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Ribosomal incorporation of backbone modified amino acids *via* an editing-deficient aminoacyl-tRNA synthetase†

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The ability to incorporate non-canonical amino acids (ncAA) using translation offers researchers the ability to extend the functionality of proteins and peptides for many applications including synthetic biology, biophysical and structural studies, and discovery of novel ligands. Here we describe the high promiscuity of an editing-deficient valine-tRNA synthetase (ValRS T222P). Using this enzyme, we demonstrate ribosomal translation of 11 ncAAs including those with novel side chains, α,α-disubstitutions, and cyclic β-amino acids.

Introduction

Life has evolved to utilize the chemistry of 20 proteinogenic amino acids (AAs) encoded by 61 codons. This genetic code, while highly conserved and safeguarded at multiple checkpoints, constrains biology to a fraction of the available chemical space. By manipulating individual translation components *in vitro* and *in vivo*, researchers have successfully created ribosomally translated peptides and proteins containing a few hundred ncAAs.[1–11]

Sense codon reassignment[1,2,4,12] is a particularly powerful strategy to introduce ncAAs when it is coupled with a reconstituted translation system, such as the Protein synthesis Using Recombinant Elements (PURE) translation system.[13] In this system, a wild type AA and aminoacyl-tRNA synthetase (AARS) pair can be withheld and substituted with a tRNA charged with an ncAA to allow for incorporation of the ncAA in response to vacated codons. To prepare this ncAA-tRNA, researchers have turned to a variety of methods. The most general strategy for the synthesis of ncAA-tRNAs is the Flexizyme system developed by Suga and coworkers.[14,15] This method utilizes activated amino acid esters and a ribozyme (Flexizyme) to catalyze the charging of an ncAA onto an acceptor tRNA. The Flexizyme system has allowed for the incorporation of a wide range of ncAAs,[15–17] yet this technique does have some drawbacks. Flexizyme recognizes the invariable 3′ end of a tRNA, and therefore cannot selectively charge a specific tRNA within a mixture. Since purified tRNAs are not readily available, *in vitro* transcribed tRNAs, which may not be as efficient as their fully modified counterparts are required.[18–20] The Flexizyme system also requires the synthesis of activated amino acids.

To address these limitations, one could potentially use a promiscuous AARS[5,21,22] in order to efficiently charge tRNAs with ncAAs. This strategy would have the advantage of using standard, non-activated amino acids and would be compatible with the readily available, wild-type *E. coli* tRNA mixture. Herein, we have focused our efforts on applying this strategy to *E. coli* ValRS. The wild-type ValRS has been shown to charge a moderate number of ncAAs so we conjectured that deleting the editing activity would broaden the substrate scope.[23–25] For example, Döring *et al.* showed that an editing deficient ValRS T222P in *E. coli* resulted in global misincorporations of threonine, cysteine, and 2-aminobutyric acid.[26] We reasoned that ValRS T222P could potentially charge additional structurally similar amino acids to further expand the genetic code.

Results/discussion

Based on this notion, we screened 29 ncAAs (Fig. S1†) for their ability to be charged by ValRS T222P using a MALDI charging assay.[27] This assay involves appending a triphenylphosphonium derivative onto the primary amino group of an AA-tRNA to enhance MALDI-TOF visualization (Fig. S2†). Eleven successful analogs (Fig. 1 and Table S1, and Fig. S3†) were charged by ValRS T222P. The first group of ncAAs included L-amino acids containing side chain modifications: *trans* alkene (crotyl glycine, **CrG**), azide (azidohomoalanine, **Aha**), nitrile (β-cyano alanine, **BCA**), as well as L-*allo*-threonine

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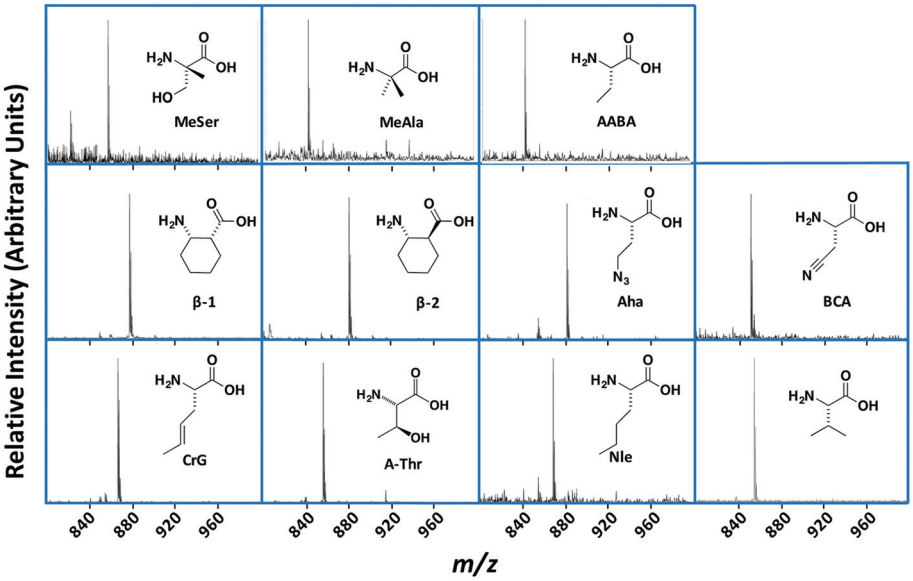

Fig. 1 AARS charging assay screen. MALDI-TOF spectra of derivatized ncAA-tRNA species after incubation with ValRS T222P. A magnified view of the region around each major peak is shown in Fig. S3.†

(**A-Thr**), norleucine (**Nle**), and aminobutyric acid (**Abu**). Side chain analogs like these are a rich source of functional diversity and are often employed in medicinal chemistry efforts to optimize peptide activity or for use as chemical biology tools.[28] The azide functional group, in particular, is a useful handle for post-translational modifications using bioorthogonal chemistry.[29]

ValRS T222P was also able to charge two α,α-disubstituted amino acids containing an α-methyl group: α-methyl alanine (2-aminoisobutyric acid, **Aib**), and α-methyl serine (**αSer**). While some of the above amino acids are near structural analogs to known ValRS T222P substrates,[26] we were surprised to find this enzyme is also able to charge constrained β-amino acids (1*R*,2*S*) 2-aminocyclohexane carboxylic acid (**β-1**), and (1*S*,2*S*) 2-aminocyclohexane carboxylic acid (**β-2**) (Fig. 1). (1*S*,2*S*) 2-aminocyclopentane carboxylic acid (**β-3**) was also charged, but less efficiently, as evidenced by the presence of a significant additional peak corresponding to charging of background valine (Fig. S4A†). We determined that the source of this contaminating valine was the ValRS T222P enzyme itself, and this background charging could be minimized by diluting the enzyme (Fig. S5†).

Because the ValRS T222P mutation only blocks the distal editing site, each of the aforementioned charged ncAAs must be recognized by the wild-type catalytic site in ValRS. By comparing those ncAAs that were charged to those that were not substrates (Fig. S1†), some trends can be gathered. ValRS rejected *N*-alkylated amino acids, amino acids that contain longer unbranched side chains, and those that contain polar groups other than on the β-carbon. Considering that **Aib**, **αSer**, **β-1**, **β-2**, and **β-3** were charged, it was surprising that the α-methyl analog of valine as well as the β^3 homolog of valine (Fig. S1†) were not.

We then sought to compare the catalytic efficiency of our ValRS T222P with flexizyme using the AARS charging assay developed by Wolfson and Uhlenbeck.[30] We followed the progress of each charging assay over 2 h. Valine and **β-1** reached maximal charging after 5 min and 20 min, respectively (Fig. S6†). Charging of the corresponding activated AAs using Flexizyme proceeded much more slowly, and only showed a small degree of charging after 2 h, even at 128-fold higher catalyst (Fig. S6†).

We then sought to determine if the ribosome, Ef-Tu, and translation factors could tolerate these amino acids in mRNA-directed peptide synthesis using the PURE system. Because ValRS T222P is promiscuous towards canonical amino acids, we cannot add it directly to a PURE translation mixture or scrambled peptides would result. To solve this problem, we supplemented our translation reactions with *E. coli* total tRNA previously charged with the amino acid of interest using ValRS T222P. This "precharged" total tRNA mixture was then added to a PURE translation reaction that lacked ValRS or ValRS T222P but included the other AAs and AARS enzymes (His/HisRS and Met/MetRS) required to read the codons in our mRNA template M<u>V</u>MH$_6$ (Fig. 2A). The fidelity of translation was verified by MALDI-TOF analysis (Fig. 2B, Fig. S7 and Table S1†), and relative translation yields were measured by ^{35}S-Methionine incorporation (Fig. 3 and Table S2†). Importantly, translations supplemented with total tRNA precharged under conditions that lacked the ncAA gave very low yields (Fig. 3, "-ncAA"), demonstrating that background readthrough of the valine codon was very low under these conditions.

We first tested the ncAAs without backbone modifications. Each of these is incorporated with moderate efficiency and high fidelity (Fig. 2, 3A, Fig. S7, and Table S2†). These ncAAs vary widely in side chain functional group diversity. Four have

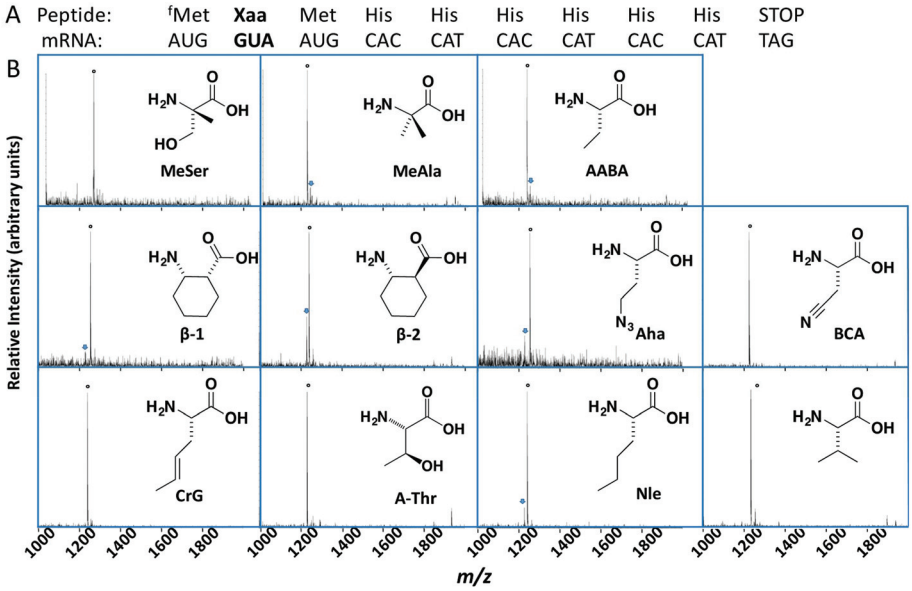

Fig. 2 MALDI-TOF spectra of *in vitro* translated peptides. (A) Sequence of the coding region of the mRNA coding region used in translation experiments. The Val codon GUA is reassigned to the ncAA shown. (B) MALDI-TOF spectra of M-ncAA-MH_6 peptides with the designated amino acids. Circles (°) indicate peptides of mass consistent with incorporation of the ncAA. Minor peaks consistent with background incorporation of valine are highlighted with an arrow. A magnified view of the region around each major peak is shown in Fig. S7.†

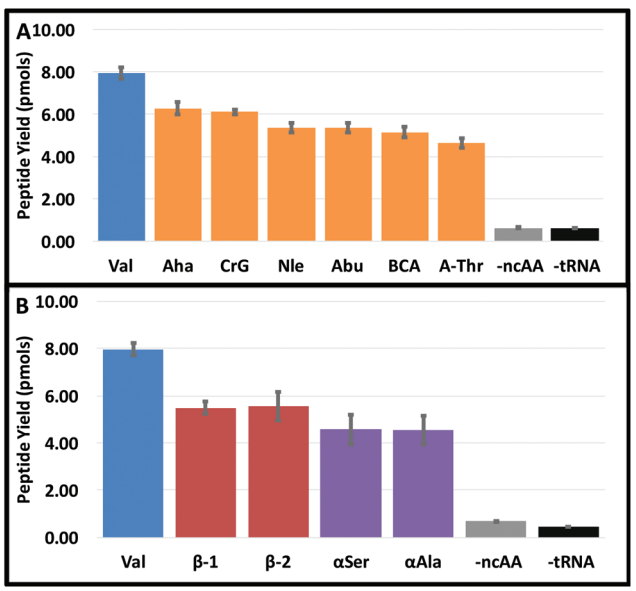

Fig. 3 Translation efficiency. Translation yields (pmoles per 50 μL reaction) as measured by ^{35}S-Methionine incorporation into Ni-NTA capturable peptides. Yields of side chain modified (A) and backbone modified (B) incorporation. Colors indicate ncAAs of the same class: orange, side chain modified; red, β-ncAA; purple, α-methyl-ncAA; gray, precharged tRNA without ncAA; black, absence of precharged tRNA. Error bars represent the standard deviation of at least 4 experiments.

previously been shown to be decent substrates for the translation apparatus, although on different tRNA bodies: **Aha**,[1] **CrG**,[31] **Nle**,[31] and **Abu**.[26] Two are novel ribosomal substrates: the nitrile containing **BCA**, and **A-Thr** which is an isomer of threonine rarely found in nature.

α-Methyl amino acids facilitate helical secondary structures in peptides.[32] This property makes them useful residues in peptide stapling techniques aiming to stabilize helical protein–protein interactions with synthetic peptides.[33] Our system incorporated **αSer** and **Aib** with high fidelity (Fig. 2 and Fig. S7†) and moderate to high efficiency (Fig. 3B). Of the two, only **Aib** has been previously shown to be ribosomally incorporated,[34] and its efficient incorporation required engineering of the translation apparatus.[35]

Initially described by Gellman and coworkers, **β-1** and **β-2**, like other constrained β-amino acids, have been shown to force peptides into constrained helical secondary structures.[36] In addition, β-amino acids confer proteolytic resistance to peptides.[37] Combining these two traits, Checco *et al.* recently inserted **β-3** into a stapled peptide known to bind to several antiapoptotic Bcl-2 family proteins.[38] The result was an α/β peptide hybrid that maintained nanomolar binding affinity towards its target with a serum stability 150 fold greater over the L-α parent peptide. The incorporation of these β amino acids into peptides *via in vitro* translation would allow for the synthesis of conformationally defined, protease stable peptide libraries. While the ribosome has recently been shown to be compatible with β-AAs, the applicable amino acids to this point have been the simple β^3 homologs of the canonical AAs that do not offer the same conformational benefits.[7,39] Our ability to charge **β-1**, **β-2**, and **β-3** onto $tRNA^{Val}$ using ValRS T222P now offered us the possibility to test these as substrates for the translation apparatus. We were delighted to find that all three were substrates (Fig. 2, 3B and Fig. S4B, S7†), although in two cases (**β-2** and **β-3**) small peaks due to background valine incorporation were also observed.

Encouraged by these results, we focused on monitoring incorporation of certain side chain analogs and backbone analogs **αSer**, **Aib**, **β-1**, and **β-2**, in response to other mRNA templates. The side chain analogs were incorporated efficiently into a longer template that required multiple incorporations (Fig. S8†). The backbone analogs proved more challenging. None of the backbone ncAAs could be incorporated into a template where we added a single Ala codon in between the initiator Met and Val codons, MAVMH$_6$. This template contains a C-terminal His-tag which prevents incompletely translated peptides from being captured and detected. To visualize if truncation was a problem for these backbone ncAAs, we used a template with an N-terminal tag, MH$_6$MVEP. The common product in these experiments was the peptide MH$_6$M (Fig. S9†), indicating the translation had terminated prior to the ncAA addition. These results with different templates show that the incorporation of the backbone ncAAs is challenging and mRNA-template dependent.

We then wondered if supplementing the translation system with additional tRNA^{Val} could solve this problem. We *in vitro* transcribed tRNA$_1^{Val}$ (Table S3†) which is which is known to recognize GUA, GUG, and GUU Val codons.[40] As a rigorous test, we created a longer template that required multiple incorporations of a particular ncAA as well as the ability to monitor for truncations, MHHHHHHMVAAAVEP. Using our standard precharging strategy and this template, we did not observe full length peptides with dual incorporation of **αSer** and **Aib** (not shown). However, when we supplemented the translation reaction with the *in vitro* transcribed tRNA$_1^{Val}$, we achieved efficient dual incorporation of **αSer** and **Aib** (Fig. 4 and Fig. S2†). These analogs could also be double incorporated into a more complicated peptide similar to those used in mRNA displayed libraries MVTNPDCFGNPVCGGGHHHHHH (Fig. S10†).[41] For **β-1** and **β-2**, however, the results were different. Supplementation of the translation with tRNA$_1^{Val}$ precharged with **β-1** or **β-2** did not lead to dual incorporations. In addition, we used a ribosomal mutant reported by Schepartz[8] that has been shown to improve the incorporation efficiency of β-amino acids. We could not observe incorporation with this ribosome mutant either. It is therefore likely that further engineering of the translation apparatus[8,35,42,43] will be required for efficient incorporation of these β-amino acids in all contexts.

Most research with editing deficient AARS enzymes has focused on their fidelity towards canonical amino acids. In this report, we exploit the inherent promiscuity of one of these enzymes to incorporate 11 different ncAAs into peptides, 8 of which have not been reported before as substrates for the translation apparatus. By precharging these ncAAs onto tRNA^{Val} in the context of total tRNA and omitting ValRS T222P from the translation reaction, we prevented loss of fidelity due to co-translational charging of competing proteinogenic AAs. This strategy, unlike Flexizyme, allows for the attachment of an unactivated ncAA onto specific tRNAs within a commercial mix of total tRNA. Moreover, the ability to incorporate novel side and backbone analogs using translation will open access to diverse, yet structurally defined peptide libraries using mRNA-display technologies.[41,44–48]

Methods

MALDI-AARS screening assay procedure

Each charging reaction contained 30 mM HEPES–KOH (pH 7.4), 15 mM $MgCl_2$, 25 mM KCl, 2 mM 2-mercaptoethanol, 6 mM ATP, 0.09 mg mL^{-1} BSA (previously dialyzed into ddH_2O), 0.02 units per μL PP$_i$ase, 350 μM deacylated tRNA, 5 μM ValRS T222P, 10 mM non-canonical amino acid, and ddH_2O to a final volume of 50 μL. Each reaction was initiated by addition of ValRS T222P to the mixture and incubated for 30 minutes at 37 °C. Each reaction was quenched by addition of 0.1 volume of NaOAc (3.0 M, pH 5.2) and tRNA was extracted with unbuffered phenol : chloroform : isoamyl alcohol solution (25 : 24 : 1). The aqueous layer was subjected to a second extraction with chloroform, followed by ethanol precipitation (3 vol.) with 0.1 volume NaOAc (3.0 M, pH 5.2). After centrifugation, the pellet was washed twice with 70% cold ethanol and resuspended in 200 mM NaOAc (pH 5.0) for use in the AARS screening assay. tRNA for use in the screening assay (6.75 μL) was added to water (3.75 μL), 30 mM (4-formylphenoxyproyl)triphenylphosphonium bromide in MeOH, 20 mM $NaCNBH_3$ in 50 mM NaOAc (pH 5.0) and tumbled at 37 °C for 2 h. The reaction was quenched with 0.1 volume 4.4 M NH_4OAc pH 5.0 and 3 volumes of ice cold 100% EtOH, pelleted and washed twice with 70% EtOH and twice with 100% EtOH. The resulting

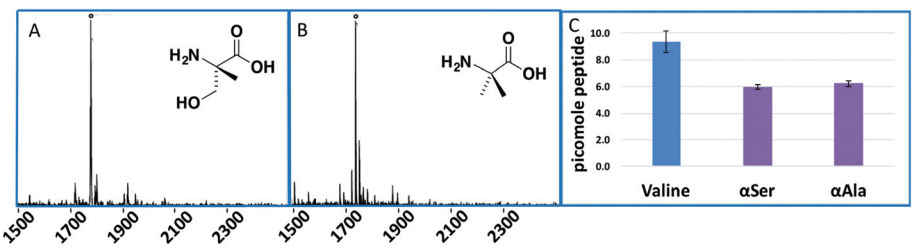

Fig. 4 Dual incorporation of ncAAs on MH$_6$MVA$_3$VEP. (A) MALDI-TOF MS demonstrating dual incorporation of α-methyl serine with 25 μM synthetic tRNA^{Val} Exp [M + H]$^+$ 1772.75 *m/z* Obs 1772.45 *m/z*. (B) MALDI-TOF demonstrating dual incorporation of α-methyl alanine (**Aib**) with 25 μM synthetic tRNA^{Val} Exp [M + H]$^+$ 1740.76 *m/z* Obs 1740.36 *m/z*. (C) Incorporation yields for dual incorporation of α-methyl serine and α-methyl alanine compared to valine.

pellet was resuspended in 2.25 μL 200 mM NH_4OAc and digested with 0.25 μL of 1 U μL^{-1} of Nuclease P1 (in 200 mM NH_4OAc, pH 5.0). After 20 minutes at room temperature, the reaction was quenched by placing on ice. One microliter was mixed with saturated CHCA (α-cyano-4-hydroxycinnamic acid) matrix in 1 : 1 MeCN : 1% TFA (9 μL). One microliter of the resulting suspension was spotted on a MALDI plate and analyzed.

tRNA pre-charging

The MALDI-AARS screening assay procedure was followed up until the first ethanol precipitation step. The precipitated tRNA was then dissolved in 20 μL 5 mM KOAc (pH 5.5) and stored at −80 °C until use.

In vitro translations

Each reaction (50 μL) contained HEPES–KOH pH 7.6 (50 mM), spermidine (2 mM), potassium acetate (10 mM), magnesium acetate (6 mM), DTT (1 mM), inorganic pyrophosphatase (1 μM), creatine kinase (40 μg mL^{-1}), nucleotide diphosphate kinase (1.08 μg mL^{-1}), (6*R*,*S*)-5,10-formyl-5,6,7,8-tetrahydrofolic acid (100 μM), myokinase (3 μg mL^{-1}), creatine phosphate (30 mM), ATP (1.5 mM), GTP (1.5 mM), *E. coli* total tRNA (2.4 mg mL^{-1}), IF-1 (2.7 μM), IF-2 (0.4 μM), IF-3 (1.5 μM), EF-G (0.52 μM), EF-Ts (8 μM), EF-Tu (10 μM), RF-1 (0.3 μM), RR-F (0.5 μM), RF-3 (0.17 μM), ribosomes (1.2 μM), MTF (0.6 μM), methionine (5 μM for radiolabeled samples, 100 μM for non-radiolabeled samples), 0.2 μM ^{35}S-Methionine (10 μCi, radiolabeled samples only), necessary amino acids and necessary AARS enzymes (0.1 μM–1.0 μM),[49] and mRNA template (1 μM). With the exception of dual incorporation of backbone modified amino acid assays, total tRNA precharged with a given amino acid (1.5 A_{260} units) was added at the start of translation and again after 30 min. For dual incorporation of backbone modified amino acids, 25 μM of *in vitro* transcribed precharged tRNA was added to the reaction. The translations were initiated by addition of the mRNA. After incubation for 1 h at 37 °C, the reactions were quenched with 150 μL TBS (50 mM Tris, 300 mM NaCl pH 8.0) with 5 mM BME. Ni-NTA resin (50 μL) was added to a 500 μL centrifugal filter (VWR) 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% TFA (50 μL). For reactions labeled with ^{35}S-Met, the yield was determined by scintillation counting of 47 μL of the elution. The non-radiolabeled reactions were purified and concentrated by Zip-Tip© C_{18} chromatography. The tips were first wetted with MeCN, followed by 1 : 1 MeCN : 0.1% TFA, 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.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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