# Chapter 7

## Introduction of an Aliphatic Ketone into Recombinant Proteins in a Bacterial Strain that Overexpresses an Editing-Impaired Leucyl-tRNA Synthetase

*Portions of this chapter are adapted from a submitted paper by Yi Tang, Pin Wang and David A. Tirrell*

**Abstract**

Bacterial hosts outfitted with engineered aminoacyl-tRNA synthetases facilitate the synthesis of recombinant proteins containing non-canonical amino acids. Oxonorvaline was incorporated into a recombinant protein in *Escherichia coli* via a rationally designed, editing-impaired leucyl-tRNA synthetase. Proteins containing the aliphatic ketone side chain of oxonorvaline can be site-specifically modified by hydroxylamine and by hydrazide reagents. The mechanism of editing of oxonorvaline by leucyl-tRNA synthetase was explored; the results indicate that a water molecule hydrogen-bonded to T252 at the editing site plays an important role in controlling editing specificity.

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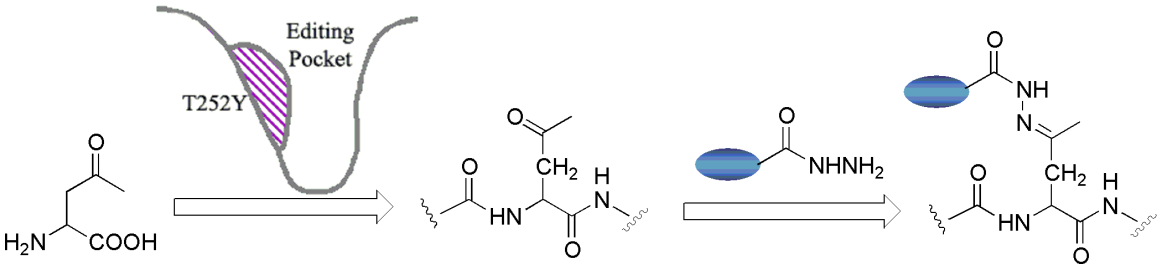


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## 1. Introduction

Relaxing the substrate specificity of the aminoacyl-tRNA synthetases (aaRSs) allows *in vivo* incorporation of non-canonical amino acids into recombinant proteins (*1-7*). We and others have demonstrated that impairing the proofreading activities of Class I aaRSs enables the *E. coli* translational machinery to insert amino acids that are normally edited following tRNA aminoacylation (*2, 8*). For example, disrupting the editing function of the *E. coli* valyl-tRNA synthetase allowed incorporation of α-aminobutyric acid into more than 20% of the valine positions in cellular proteins (*2*). An *E. coli* leucyl-tRNA synthetase (LeuRS) carrying the mutation T252Y, a residue critical in modulating the editing site geometry (*9, 10*) was unable to proofread amino acids with unbranched side chains, such as norvaline (**4**), norleucine, and allylglycine (*8*). Consequently, a series of non-canonical amino acids was successfully inserted into recombinant proteins in place of leucine (**1**) (*8*). In this chapter, we demonstrate further application of this mutant to effect residue-specific incorporation of oxonorvaline (**3**). Introduction of the aliphatic ketone group of **3** allows chemoselective modification of proteins under mild conditions. We also show that residue T252 controls editing site specificity not only through steric, but probably also via hydrogen-bonding interactions with the substrate side chain.

## 2. Materials and Methods

### 2.1. Materials

Amino acids **1** and **4** were purchased from Sigma. Amino acids **2** and **3** were prepared as described by Tang et al (*8*), i.e., by alkylation of diethyl acetamidomalonate

by the alkyl tosylate, followed by base hydrolysis, decarboxylation and enzymatic deacylation. [$^{32}$P]-labeled sodium pyrophosphate was purchased from NEN Life Sciences. Oligonucleotides were synthesized at the Caltech Biopolymer Synthesis Center. General cloning was performed in XL-1 blue cells. Expression strain SG13009 was purchased from Qiagen.

### 2.2. Synthetase Expression and Purification

SG13009 cells carrying the pREP4 repressor plasmid were transformed with p32leus (*8*) to yield the expression strain. Protein expression was induced at $OD_{600}$ = 0.5 with 1 mM IPTG. After three hours, the cells were harvested and lysed with sonification. The enzymes were purified using Ni-NTA agarose resins under native conditions according to the manufacturer's instructions (Qiagen). The proteins were purified to >95% as indicated by SDS-PAGE. Proteins were stored in Buffer A (50 mM Tris-HCl, 1 mM DTT)/50% glycerol. Aliquots were flash frozen in liquid nitrogen and stored at -80°C. The concentration of each enzyme was determined by measuring the absorbance at 280 nm under denaturing conditions.

### 2.3. ATP-$PP_i$ Exchange Assay

The assay was performed according to literature procedures (*8*). The assay buffer contained 50 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 1 mM DTT, 2 mM ATP and 2 mM [$^{32}$P]-$PP_i$ (0.5 TBq/mol). The concentration of enzyme was 75 nM. The amino acid concentration ranges varied depending on the activity of the enzyme toward the substrate. Aliquots (15 µL) were quenched in 500 µL quench solution (200 mM $PP_i$, 7% w/v

$HClO_4$ and 3% w/v activated charcoal). The charcoal was washed twice with 10 mM $PP_i$, 0.5% $HClO_4$ and counted. The results reported in Table 7-1 are averages from triplicate experiments.

### 2.4. Expression Plasmids

Plasmid pA1EL (a derivative of pQE9 (Qiagen)) was used as the template for site-directed mutagenesis (*8*). The synthetic leucine zipper protein A1 (*8*) was inserted at the *BamHI* restriction site of pQE9 to yield pQEA1. The *E. coli leuS* gene with its endogenous promoter was cloned from *E. coli* genomic DNA and inserted into the *NheI* site of pQEA1 to yield the template pA1EL. The Quickchange protocol (Stratagene) was used to introduce the mutations at position T252 in *leuS*. The pair primers were used in the reaction to generate the tyrosine, isoleucine and valine mutations. The integrity of the entire plasmid was verified through DNA sequencing. The plasmids carrying the T252Y, T252I, T252V mutants were designated as pA1T252Y, pA1T252I, pA1T252V respectively. Overexpression of LeuRS was verified by SDS-PAGE of whole cell lysates from overnight cultures.

### 2.5. Analog Incorporation Assay

The leucine auxotrophic strain LAM1000 (*11*) was transformed with pA1EL, pA1T252Y, pA1T252I, pA1T252V and with pREP4 to yield the A1 expression strains LAM1000/(pA1EL, or pA1T252Y, or pA1T252I, or pA1T252V). Growth and expression were performed in supplemented M9AA media (M9 medium, amino acids at 40 mg/L, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.4 wt% glucose, 5 μg/ml thiamin, 200 μg/ml

ampicillin and 25 μg/ml kanamycin). M9AA (200 mL) was inoculated with 1 mL of an overnight culture of the expression strain. The cells were grown to an $OD_{600}$ between 0.9 and 1.0, pelleted and washed with cold 0.9% NaCl three times. The cells were then resuspended in fresh M9AA medium with 16 natural amino acids (Leu, Met, Ile and Val were not added). Aliquots (10 mL) of the resuspended cells were added to different test tubes, each containing one of the amino acid analogs at a concentration of 320 mg/L. Leucine was added to a separate 10 mL solution at 40 mg/L as a positive control. After 10 min, 1 mM IPTG was added to induce protein expression. After three hours, the cells were collected by centrifugation (5000 g, 10 min, 4°C), resuspended in 600 μL of Buffer B (8.0 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris, pH 8.0) and frozen at -80°C. Whole cell lysates were analyzed by SDS-PAGE.

### 2.6. Protein Composition Analysis

The target protein A1 in 600 μL of whole-cell lysate was purified on a Ni-NTA spin column (Qiagen) according to the manufacturer's instructions. A1 protein was eluted in 400 μL Buffer B, pH 4.5. A portion of the eluent (10 μL) was diluted in 450 μL of 50 mM $(NH_4)_2CO_3$. The pH was adjusted to optimal trypsin working pH (8.0). Trypsin stock solution (5μL, 20 μg/200 μL) was added and the sample was incubated at room temperature overnight. The reaction was quenched by addition of 2 μL trifluoroacetic acid (TFA). The reaction mixture was subjected to C18 ZipTip (Millipore) purification and peptide fragments were eluted with 3 μL of 0.1 TFA, 50% $CH_3CN$. One μL of eluent was used for tryptic MALDI analysis. The remaining 350 μL

of spin column eluent was dialyzed against water extensively and lyophilized to a fluffy powder. The powder was sent directly for MALDI and amino acid analysis.

### 2.7. Chemical Modification of Ketone in Protein

Purified proteins containing ketone functionality were dissolved in 200 µl of PBS buffer (pH=6.0) and added to 20 µl of 5mM biotin hydrazide (BH, dissolved in PBS). Protein/BH mixtures were incubated at room temperature for 1 to 1.5 h. Reaction solutions were then washed twice with distilled water using a buffer-exchange column (Millipore, MWCO=5 kDa). Standard western blotting procedures were used to identify proteins modified with BH.

## 3. Results and Discussion

We first tested the translational activities of **3** and its hydrocarbon isostere didehydroleucine (**2)** in an *E. coli* host (LAM1000/pA1EL) that overexpresses wild type LeuRS. The target protein is a synthetic leucine zipper designated A1, the properties of which have been discussed previously (*12*). As shown in Figure 7-1A, **2** supported protein expression at a level comparable to that observed in cultures supplemented with **1**, whereas **3** did not. We determined the rates of activation of **2** and **3** by LeuRS by means of the ATP-$PP_i$ exchange assay (Table 7-1). The specificity constant $k_{cat}/K_m$ for the alkenyl substrate **2** is reduced approximately 100-fold compared to **1**, while that of **3** is reduced 4500-fold. The slower activation rate of **3**, however, is not sufficient to rationalize the lack of translational activity. We have previously described the efficient incorporation of hexafluoroleucine (Hfl) into recombinant proteins, despite the fact that

the specificity constant for Hfl is nearly equal to that for **3** (*12*). We therefore attribute the lack of incorporation of **3** to the editing activity of wild type LeuRS.

Consistent with this conjecture, we found that a host strain outfitted with the T252Y mutant afforded good yields of A1 in media supplemented with **3** (Figure 7-1A, lane 8). The electrophoretic mobility of the substituted protein was reduced, an effect observed previously in variants of A1 containing leucine surrogates. The purified protein from this sample was characterized by a molar mass indistinguishable (by MALDI mass spectometry, Figure 7-1B) from that of the wild-type protein, since the masses of **1** and **3** are nearly identical. To confirm the incorporation of **3**, we treated tryptic fragments of A1 samples containing **1**, **2** or **3**, with hydroxylamine for two hours at room temperature. As indicated in Figures 7-2A and 6-2B, fragments containing **1** and **2** were not modified. The same fragment (which contains three residues encoded as leucine) derived from the protein containing **3** yielded a ladder of signals separated by 15 mass units (Figure 7-2C), indicating conversion of the ketone side chain to the oxime. Amino acid analysis indicated approximately 60% replacement of **1** by **3**; the heterogeneity arising from partial substitution is the likely source of band-broadening in lane 8 of Figure 7-1A. Modification of the protein by hydrazide reagents was also demonstrated. Wild type and **3-**A1 were treated with biotin hydrazide, subjected to western blot analysis and visualized with a biotin-specific streptavidin-HRP conjugate. The reaction is specific for **3** (Figure 7-2D, lane 4).

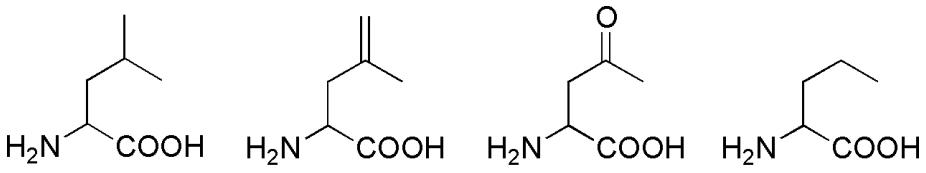
Our *in vivo* assays demonstrate that incorporation of oxonorvaline into recombinant proteins requires an *E. coli* strain outfitted with an editing-attenuated form of LeuRS. But proofreading of **3** by the wild type LeuRS is somewhat surprising.

Previous studies have suggested that amino acids (including **1**) branched at the γ̃-carbon are sterically excluded from the LeuRS editing cavity, at least in part by the gatekeeping residue T252. Why should **3** suffer editing when its isostere **2** does not? The explanation may lie in the fact that a water molecule is hydrogen-bonded to the hydroxyl group of T252 (*13*), and for hydrophobic substrates, defines the size of the editing cavity. But the ketone function of **3** can also form a hydrogen bond with T252, and may thereby displace the bound water. According to this view, hydrogen bonding to T252 secures **3** in the editing cavity and facilitates subsequent proofreading.

To test this hypothesis, we generated two additional mutants (T252I and T252V) of LeuRS. The four LeuRS variants examined here are characterized by residue volumes (at position 252) of 194 (Y), 167 (I), 140 (V) and 116 (T) $Å^3$, respectively (*14*). If one estimates the effective side-chain volume of residue 252 of the wild type synthetase by adding the molar volume of water (30 $Å^3$), the T252 and T252V variants would be expected to form editing cavities roughly equal in size. We examined the capacity of each of the four synthetase variants to edit **1**, **3**, and norvaline (**4**). As expected, none of the four edits the natural substrate (Figure 7-3, lanes 1-4), while norvaline is edited by both the wild type synthetase and the T252V variant (Figure 7-3, lanes 7 and 8). In contrast, **3** is edited only by the wild type synthetase, and not by the T252V mutant, despite the near equivalence in the volumes of the editing cavities of these two variants. We suggest that the most plausible explanation of these results involves anchoring of **3** in the editing cavity through formation of a hydrogen bond to the side-chain hydroxyl group of T252. An analogous mechanism has been proposed by Fersht to explain the editing specificity of valyl-tRNA synthetase toward aminobutyric acid and threonine (*15*).

## 4. Conclusion

In conclusion, we report the sucessful incorporation of oxonorvaline into recombinant proteins by rational redesign of the editing domain of the *E. coli* leucyl-tRNA synthetase. Coupled with previous experiments (*1, 4, 5, 8*), these results demonstrate that manipulation of the synthetic and editing sites of the aminoacyl-tRNA synthetases constitute complementary strategies for incorporation of non-canonical amino acids into proteins *in vivo*.




**1** **2** **3** **4**

**Table 7-1**. **Amino Acid Activation by Wild-type LeuRS**[a].

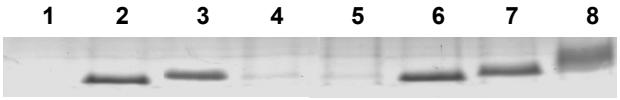
| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM)[b] | $k_{cat}/K_m$ (*rel*) |
|---|---|---|---|
| **1** | 2.2±0.02 | 18±1.7 | 1 |
| **2** | 1.08±0.12 | 1034±260 | 1/117 |
| **3** | 0.06±0.01 | 2245±804 | 1/4573 |

[a] 6xHis fusion protein. [b] Concentration of L-isomer.

**Figure 7-1**

(A) Incorporation of **2** and **3** into target protein A1. Lanes 1-4: expression in host LAM1000/pA1EL. Lanes 5-8: expression in host LAM1000/pA1EL_T252Y. Lanes 1 and 5: induction without **1**; lanes 2 and 6: Induction with **1** at 40 mg/L; lanes 3 and 7: induction with **2** at 100 mg/L; lanes 4 and 8: induction with **3** at 200 mg/L. All concentrations are of the L-isomer. (B) MALDI-MS of protein shown in lane 8. The single peak confirms the identity of the protein.

A




B

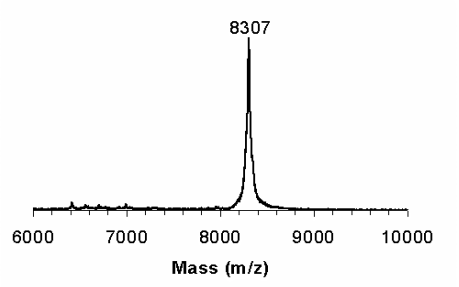
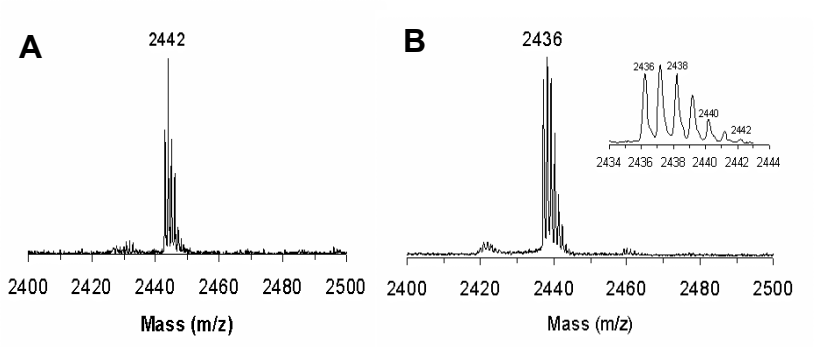
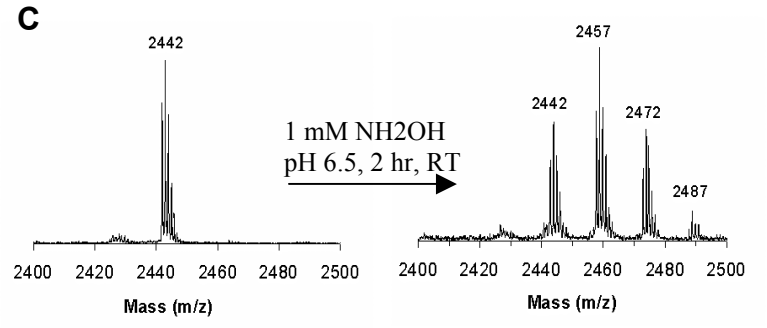


**Figure 7-2**

Chemoselective modification of the ketone moiety in target protein A1. (A)-(C) show the mass spectra of the tryptic fragment **L**KNEIED**L**KAEIGD**L**NNTSGIR (mwt: 2442); (A) Wild type; (B) **2-**A1; (C) **3-**A1. Before hydroxylamine treatment, the mass is nearly identical to that of wild type. After treatment with hydroxylamine for two hours at room temperature, mass peaks separated by 15 mass units were observed, corresponding to the conversion from ketone to oxime. Fragments shown in (A) and (B) were unmodified after treatment. (D) Biotin-specific western blot analysis of wild type and **3**-A1 treated with biotin-hydrazide. Lane 1: wild type A1 before treatment; 2: **3**-A1 before treatment; 3: wild type after treatment; and 4: **3**-A1 after treatment.

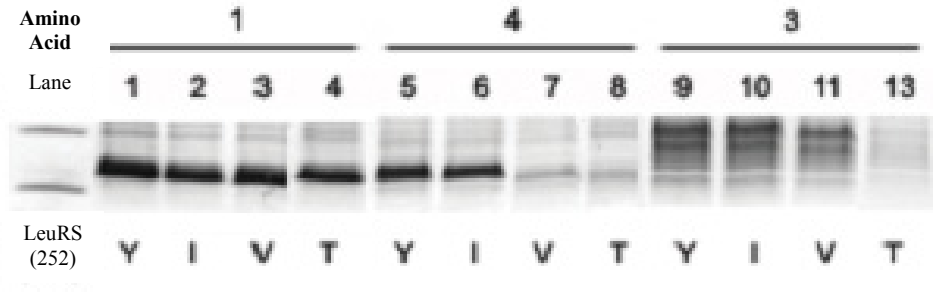

A
2442
2400 2420 2440 2460 2480 2500
Mass (m/z)
B
2436
2436 2438 2440 2442
2434 2436 2438 2440 2442 2444
2400 2420 2440 2460 2480 2500
Mass (m/z)


C
2442
2400 2420 2440 2460 2480 2500
Mass (m/z)
1 mM NH2OH
pH 6.5, 2 hr, RT
2457
2442
2472
2487
2400 2420 2440 2460 2480 2500
Mass (m/z)


D
1 2 3 4

**Figure 7-3**

Incorporation of unnatural amino acids by different LeuRS editing mutants. The mutations were all at the gatekeeping residue T252. For each mutant, the natural substrate (**1)** and two unnatural substrates (**3**) and (**4**) are shown.


Amino Acid
1
4
3
Lane
1 2 3 4 5 6 7 8 9 10 11 13
LeuRS (252)
Y I V T Y I V T Y I V T

## 5. References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