Published in final edited form as:
Chembiochem. 2009 September 4; 10(13): 2188–2190. doi:10.1002/cbic.200900407.

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

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Abstract

A leucine analog containing a ketone has been incorporated into proteins in *E. coli*. Only *E. coli* strains overexpressing an editing-deficient leucyl-tRNA synthetase were capable of synthesizing proteins with the aliphatic ketone amino acid. Modification of ketone-containing proteins under mild conditions has been demonstrated.

Keywords

non-canonical amino acids; ketones; protein modification; aminoacyl-tRNA synthetases

Relaxing the substrate specificity of the aminoacyl-tRNA synthetases (aaRSs) allows in vivo incorporation of non-canonical amino acids into recombinant proteins. 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.[1–7] Furthermore, recent work has shown that manipulating aaRSs by altering editing activity[8] or by transplanting editing domains[9] can improve the fidelity with which non-canonical amino acids are incorporated into proteins. We reported previously that an *E. coli* leucyl-tRNA synthetase (LeuRS) carrying the mutation T252Y, a residue critical in modulating the editing site geometry,[2, 3, 10] is unable to proofread amino acids with unbranched side chains, and enables insertion of a variety of non-canonical amino acids into recombinant proteins in place of leucine (**1**).[1] In this report, 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 first tested the translational activities of **3** and its hydrocarbon isostere didehydroleucine (**2**) in a leucine-auxotrophic *E. coli* host strain (LAM1000/pA1EL/pREP4) that overexpresses wild-type LeuRS. The target protein was a hexahistidine-tagged synthetic

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leucine zipper designated A1, the properties of which have been discussed previously.[11] As shown in Figure 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 1).[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 lower activation rate, however, is not sufficient to rationalize the lack of translational activity of **3**. We have previously described the efficient incorporation of hexafluoroleucine (Hfl) into recombinant proteins expressed in *E. coli* LAM1000/pA1EL/pREP4, despite the fact that the specificity constant for Hfl is nearly equal to that for **3** (k_{cat}/K_m (*rel*) of LeuRS for Hfl: 1/4100).[11] We therefore hypothesized that the lack of incorporation of **3** was attributable to the editing activity of wild-type LeuRS. Consistent with this conjecture, we found that a host strain (*E. coli* LAM1000/pA1T252Y/pREP4) outfitted with the T252Y LeuRS mutant afforded good yields of A1 in M9 medium depleted of leucine, isoleucine, valine, and methonine (M9 –LIVM medium)[1] and supplemented with **3** (Figure 1A, lane viii). The electrophoretic mobility of the substituted protein was reduced, an effect observed previously in variants of A1 containing leucine surrogates.[1] The purified protein from this sample was characterized by a molar mass indistinguishable (by MALDI mass spectometry, Figure 1B) from that of the leucine form of the protein (**1**-A1), since the masses of **1** and **3** are nearly identical.

To confirm incorporation of **3**, we treated tryptic fragments of A1 samples containing **1, 2** or **3**, with hydroxylamine (1 mM) for two hours at room temperature. As indicated in Figures 2A and 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** (**3**-A1) yielded a ladder of signals (Figure 2C) in which each 15-unit mass shift indicates replacement of **1** by **3** and subsequent 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 most likely source of band-broadening in lane viii of Figure 1A. Modification of the protein by hydrazide reagents was also demonstrated. Samples of **1**-A1 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**-A1 (Figure 2D, lane iv).

These results show that incorporation of oxonorvaline into recombinant proteins requires an expression 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 γ-carbon are sterically excluded from the LeuRS editing cavity, at least in part by the gatekeeping residue T252.[2, 3, 5, 10] Why should **3** be edited when its isostere **2** is not? The explanation may lie in the hydrogen-bond acceptor character of the ketone function. Previous work has demonstrated that wild-type *E. coli* LeuRS edits exclusively through a posttransfer mechanism.[12, 13] Therefore, after the enzymatic ligation of **3** and $tRNA^{Leu}$ in the aminoacylation active site, the 3' end of newly formed **3**-$tRNA^{Leu}$ changes conformations to allow **3** to enter the posttransfer editing active site of LeuRS. If **3** forms a hydrogen bond with one or more residues in the editing site, it may be subjected to hydrolytic cleavage from $tRNA^{Leu}$.

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] An ordered water molecule appears within 2.83 Å of residue T252 in a crystal structure of LeuRS determined in the absence of ligand.[15] 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 wild-type and T252V variants would be expected to form editing cavities roughly equal in size (assuming

that the water molecule is displaced in T252V). We examined the capacity of each of the four synthetase variants to incorporate **1, 3**, and norvaline (**4**) (a known substrate of the wild-type LeuRS editing site), into proteins. As expected, cells harboring each of the four variants were able to synthesize full-length protein in M9 –LIVM medium supplemented with the natural substrate leucine (Figure 3, lanes i–iv). Supplementing the expression medium with norvaline leads to expression of full-length protein only when bulkier amino acids (Y and I) are substituted at position 252 (Figure 3, lanes v–viii); **4** is edited both by the wild-type synthetase and by the T252V mutant. In contrast, **3** supports protein synthesis in M9 –LIVM medium when the threonine residue at position 252 is mutated to valine, isoleucine, or tyrosine (Figure 3, lanes ix–xii). Even introducing the subtle T252V mutation, in which the side-chain hydroxyl group of residue T252 is replaced by a methyl group, yields an expression system in which **3** is translationally active. We suggest that the most plausible explanation of these results involves anchoring of **3** in the editing cavity of the wild-type enzyme through formation of a hydrogen bond to the side-chain hydroxyl group of T252. Because **2** forms no such hydrogen bond, it is excluded from the editing site of the wild-type synthetase and is translationally active in wild-type cells. Removal of the hydrogen-bond donor site through introduction of the T252V mutation reduces the affinity of **3** for the editing site and allows **3** to escape proofreading by the synthetase. An analogous mechanism has been proposed by Fersht to describe the editing specificity of valyl-tRNA synthetase toward threonine.[16]

In conclusion, we report successful incorporation of oxonorvaline into recombinant proteins through redesign of the editing domain of the *E. coli* leucyl-tRNA synthetase. The selective chemistry of the oxonorvaline side chain promises to provide useful new approaches to protein engineering and proteomic analysis.[17–19]

Experimental Section

Detailed experimental protocols can be found in the supporting information.

Acknowledgments

We thank Dr. Mona Shahgholi for assistance with MALDI-TOF mass spectrometry. This work was supported by NIH Grant R01-GM62523 and by the NSF Center for the Science and Engineering of Materials at Caltech. Y. Tang was supported by a fellowship from the Whitaker Foundation, J. A. Van Deventer was supported by a National Defense Science and Engineering Graduate Fellowship, and A. J. Link was supported by a National Science Foundation Graduate Research Fellowship.

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Supporting information for this article (including detailed experimental protocols) is available on the WWW under http://www.chembiochem.org or from the author.

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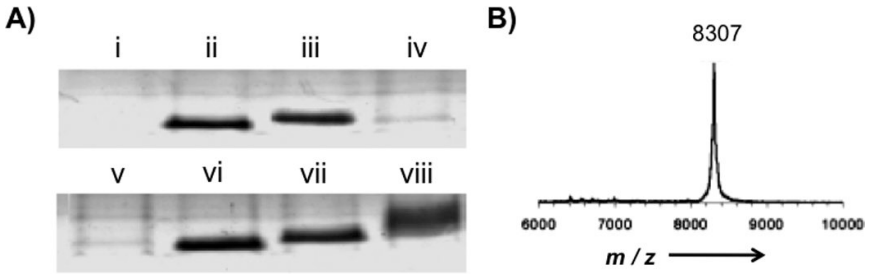


Figure 1.

Incorporation of **2** and **3** into target protein A1. A) Coomassie Brilliant Blue-stained SDS-PAGE gels of whole cell lysates after expressions in M9 –LIVM medium. Lanes i–iv: expression in host LAM1000/pA1EL/pREP4. Lanes v–viii: expression in host LAM1000/pA1T252Y/pREP4. Lanes i and v: induction without **1**; lanes ii and vi: Induction with **1** at 40 mg L^{-1}; lanes iii and vii: induction with **2** at 100 mg L^{-1}; lanes iv and viii: induction with **3** at 200 mg L^{-1}. All concentrations are of the L-isomer. B) MALDI-MS of protein shown in lane viii. The single peak (found: 8307 Da; calculated: 8307 Da) confirms the identity of the protein.

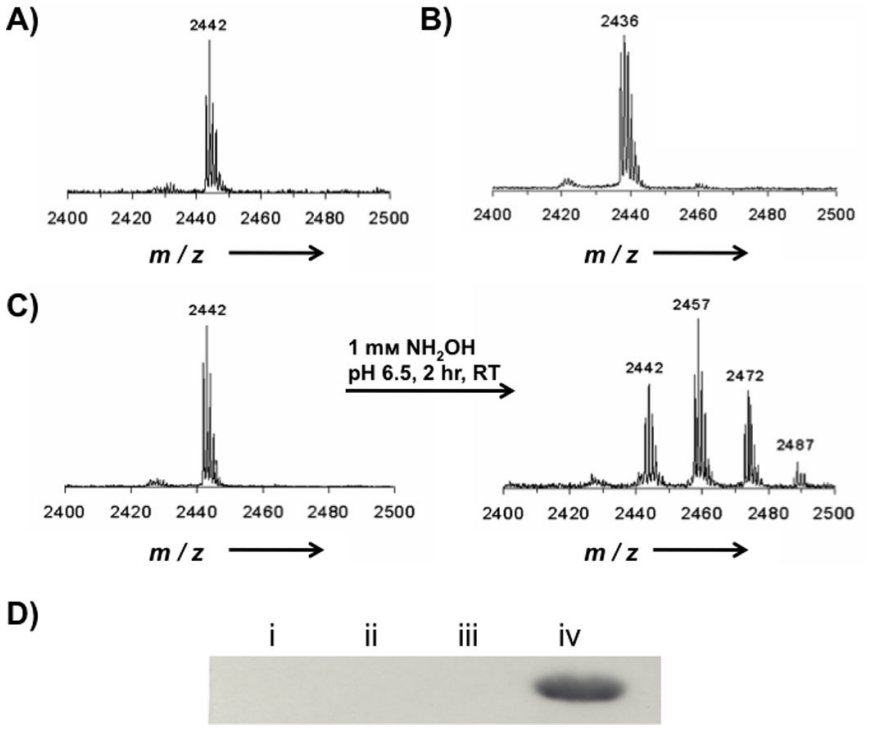


Figure 2.

Chemoselective modification of the ketone moiety in target protein A1. A)–C) Mass spectra of the tryptic fragment **L**KNEIED**L**KAEIGD**L**NNTSGIR derived from A1; A) **1**-A1 (found: 2442 Da; calculated: 2442 Da); B) **2**-A1 (found: 2436 Da; calculated: 2436 Da); C) **3**-A1 (found: 2442 Da; calculated: 2442 Da). Before hydroxylamine treatment, the mass of **3**-A1 is nearly identical to that of **1**-A1. After treatment with hydroxylamine (1 mM) in PBS (pH 6.5) 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 analyses of **1**-A1 and **3**-A1 treated with biotin-hydrazide. Lane i: **1**-A1 before treatment; ii: **3**-A1 before treatment; iii: **1**-A1 after treatment; iv: **3**-A1 after treatment.

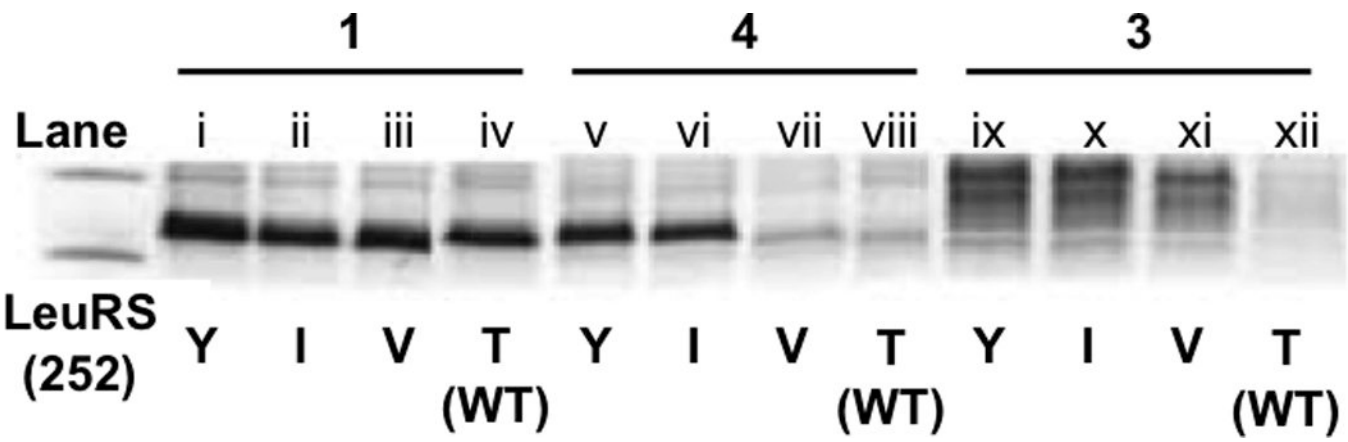
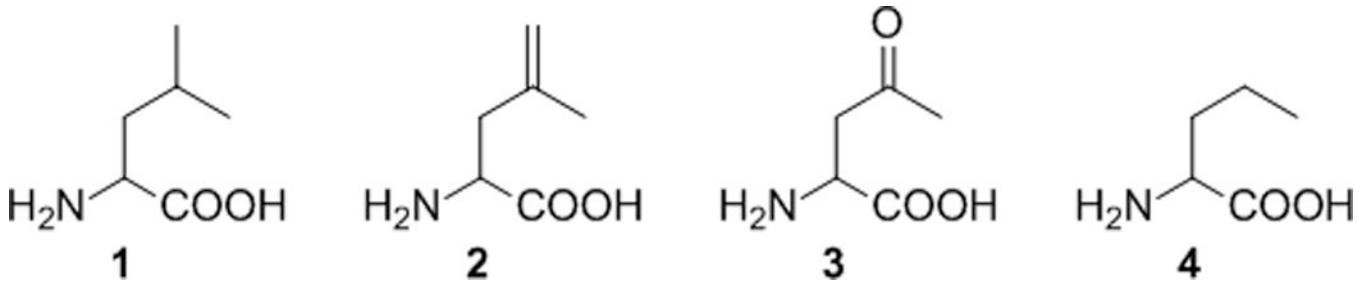


Figure 3.

SDS-PAGE analysis of protein expression in cells overexpressing editing mutants of LeuRS. Expression in M9 –LIVM medium supplemented with **1, 3**, or **4**, was performed with *E. coli* cells overexpressing LeuRS containing Y, I, V, or T at position 252. After expression, whole cell lysates were subjected to SDS-PAGE, and proteins were visualized with Coomassie Brilliant Blue stain.



Scheme 1.
Amino acids used in this study. **1**: leucine; **2**: didehydroleucine; **3**: oxonorvaline; **4**: norvaline.

Table 1

Amino Acid Activation by Wild-type LeuRS.[a]

Substrate	k_{cat} (s^{-1})	K_m (μM)[b]	k_{cat}/K_m (*rel*)
1[c]	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.[1]

[b] Concentration of L-isomer.

[c] The kinetic parameters of the T252Y mutant were determined to be essentially the same as those for the wild-type enzyme for leucine (k_{cat} = 1.92 ± 0.03 s^{-1}, K_m = 20 ± 2.3 μM).[1]