptide product and all of the translation proteins, since the PURE system uses His-tagged proteins. We have noticed that in short templates containing a His tag, we often observe glutamine misincorporations, presumably due to depletion of the His-$tRNA^{His}$ and competition with the near cognate Gln-$tRNA^{Gln}$. These misincorporations lead to host of peaks which make interpretation of UAA incorporation by MS challenging. A disadvantage of the FLAG tag is that it is highly negatively charged, rendering peptides containing it much harder to detect when using positive mode MALDI. Finally, methionine analogs represent a special case, since methionine is required for initiation of all translation sequences. To measure the yield of methionine analog translations, another radioactive amino acid, such as histidine is used.

4. Results and discussion

4.1. PURE translation with test templates and single UAA optimization

The concentrations of the amino acids need to be optimized individually before any combinations are tried. The analogs are initially utilized at 400 μM in translations performed on a 50 μL scale. Two translations are attempted; one unlabeled and used for MALDI-TOF analysis, and one labeled with ^{35}S-Met for quantification of yield.

Table 1
mRNA test templates. List of peptide-encoding sequences used to analyze analog translation. Epitope tags are usually C-terminal, but can be utilized on the N-terminus to analyze for truncations. The full length mRNA sequences can be found in [27].

Epitope tag	Coding region	Epitope tag
	MHFSW	DYKDDDDK
	MVACG	DYKDDDDK
	MTINR	DYKDDDDK
	MLEPQ	DYKDDDDK
	MDYKM	HHHHHH
MHHHHHH	VACG	DYKDDDDK

4.2. Troubleshooting yield and fidelity

The data from this initial translation provides information about the yield of the reaction, which can be compared to similar reactions with a natural amino acid control. The MALDI-TOF MS analysis provides information about the fidelity of the translation. If the anticipated peptide mass with the UAA is not observed, there are two possibilities: misincorporation or truncation. Use of a misincorporation mass table (S1) can be useful to quickly determine the identity of the misincorporated amino acid. Misincoporations typically are observed in when either (1) a near-cognate AA-tRNA is able to compete effectively at the ribosomal A-site with the UAA-tRNA containing a cognate codon, or (2) when residual natural amino acid competes with the unnatural variant for the AARS. Truncation occurs when the UAA is not an efficient translation substrate, and there are no competing AAs or AA-tRNAs. Truncations can only be visualized by MS if an N-terminal tag is used (Table 1 includes one that we have used); however, a low translation yield with C-terminally tagged peptide is also typically indicative of truncation. If these problems are observed, there are several strategies to enhance UAA translation efficiency and fidelity.

4.2.1. Increase UAA/AARS concentrations

Increasing the concentration of the UAA is typically the first strategy we use to improve fidelity and efficiency. Limiting factors to this strategy include the solubility of the UAA, as well as the possibility that very high concentrations may lead to competition with other AARS (if the UAA is charged onto tRNA by two different AARS). If increasing the concentration does not dramatically improve yield, we also typically try to increase the concentration of the appropriate AARS to enhance the rate of formation of the UAA-tRNA.

4.2.2. Remove contamination

If the UAA is a relatively poor substrate for an AARS, even trace amounts of the contaminating natural AA can be a problem. There are several potential sources of contamination. A common source is the amino acids themselves. For example, we have found that Gln is contaminated with Glu and Asn is contaminated with Asp. It is often helpful in these situations to lower the concentration of the natural amino acid that contains the contaminant amino acid. Another contaminant source is the *E. coli* total tRNA which does contain aminoacylated-tRNA species. These residual AA-tRNAs can be removed by deacylation at pH 8.8 followed by dialysis.

4.2.3. 1-Aminocyclopentanoic acid (V_3) as a case study

One UAA that we have shown can be incorporated with reasonable efficiently into peptides via translation is 1-aminocyclopentanoic acid (V_3). To test its incorporation we used the template encoding MVHMH_6M. We first performed three ^{35}S-Met labeled translations, and measured the yield of His-tagged peptide products (Fig. 1). Notably, by leaving out valine and adding V_3, the yield remained approximately the same (Fig. 1A). However, when the two assays were analyzed by MALDI-MS, a different picture emerged. The first translation containing valine showed the expected mass (Fig. 1B), but the assay with V_3 showed two peaks – one minor peak of a peptide containing V_3, and one intense peak corresponding to the peptide containing valine (Fig. 1C). Therefore, the issue with V_3 incorporation is the presence of competing valine. Using deacylated tRNA significantly improved incorporation

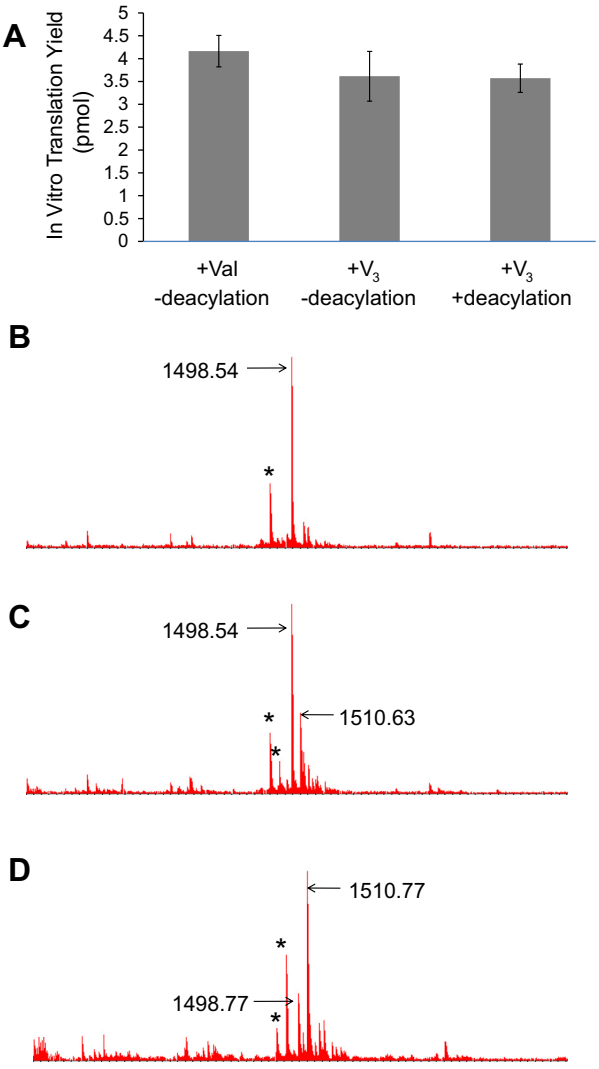

Fig. 1. Valine analogue incorporation. Efficiency and fidelity of 1-amino-cylcopentanoic acid (V_3) incorporation in place of valine in a template encoding $MVHMH_6M$. Each reaction was conducted on a 50 μL scale and contained only the necessary amino acids and AARS needed to synthesize the peptide. (A) Comparison of the effect on translation yield of V_3 incorporation and tRNA deacylation. Error bars indicate ±1 standard deviation. (B–D) MALDI-TOF mass spectra of translation reaction products. (B) Translation conducted with all natural amino acids. Expected $[M+H]^+$ of $MVHMH_6M$ product is 1498.62. (C) Translation conducted with V_3 in lieu of valine. Expected $[M+H]^+$ of MV_3HMH_6M is 1510.77. (D) Translation conducted with V_3 and deacylated tRNA. The asterisks (*) indicate peaks corresponding to peptides lacking the formyl group from the N-terminal methionine.

efficiency (Fig. 1D), suggesting that Val-$tRNA^{Val}$ present in the commercial tRNA mix was the culprit. Use of deacylated tRNA did not lower the yield. This study highlights the importance of careful monitoring of UAA translation fidelity by MS.

4.3. Combining UAAs into a single translation

Efficient incorporation of the UAA into a single template does not guarantee that it will be efficiently incorporated into peptides when combined with other analogs. Therefore, we take a systematic approach optimizing incorporation of combinations of UAAs. We typically first test each UAA in a translation reaction that contains each of the test template mRNAs (excluding MDYKM-His-6, which would complicate the purification and must be conducted in a separate reaction). Since the expected products from each of the templates are different in mass, MALDI analysis typically allows a quick verification of fidelity. If there is a lowering of yield or any misincorporations after this initial test, additional adjustments to UAA/AARS concentrations or contaminant removal may be required. Once each UAA is satisfactorily incorporated in the presence of every test template, we then begin combining it with other UAAs. Typically this is done by adding a single additional UAA at a time, which simplifies any potential troubleshooting of both yield and fidelity. If problems are observed, we adjust the concentrations of the UAA or AARSs and check for enhanced yield and fidelity. To aid in troubleshooting, we have found it helpful to perform translation experiments where certain natural amino acids are added to the translation reactions as competitors. If the yield or fidelity is dramatically enhanced, then the UAA that competes with that natural AA is likely the problem. Using this systematic approach we have prepared peptide libraries containing up to 12 different unnatural amino acids with good fidelity and yield.

5. Conclusion

This article lays out an approach to readily define a group of compatible UAAs for incorporation into peptides or proteins using PURE translation. Because it uses the natural ribosomal machinery, this method can be a very quick way of synthesizing peptides with non-natural functional groups as it does not require any protein engineering or chemical synthesis. Moreover, incorporation of multiple UAAs can be easily achieved with minimal troubleshooting. Use of this method enables synthesis of diverse peptide libraries with drug-like properties that may hasten the discovery of new bioavailable peptide therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2012.03.015.

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