Attenuation of the Editing Activity of the *Escherichia coli* Leucyl-tRNA Synthetase Allows Incorporation of Novel Amino Acids into Proteins in Vivo†

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Received May 15, 2002; Revised Manuscript Received June 24, 2002

ABSTRACT: The fidelity of translation is dependent on the specificity of the aminoacyl-tRNA synthetases (aaRSs). The aaRSs that activate the hydrophobic amino acids leucine, isoleucine, and valine employ a proofreading mechanism that hydrolyzes noncognate aminoacyl adenylates and misaminoacylated tRNAs. Discrimination between structurally similar amino acids by these AARSs is believed to operate by a double-sieve principle, wherein a separate editing domain governs hydrolysis on the basis of the size and hydrophilicity of the amino acid side chain. Leucyl-tRNA synthetase (LeuRS) relies on its editing function to correct misaminoacylation of $tRNA^{Leu}$ by isoleucine and methionine. Thr252 of *Escherichia coli* LeuRS has been shown previously to be important in defining the size of the editing cavity. Here we report the isolation and characterization of three LeuRS mutants with point mutations at this position (T252Y, T252L, and T252F). The proofreading activity of the synthetase is significantly impaired when an amino acid bulkier than threonine is introduced. The rate of misaminoacylation of $tRNA^{Leu}$ by isoleucine and valine increases with the increasing size of the amino acid substituent at position 252, and the noncognate amino acids norvaline and norleucine are inserted efficiently at the leucine sites of recombinant proteins under conditions of constitutive overexpression of the T252Y mutant in *E. coli*. In addition, the unsaturated amino acids allylglycine, homoallylglycine, homopropargylglycine, and 2-butynylalanine all support protein synthesis in *E. coli* hosts harboring the mutant synthetase. These results demonstrate that programmed manipulation of the editing cavity can allow in vivo incorporation of novel protein building blocks.

The fidelity of protein biosynthesis is controlled in large part by the aminoacyl-tRNA synthetases (aaRSs)[1] (*1*−*3*). The aaRSs catalyze the joining of tRNAs and amino acids in two steps as outlined in Scheme 1: the cognate amino acid is first converted to the aminoacyl adenylate and subsequently transferred to the 3′-end of the corresponding tRNA. The substrate specificity of the aaRSs is remarkable, as each enzyme must distinguish small differences among amino acid side chains to maintain accurate translation and cell viability.

The synthetases associated with the aliphatic hydrophobic amino acids, including leucine (**1**), isoleucine (**2**), and valine (**3**), must distinguish among nearly isosteric substrates. All three synthetases are multidomain, monomeric enzymes, and all belong to the class I synthetase family (*2*). Isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS) carry out amino acid discrimination by a "double-sieve" mechanism (*4*−*7*). IleRS rejects isomeric (e.g., leucine) or larger amino acids at its active site and rejects smaller amino acids such as valine at its editing site (*5*). As a result, IleRS mischarges valine at a frequency of 1 in 3000, even though

Scheme 1

$$\text{ATP + aa} \xrightleftharpoons{\text{aaRS}} \text{aa-AMP + PP}_i \quad (1)$$

$$\text{aa-AMP + tRNA} \xrightarrow{\text{aaRS}} \text{aa-tRNA + AMP} \quad (2)$$

valine is activated just 180 times more slowly than isoleucine (*8*). In an analogous fashion, ValRS rejects larger amino acids such as leucine and isoleucine at its synthetic site and proofreads threonine at its editing site on the basis of the hydrophilicity of the threonine hydroxyl group (*7*). The crystal structures of IleRS (*9*, *10*), ValRS (*11*), and LeuRS (*12*) have been determined, and each shows the editing site embedded in a readily identified editing domain. In all three structures, the synthetic active sites adopt the characteristic Rossmann fold found in all class I synthetases. The editing domains are inserted into the Rossmann folds via flexible linkers and form so-called connective polypeptide 1 (CP1) domains (*13*). Both pretransfer editing of misadenylated amino acids and posttransfer editing of misaminoacylated tRNAs by the editing domains of IleRS and ValRS have been observed (*7*, *14*). The CP1 domain in ValRS is outfitted with two subsites for recognition of threonine (*11*): one specifically for Thr-$tRNA^{Val}$ in posttransfer editing and a putative pocket for binding Thr-AMP in pretransfer editing. Bishop et al. recently suggested a postpre−prepre editing mechanism (*15*), in which posttransfter editing primes the synthetase toward proofreading and is the dominant pathway under in vivo conditions. Mutagenesis of residues involved

† This work was supported by the NSF Center for the Science and Engineering of Materials at Caltech and by NIH Grant R01-GM62523. Y.T. is supported by a graduate fellowship from the Whitaker Foundation.

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; CP1, connective peptide domain 1; αbu, α-aminobutyric acid; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

10.1021/bi026130x CCC: $22.00
Published on Web 07/27/2002

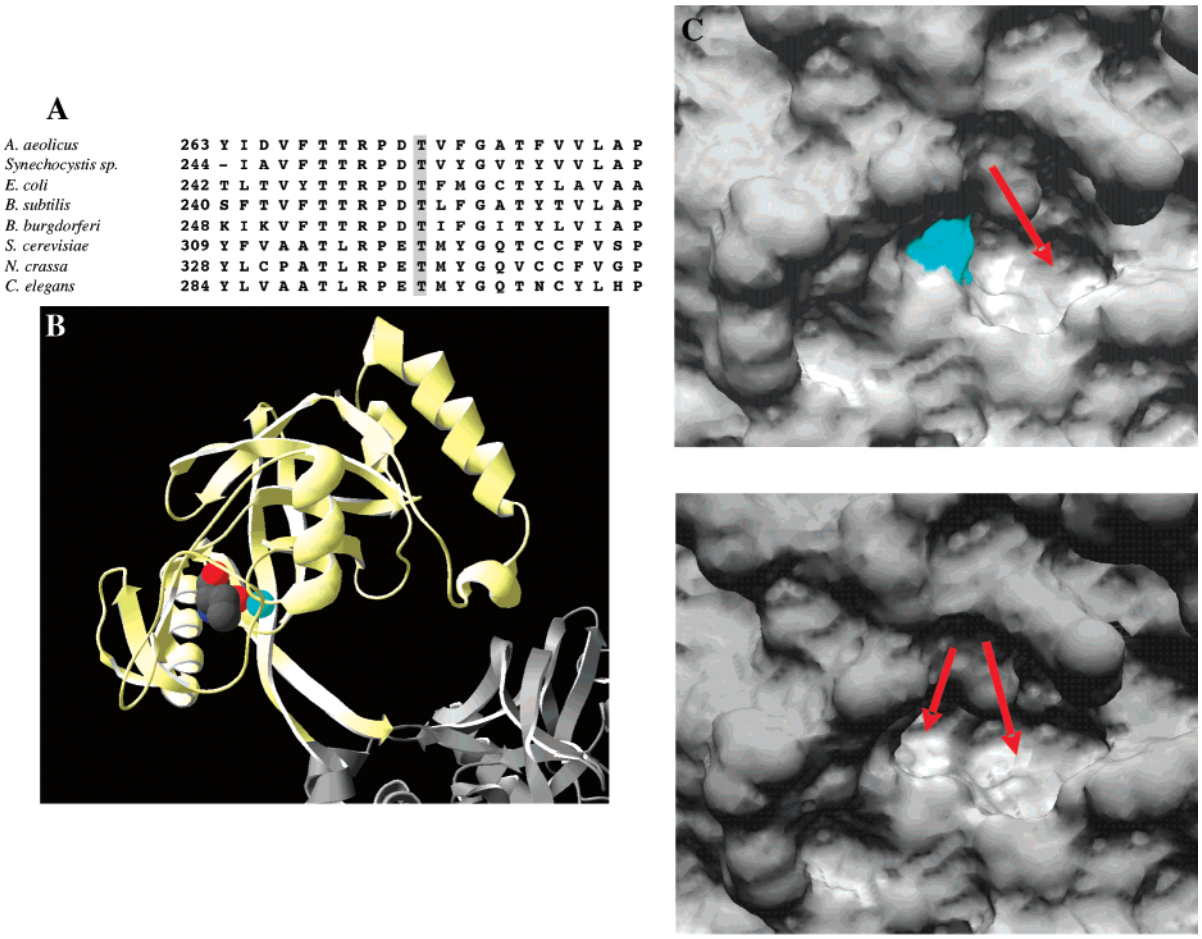


FIGURE 1: (A) Alignment of sequences flanking the T252 position in LeuRS variants derived from eight different organisms. The shaded threonine residue is conserved across species. Sequences immediately adjacent to T252 are also highly conserved. Sequence source, http://www.expasy.ch. (B) Crystal structure of the *T. thermophilus* LeuRS CP1 domain. The CP1 domain is shown in yellow. The rest of the enzyme is visible at the bottom right in gray. The conserved T252 residue is shown in the space-filling representation. The oxygen of the T252 side chain is shown in red. The water molecule that hydrogen bonds to this oxygen is shown in cyan. In this view, the T252 side chain is oriented toward the synthetic active site (gray portion). (C) Closeup view of the *T. thermophilus* LeuRS putative editing pocket. Molecular surfaces are generated using Swiss-Pdbviewer. The top panel shows the wild-type LeuRS cavity. The water molecule that hydrogen bonds to T252 is shown in cyan. T252 is not visible in this representation. The groove adjacent to the water molecule (red arrow) provides a possible binding site for straight chain analogues such as methionine. The bottom panel shows the T252A LeuRS cavity. This representation is generated by deleting the coordinates of the T252 γ-carbon and γ-oxygen atoms and the water molecule. This mutation creates a second binding groove (additional red arrow) where the isobutyl side chain of leucine can be accommodated. Mursinna et al. (*22*) showed that this mutant hydrolyzes leucine from Leu-tRNA^{Leu}.

in the proofreading processes of both IleRS and ValRS results in the accumulation of misaminoacylated cognate tRNAs (*9*, *15*, *16*). Valine was appended to tRNA^{Ile} in vitro when D342 of *Escherichia coli* IleRS, a residue critical for the translocation of Val-tRNA^{Ile} to the editing site, was mutated (*15*). The nonnatural amino acid 2-aminobutyric acid (αbu) was inserted into more than 20% of valine positions in cellular proteins in vivo, by ValRS mutants that were isolated by screening mutagenized *E. coli* strains (*17*). The mutations were all located within the CP1 region of the synthetase.

Leucyl-tRNA synthetase (LeuRS) is an 860-amino acid monomeric protein (*18*). Unlike the well-studied proofreading mechanisms of IleRS and ValRS, the "double-sieve" behavior of LeuRS is poorly understood. The CP1 domain in LeuRS is approximately 200 amino acids in length (*12*), and the residues involved in proofreading have not been identified. The relative contributions of pre- and posttransfer proofreading in LeuRS are also not known (*19*). Chen et al. found that disruption of the editing domain, either by site-directed mutagenesis of alanine 293 (*20*) or by PCR domain insertion (*21*), results in formation of Ile-tRNA^{Leu} and Met-tRNA^{Leu} in vitro.

Residues in a threonine-rich region in *E. coli* IleRS CP1 are crucial to IleRS proofreading activity (*9*). Using alanine scanning mutagenesis of a homologous region in the CP1 domain of *E. coli* LeuRS, Mursinna et al. (*22*) provided the first insight into the structural basis of LeuRS editing. They identified T252 as an essential residue in the LeuRS posttransfer editing pathway. Alignment of LeuRS CP1 domain sequences from different organisms shows that this threonine is rigorously conserved among species (Figure 1A). The hydroxyl group of the side chain of T252 forms a hydrogen bond with a water molecule, as observed in the crystal structure (*12*). The editing domain is shown in Figure 1B with the T252−water complex shown in the space-filling representation. The oxygen atom of the threonine side chain is shown in red, and the oxygen atom of the hydrogen-bonded water molecule is shown in cyan. The side chain of T252 and the putative posttransfer editing cavity are oriented toward the synthetic active site (partially shown in gray).

Figure 1C shows the molecular surface representation of the cavity. It can be seen that the cavity consists of a groove in which a linear chain, such as the thioether side chain of methionine (**4**), can be accommodated. The water molecule (shown in blue) occupies one side of this groove. The importance of T252 (not visible in this representation) in preventing leucine from binding in the editing pocket can be visualized by manually removing the coordinates of the γ-carbon and oxygen atoms of T252, along with the water molecule in the structure file. The remodeled surface (Figure 1C, bottom) reveals an additional groove juxtaposed on the existing cavity, which could accommodate the isobutyl side chain of leucine. Indeed, purified LeuRS-T252A rapidly hydrolyzes Leu-tRNA^{Leu} in vitro (*22*). Wild-type LeuRS proofreads against isoleucine and methionine, both of which lack branching at the γ-carbon atom. T252 thus facilitates substrate recognition at the editing site on the basis of the sizes of substituents at the γ-carbons of the amino acid side chains. Amino acids with a tertiary γ-carbon, such as leucine, are too bulky to fit in the pocket, while those with secondary γ-carbons can enter the pocket and are hydrolyzed from misaminoacylated tRNA^{Leu}.

Our laboratory is interested in the use of nonnatural amino acids for applications in protein engineering and biomaterials synthesis (*23*−*32*). Of special interest are amino acids with novel physical (*24*, *25*) and chemical properties (*27*, *31*, *32*). For example, fluorinated analogues of leucine, such as trifluoroleucine and hexafluoroleucine, have been shown to stabilize coiled-coil domains with respect to thermal or chemical denaturation (*24*, *25*, *33*). In addition, we have tested the in vivo translational activities of several other amino acids that are structurally similar to leucine, including norvaline (**5**) and norleucine (**6**). In vivo protein expression studies showed that neither of these analogues supported protein synthesis in cultures depleted of leucine. On the basis of our interpretation of the LeuRS editing mechanism, we attribute the lack of translational activity of **5** and **6** to proofreading by LeuRS**,** similar to that observed for methionine. An analogous mechanism controls the editing specificity of ValRS, which rapidly hydrolyzes αbu-tRNA^{Val}, while leaving Val-tRNA^{Val} intact (*5*).

In this report, we present a successful attempt to disrupt the editing function of LeuRS through directed mutagenesis of the key residue T252. The premise of this work was that the editing activity of LeuRS should be attenuated by replacement of T252 with a bulkier amino acid residue. We studied in vitro aminoacylation kinetics of three T252 LeuRS mutants and discovered that these enzymes indeed exhibit impaired editing activity toward natural amino acids isoleucine and valine. One such mutant, T252Y, was chosen for subsequent in vivo studies. When this mutant was overexpressed in *E. coli*, the previously translationally silent amino acids **5** and **6** were incorporated into recombinant proteins with high efficiency. In addition, we tested the translational activities of the unsaturated analogues allylglycine (**7**), propargylglycine (**8**), α-cyanoalanine (**9**), homoallylglycine (**10**), homopropargylglycine (**11**), and 2-butynylglycine (**12**). Analogues **10**−**12** have been tested previously as methionine surrogates (*30*, *32*). We find that the T252Y LeuRS mutant enables incorporation of **7** and **10**−**12** into *E. coli* proteins (*34*).

MATERIALS AND METHODS

Materials. Amino acids **1**−**8** were purchased from Sigma; **9** was purchased from ICN (Irvine, CA). Amino acids **10**−**12** were prepared as described by van Hest et al. (*32*), i.e., by alkylation of diethyl acetamidomalonate by the alkyl tosylate, followed by base hydrolysis, decarboxylation, and enzymatic deacylation. ^{3}H-labeled amino acids were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). ^{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.

Cloning and Mutagenesis. *LeuS* was cloned directly from *E. coli* genomic DNA using four-primer PCR. The flanking primers were 53Sph (5′-GGACCACTGGCTGGCATGC-AAGAGCAATAC-3′) and 35Hind (5′-CGCTTCCTCCC-AAGCTTAGCCAACGACC-3′). The introduced restriction sites are underlined. The remaining pair of complementary oligos was designed to carry the desired mutations at position 252, 53*NNN* (5′-CTACCCGCCCGGAC*NNN*TTTATGGGT-TGTAC-3′), where *NNN* encodes the desired amino acid (ACC in WT, TAC in T252Y, CTC in T252L, and TTC in T252F). 35*NNN* is the oligonucleotide that is complementary to 53*NNN*. In the first reaction, primers 53Sph and 35*NNN* were used to yield a 780 bp product. In the second reaction, primers 53*NNN* and 35Hind were used to yield a 1900 bp product. The products were then mixed and subjected to further amplification with 53Sph and 35Hind. *Pwo* polymerase was used throughout to minimize random mutations. The resulting 2600 bp DNA fragment was gel-purified and digested overnight with *Sph*I and *Hin*dIII. The digested fragment was ligated into the expression plasmid pQE32 to yield p32leus, p32T252Y, p32T252L, and p32T252F, which encode the wild-type, T252Y, T252L, and T252F enzymes, respectively. The cloned enzymes contained the N-terminal leader sequence MRGSHHHHHGIR. The fidelity of PCR cloning and the presence of the desired mutations were checked by DNA sequencing. An *Msl*I restriction site is removed upon successful mutagenesis and was used as a simple indicator of mutation at position T252.

Synthetase Purification. SG13009 cells carrying the pREP4 repressor plasmid were transformed with p32leus, p32T252Y, p32T252L, and p32T252F to yield the expression strains. Protein expression was induced at an OD_{600} of 0.5 with 1 mM IPTG. After 3 h, the cells were harvested and lysed by 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 and 1 mM DTT) and 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.

ATP−*PP$_i$ Exchange Assay*. The assay was performed according to literature procedures (*16*). 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 the enzyme was 75 nM. The amino acid concentration ranges varied depending on the activity of the

Table 1: ATP−PP_i Exchange Kinetic Parameters of Wild-Type Six-His LeuRS toward Canonical (**1**−**4**) and Noncanonical (**5** and **6**) Amino Acids

amino acid	K_m (μM)[a]	k_{cat} (s^{-1})	k_{cat}/K_m (rel)
1[b]	18 ± 1.7	2.2 ± 0.02	1
2	2568 ± 555	0.06 ± 0.002	1/5231
3	2356 ± 320	0.03 ± 0.0008	1/9599
4	2178 ± 554	0.08 ± 0.003	1/3327
5	1155 ± 368	1.24 ± 0.06	1/114
6	2516 ± 659	0.22 ± 0.02	1/1397

[a] Concentrations are quoted for L-amino acids. [b] The kinetic parameters of the T252Y mutant were determined to be essentially the same as those for the wild-type enzyme for leucine ($K_m = 20 \pm 2.3$ μM, $k_{cat} = 1.92 \pm 0.03$ s^{-1}).

enzyme toward the substrate (10−500 μM for leucine, 50−5000 μM for **5** and **6**, and 200−8000 μM for **2**−**4**). Aliquots (15 μL) were quenched in 500 μL of 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 and 0.5% $HClO_4$ and counted. The results reported in Table 1 are averages from triplicate experiments.

Aminoacylation Assay. Aminoacylation assays were carried out as described previously (*16*, *35*, *36*). Assays were performed at 37 °C in 30 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, and 2 mM ATP. A purified *E. coli* tRNA mixture (Roche Biochemical) was used in the assay at a final concentration of 12 mg/mL. The tRNA solution was treated at 80 °C for 3 min and allowed to cool slowly to 37 °C before use. For reactions involving leucine, 20 μM [^{3}H]Leu (2000 dpm/pmol) and 10 nM LeuRS were added. For assays involving noncognate substrates isoleucine and valine, 20 μM [^{3}H]aa (6000 dpm/pmol) and 500 nM LeuRS were added. Reactions were initiated by adding enzyme; 10 μL aliquots were quenched on filter disks pretreated with 5% trichloroacetic acid (TCA) and the corresponding amino acid (100 μM). The filter disks were washed with cold 5% TCA three times and counted.

Expression Plasmids. Plasmid pA1EL [a derivative of pQE9 (Qiagen)] was used as the template for site-directed mutagenesis (*25*). The synthetic leucine zipper protein A1 (*37*) was inserted at the *Bam*HI 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 *Nhe*I site of pQEA1 to yield the template pA1EL. The Quickchange protocol (Stratagene) was used to introduce the mutation at position T252 in *leuS*. Primers 53TAC and 35GTA were used in the reaction to generate the tyrosine mutation. The integrity of the entire plasmid was verified through DNA sequencing. The plasmid carrying the T252Y mutant was designated pA1T252Y. Overexpression of LeuRS was verified by SDS−PAGE of whole cell lysates from overnight cultures.

Analogue Incorporation Assay. The leucine auxotrophic strain LAM1000 (*24*) was transformed with pA1EL or pA1T252Y, and with pREP4 to yield the A1 expression strain LAM1000/pA1EL or LAM1000/pA1T252Y. Growth and expression were performed in supplemented M9AA medium (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 analogues 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 3 h, the cells were collected by centrifugation (5000*g* for 10 min at 4 °C), resuspended in 600 μL of buffer B [8.0 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris (pH 8.0)], and frozen at −80 °C. Whole cell lysates were analyzed by SDS−PAGE.

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 of buffer B at 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 the optimal trypsin working pH (8.0). A trypsin stock solution (5 μL, 20 μg/200 μL) was added, and the sample was incubated at room temperature overnight. The reaction was quenched by addition of 2 μL of 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 and 50% CH_3CN. One microliter 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.

RESULTS AND DISCUSSION

As shown in Figure 1C, the T252−H_2O complex is positioned at the entrance of the putative editing site of LeuRS. This work was motivated by the conjecture that replacing T252 with a larger amino acid could block the binding of misaminoacylated tRNA species and result in relaxed LeuRS amino acid specificity. We replaced T252 with the bulkier residues leucine, phenylalanine, and tyrosine. The van der Waals volumes of the these amino acids are 124, 135, and 141 Å^3, respectively; the volume of threonine is 93 Å^3 (*38*). These mutations introduce different degrees of steric hindrance to the editing groove, and were expected to yield varying rates of $tRNA^{Leu}$ misaminoacylation.

Cloning and Mutagenesis. Genes encoding wild-type LeuRS and the mutants T252L, T252F, and T252Y were obtained from four-primer PCR with *E. coli* genomic DNA as the template. Enzymes were expressed in *E. coli* by construction of individual pQE32 derivatives containing the gene in frame with the N-terminal six-His tag derived from the vector. The enzymes were purified under native conditions using Ni^{2+} affinity chromatography to >95% purity as estimated by SDS−PAGE. The typical yields of enzymes were ca. 4 mg/L.

Amino Acid Activation in Vitro. We examined the kinetics of the ATP-dependent amino acid activation reaction using purified, wild-type His_6-synthetase. We compared the rates of activation of canonical amino acids **1**−**4** and noncanonical amino acids **5** and **6** by using an ATP−PP_i exchange assay. The kinetic parameters are listed in Table 1. The K_m reported

for leucine is consistent with that reported in the literature (*39*), while the k_{cat} value is slightly lower. Compared to that of leucine, the catalytic efficiencies of LeuRS toward natural, noncognate substrates isoleucine, valine, and methionine were decreased by factors of 3300, 9600, and 5200, respectively. Under normal cellular conditions, these amino acids do not pose significant threats to LeuRS fidelity. In contrast, LeuRS displayed higher activity toward the noncanonical analogues **5** and **6**. Replacing the isobutyl side chain of leucine with the *n*-propyl group of **5** decreased the activation rate only 120-fold. A comparable (200-fold) decrease was noted in ValRS activity when the isopropyl side chain of valine was replaced with the ethyl group of αbu (*5*). The proofreading mechanism of ValRS increases selectivity for valine over αbu by an additional factor of 60, resulting in a final discrimination factor of 12 000 (*40*). In vivo translation studies have shown that trifluoroleucine, which is activated by LeuRS 250-fold more slowly than leucine, can replace leucine in proteins quantitatively (*24*). In contrast, we have found no conditions under which **5** will support protein synthesis in bacterial cultures depleted of leucine. The comparable activation rates of **5** and trifluoroleucine in vitro, coupled with the differences in their translation activities in vivo, strongly indicate that LeuRS carries out additional discrimination against **5** through an editing step. The linear, four-carbon side chain analogue **6** is also subject to proofreading by LeuRS. The activities of LeuRS mutants toward leucine, **5**, and **6** were essentially identical to those reported for the wild-type enzyme, in agreement with a previous conclusion that activities at the synthetic site and the editing site are mutationally exclusive (*16*).

Aminoacylation Kinetics of Mutants. The abilities of the LeuRS mutants to aminoacylate $tRNA^{Leu}$ with amino acids **1**−**3** were measured by standard TCA precipitation assays. The results are shown in Figure 2. All four enzymes aminoacylate $tRNA^{Leu}$ with leucine at comparable rates, indicating that replacement of T252 with bulkier residues has no effect on the rate of synthesis of Leu-$tRNA^{Leu}$. For reactions of isoleucine or valine, high concentrations (500 nM) of LeuRS were used to facilitate detection. The wild-type enzyme yielded no detectable Ile-$tRNA^{Leu}$ or Val-$tRNA^{Leu}$. This is expected, as any misaminoacylation by the synthetic active site should be corrected by the proofreading mechanism of the CP1 domain. The three mutants in this study all displayed impaired editing activity toward isoleucine and valine, confirming the importance of T252 in the editing process. Poor activation of valine in the synthetic active site results in rates of synthesis of Val-$tRNA^{Leu}$ that are lower than that of Ile-$tRNA^{Leu}$. The rate of misaminoacylation increases as the size of residue 252 increases. The T252Y mutant yields the highest rate of misaminoacylation, followed by T252F and T252L. The observed correlation between side chain volume (Tyr > Phe > Leu > Thr) and misaminoacylation rate supports our initial hypothesis that a larger amino acid at position 252 would block binding of misaminoacylated $tRNA^{Leu}$ at the editing site and reduce the efficiency of proofreading.

In Vivo Properties of Mutant Synthetases. The T252Y mutant displayed the highest activity in misaminoacylating $tRNA^{Leu}$ based on the in vitro aminoacylation assay. Overexpression of this mutant could lead to accumulation of

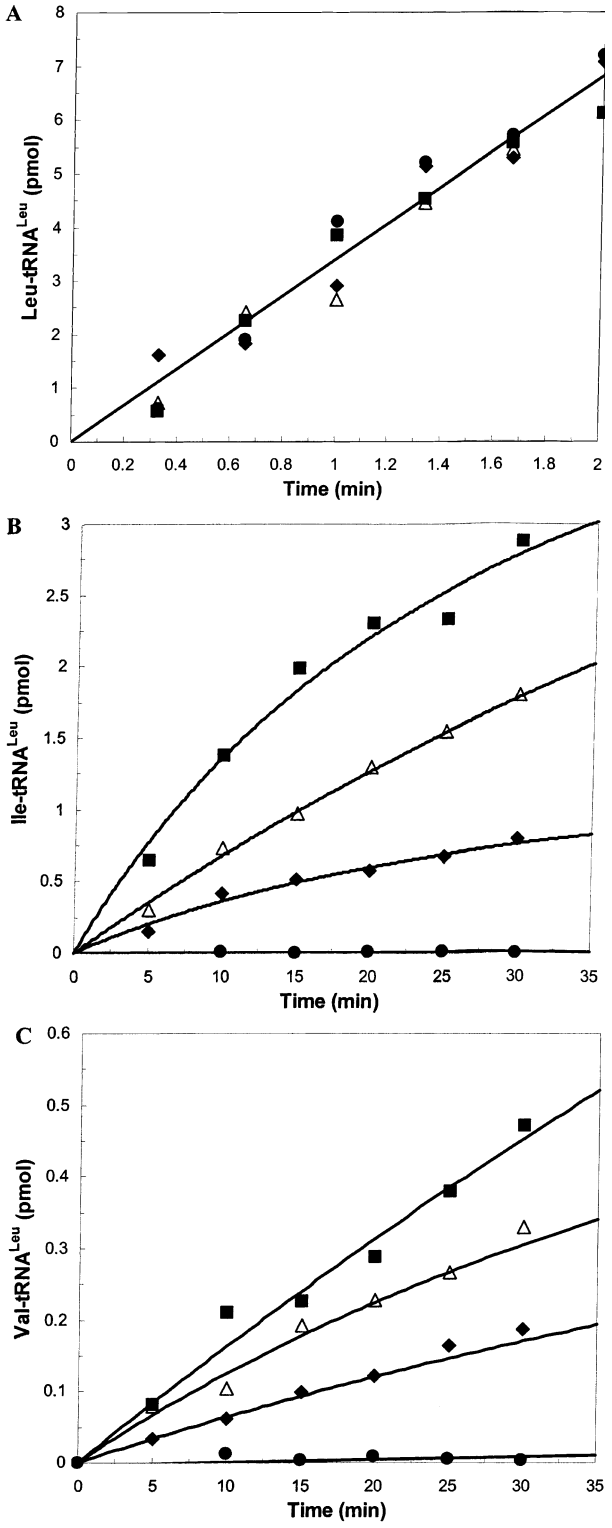


FIGURE 2: Aminoacylation of $tRNA^{Leu}$ by wild-type and mutant LeuRS enzymes at 37 °C. Each enzyme was evaluated for its ability to aminoacylate $tRNA^{Leu}$ with leucine (A), isoleucine (B), and valine (C): (●) wild-type enzyme, (■) T252Y, (△) T252F, and (◆) T252L. All enzymes displayed comparable rate of leucine aminoacylation (10 nM enzyme and 20 μM leucine), demonstrating that point mutations in the editing cavity do not affect the kinetics of this reaction. While the wild-type LeuRS does not misaminoacylate $tRNA^{Leu}$ with isoleucine or valine, all three mutant enzymes catalyze misaminoacylation under these conditions (500 nM enzyme and 20 μM analogue). The rate of misacylation increases as larger amino acids are introduced at position 252. The mutant T252Y was chosen for in vivo incorporation studies. The data are fitted to the Michaelis−Menten equation.

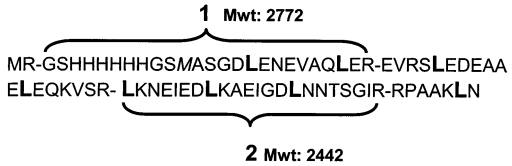


FIGURE 3: Amino acid sequence of target protein A1. The protein contains 74 residues, including eight leucines. Two tryptic fragments were used for MALDI analyses and are labeled as shown. Fragment 1 contains two leucine sites and one methionine site and has an expected mass of 2772. Fragment 2 contains three leucine sites and has an expected mass of 2442.

misaminoacylated $tRNA^{Leu}$ in vivo, and result in the incorporation of amino acid analogues that are edited by the wild-type enzyme. The expression plasmid pA1EL was the template for site-directed mutagenesis. The construction and application of this plasmid have been reported previously (*25*). The plasmid carries a gene encoding a 74-residue synthetic leucine zipper protein; the protein contains eight leucine residues, of which six can be detected easily by tryptic mass spectrometry (Figure 3). The plasmid also carries a copy of the wild-type *leuS* gene, which encodes *E. coli* LeuRS under control of its endogenous, constitutive promoter. *E. coli* hosts transformed with pA1EL exhibit LeuRS activity higher than the activities of untransformed hosts. The T252Y mutation was introduced via site-directed mutagenesis. The resulting plasmid, pA1T252Y, was transformed into the leucine auxotrophic strain LAM1000/pREP4 to yield the expression strain LAM1000/pA1T252Y/pREP4. LAM1000 contains the wild-type *leuS* gene on its chromosome.

We compared levels of protein expression in host strains carrying either pA1EL or pA1T252Y. To test the ability of an analogue to serve as a leucine surrogate, we adopted a medium shift procedure for depleting the expression medium of leucine. Cells were first grown in M9 medium supplemented with all 20 natural amino acids. At the time of protein induction, cells were transferred to a new medium containing 19 amino acids, either without leucine (negative control), with leucine (positive control), or with one of the analogues. Protein expression was induced with IPTG and continued for 3 h. The levels of target protein accumulation were visualized by SDS−PAGE and are shown in Figure 4. As expected, when the wild-type synthetase was overexpressed, only cognate substrate leucine supported protein synthesis (lane 2 in Figure 4A). Neither the canonical nor the noncanonical analogues (lanes 3−6) supported detectable levels of A1 expression.

The background level expression (lane 1) in Figure 4A is nearly undetectable. This is expected since in the absence of leucine, none of the other 19 amino acids is joined to $tRNA^{Leu}$ at a substantial rate. In contrast, when we performed the same experiments with strains that overexpress the T252Y mutant, significant target protein expression was observed, even in medium depleted of leucine (not shown). Composition analysis of the protein expressed under these conditions shows that natural amino acids **2**−**4** are incorporated at positions normally occupied by leucine, indicative of the inability of the LeuRS mutant to proofread misaminoacylated $tRNA^{Leu}$ in vivo. To decrease the level of background expression, we opted to supplement the M9 expression medium with only 16 natural amino acids (without **1**−**4**), in addition to the analogues of interest.

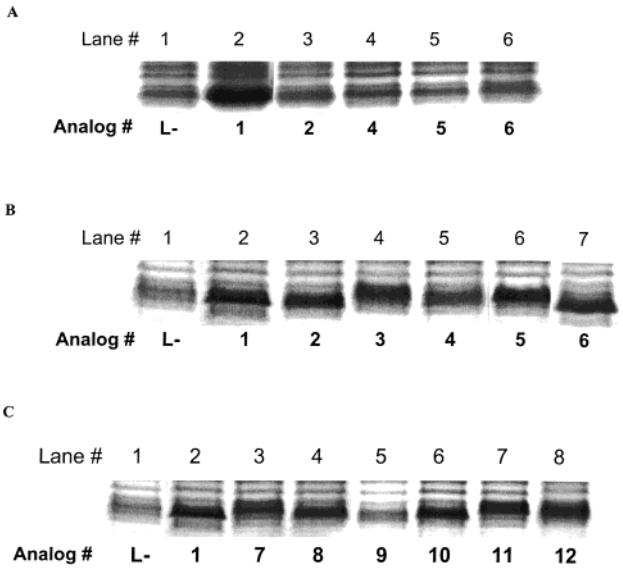


FIGURE 4: In vivo incorporation of unnatural amino acids by *E. coli* host strains equipped with the wild type and mutant LeuRS variants. Proteins were visualized by Coomassie staining of SDS−PAGE gels. The amino acids of interest were supplemented at 320 mg/L of the L-isomer. Lane numbers are indicated at the top of the gel, and analogue numbers are shown below the gel. L^- represents induction without any of the amino acids in Scheme 2. (A) Protein expression profiles for the wild-type overexpression strain LAM1000/pA1EL; none of the supplied amino acids supported protein synthesis except leucine (lane 2). (B) Protein expression profiles for the LAM1000/pA1T252Y overexpression strain. Previously inactive analogues **2**−**6** (lanes 3−7, respectively) yield bands indicating accumulation of the target protein. (C) Protein expression profiles for unsaturated analogues **7**−**12**. Analogues **7** and **10**−**12** support significant levels of protein synthesis in the absence of leucine. The culture supplemented with **8** (lane 4) shows protein expression, but this occurs via incorporation of methionine (see the text). Analogue **9** (lane 5) does not support detectable levels of protein expression under these conditions.

Figure 4B shows the levels of protein expression supported by the different analogues in the host LAM1000/pA1T252Y/pREP4. Exclusion of amino acids **2**−**4** from the expression medium diminished the levels of background expression (lane 1). We attribute the residual expression observed in this experiment to the incorporation of **2**−**4** accumulated via cellular biosynthesis (LAM1000 is only auxotrophic in leucine). Analogues **2**−**6** all supported substantial levels of protein synthesis in the absence of leucine when the mutant LeuRS was overexpressed. The amounts of protein that were obtained correlate well with the results of in vitro ATP−PP_i exchange experiments. The most robust analogues **5** and **6** supported protein synthesis at almost the same level as leucine. Protein expression levels in media supplemented with more sluggish analogues **2**−**4** were visibly reduced.

We do not fully understand the differences in the electrophoretic mobilities of the modified A1 proteins shown in Figure 4. We have previously observed similar changes in the electrophoretic mobility of A1 upon incorporation of trifluoroleucine (*24*). The integrity of each purified protein was confirmed by MALDI-TOF mass spectrometry (data not shown) and excluded the possibilities of premature translational termination and posttranslational modification. Interestingly, the substituted proteins migrated more slowly with decreased amino acid side chain hydrophobicity. For example, the hydrophobicities (defined as the free energy required to transfer the amino acid from a hydrophobic to

Table 2: Results of Amino Acid Analyses of Target Proteins Expressed in Media Supplemented with **1**, **2**, **3**, **4**, **5**, or **6**[a]

amino acid in protein	Exp[c]	**1**[b] Obs[d]	**1**[b] Δ%[e]	**2**[b] Obs	**2**[b] Δ%	**3**[b] Obs	**3**[b] Δ%	**4**[b] Obs	**4**[b] Δ%	**5**[b] Obs	**5**[b] Δ%	**6**[b] Obs	**6**[b] Δ%
1	10.8	10.9	+**0.9**	5.9	−45	6.0	−44	5.0	−54	2.3	−79	0.97	−91
2	4.1	3.8	−7.3	7.2	+76	3.8	−7.3	4.2	+2.4	4.1	0	3.8	−7.3
3	4.1	4.2	+2.4	4.5	+9.7	6.7	+63	4.7	+14	4.**3**	+4.9	4.1	0
4	2.7	2.4	−11	2.5	+7.4	2.6	−3.7	6.3	+133	0.87	−68	0.22	−92
5	0	ND[f]	–	ND[f]	–	ND[f]	–	ND[f]	–	10.6	–	ND[f]	–
6	0	ND[f]	–	ND[f]	–	ND[f]	–	ND[f]	–	ND[f]	–	12.4	–
total[g]	21.6	21.3	−1.4	20.1	−6.9	20.2	−5.2	20.2	−5.2	22.1	+2.3	21.4	−0.9

[a] The reported values are mole fractions of amino acids. [b] **1** was supplemented at 40 mg/L; other amino acids were supplemented at 320 mg/L. [c] Expected mole fractions based on the composition of **1**−**4** in A1. [d] Observed mole fractions. [e] Percentage deviation between observed and expected mole fractions. [f] Not detected. [g] Sum of mole fractions corresponding to **1**−**6**.

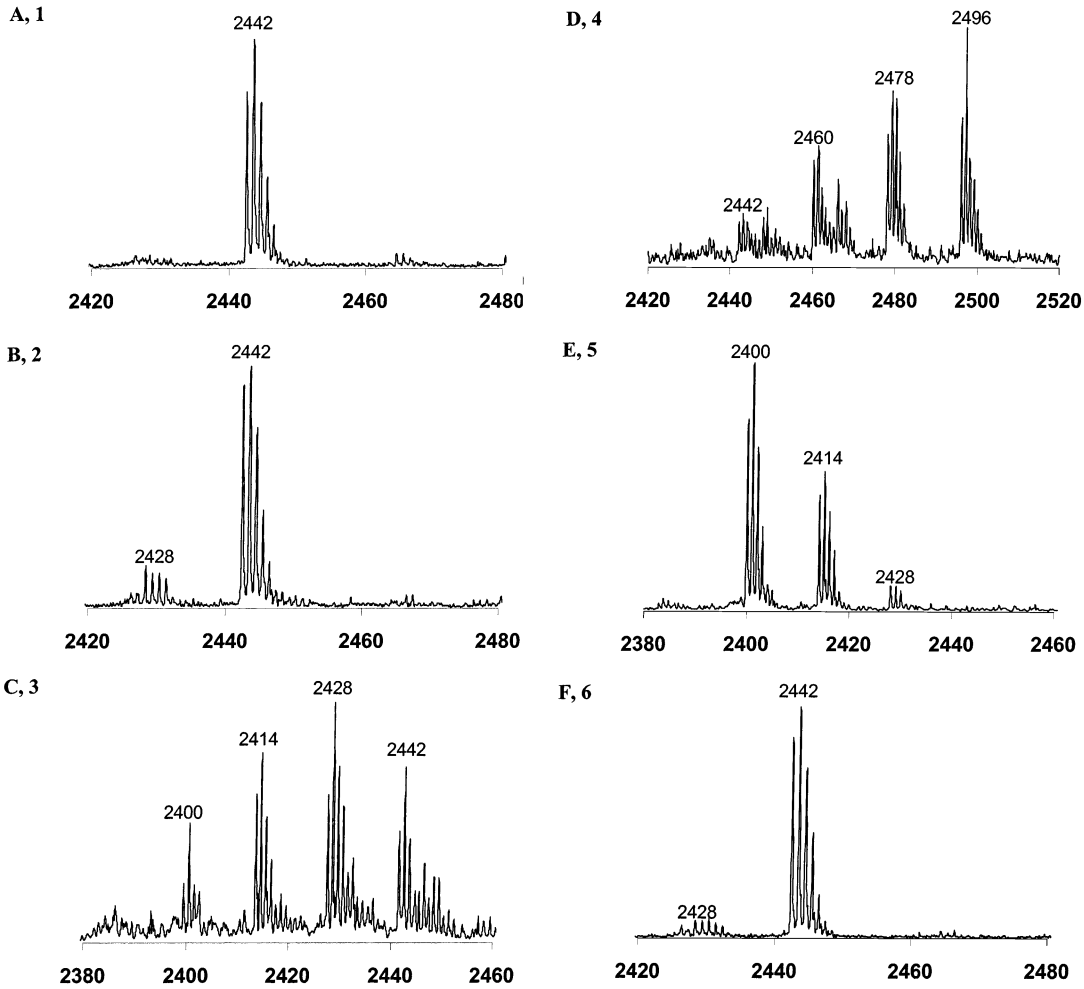


FIGURE 5: MALDI-MS of tryptic peptide fragment 2 containing (A) **1**, (B) **2**, (C) **3**, (D) **4**, (E) **5**, and (F) **6**. The peptide has the sequence **L**KNEIED**L**KAEIGD**L**NNTSGIR. The expected mass of the fragment is 2442. Three sites of substitutions are possible. The minor peaks at a mass of 2428 found in panels B and F correspond to fragments containing one valine substitution. The shoulder peaks observed in panel D have masses of 2446 and 2464. The peak at a mass of 2446 corresponds to fragment 2 containing one leucine (or isoleucine), one valine, and one methionine (mass change of 4) at the leucine positions. The shoulder at a mass of 2464 corresponds to fragment 2 containing one valine and two methionines (mass change of 22) at the leucine positions.

an aqueous environment, relative to that of glycine) of leucine, isoleucine, and valine are 2.4, 2.9, and 1.7 kcal/residue, respectively (*41*). Figure 4B shows that the relative mobilities of A1 containing these amino acids correlate directly with these values (Ile-A1 > Leu-A1 > Val-A1). A1 proteins substituted with the more hydrophobic analogue trifluoroleucine or hexafluoroleucine also migrate faster than the wild-type protein (*25*).

Protein Amino Acid Analysis. Compositions of A1 protein expressed under different conditions of amino acid supplementation were determined by amino acid analysis. The proteins were purified via Ni−NTA columns under denaturing conditions. Table 2 lists results for proteins expressed in media containing **1**−**6**. The expected individual mole fractions of **1**−**4** are listed and compared to the observed mole fractions of these amino acids. The combined mole fractions of these amino acids and the nonnatural amino acids (in samples containing **5** or **6**) are also compared to the expected total value. Canonical amino acids isoleucine, valine, and methionine were inserted at leucine positions in

the T252Y mutant strain at substitution rates of 45, 54, and 44%, respectively. These numbers are calculated by noting the percentage decrease in the amount of leucine in the purified protein. Corresponding increases in the mole fractions of isoleucine, valine, or methionine are observed, confirming the presence of these natural amino acids at positions other than those specified by their respective codons. The mole fraction totals of **1**−**4** decreased slightly in proteins synthesized in media supplemented with isoleucine, valine, or methionine. We initially suspected the observed decrease in total mole fractions might be due to substitution of leucine with other natural amino acids, such as threonine. However, no evidence of other amino acids was detected by mass spectrometry.

The amino acid analysis results for proteins containing the noncanonical amino acids **5** and **6** are also shown in Table 2. Both amino acids elute separately during analysis and can be quantified directly. As expected from the intensities of the SDS−PAGE bands, most of the leucine positions are occupied by the unnatural surrogates. On the basis of the levels of leucine depletion, **5** and **6** are incorporated at levels of 79 and 91%, respectively. Although the observed mole fractions of isoleucine and valine in these samples match the expected values, the mole fraction of methionine is significantly lower. The total mole fractions of **1**−**6** in the protein, however, matched the expected total mole fractions of **1**−**4** in the wild-type protein (errors of 2.3 and 0.9%, respectively). These results suggest that in addition to occupying the leucine positions in A1, **5** and **6** substituted for methionine at ATG codons with rates of 68 and 92%, respectively. This result is not surprising since we have reported previously that both amino acids serve as methionine surrogates in media depleted of methionine (*30*). Tryptic mass spectrometry provided additional evidence that these two noncanonical amino acids replace both leucine and methionine in the target protein, as discussed below.

Protein Tryptic Mass Spectrometry. Tryptic digestion of A1 yields several peptide fragments that can be easily detected by MALDI mass spectrometry as shown in Figure 3. For most of these studies, we focused on fragment 2 (N-**L**KNEIED**L**KAEIGD**L**NNTSGIR-C). There are three leucine sites in this fragment; the unsubstituted mass is 2442. The spectra of peptide fragments that contained amino acids **1**−**6** are shown in Figure 5. Peptides containing isoleucine and **6**, which have the same mass as leucine, displayed a single peak in MALDI analysis. The anticipated differences in peptide mass can be clearly detected for the other analogues. For example, each substitution of leucine with methionine yields a mass increment of 18 mass units. Four peaks separated by 18 mass units are observed by MS (Figure 5D), corresponding to 0, 1, 2, and 3 sites of substitution by methionine. Similar patterns are observed for valine (mass difference of −14, Figure 5C) and **5** (mass difference of −14, Figure 5E). Figure 6 shows the tryptic MS patterns for peptide fragments 1 containing leucine, **5**, and **6**. This peptide contains two leucine sites and one methionine site, and has a wild-type mass of 2772. The peak with the exact mass was observed in the wild-type fragment (Figure 6A). When either **5** or **6** is added to the expression medium, the mass shift observed of this fragment indicates that the analogues replace both leucine and methionine in the target protein, in accord with the results of amino acid analysis.

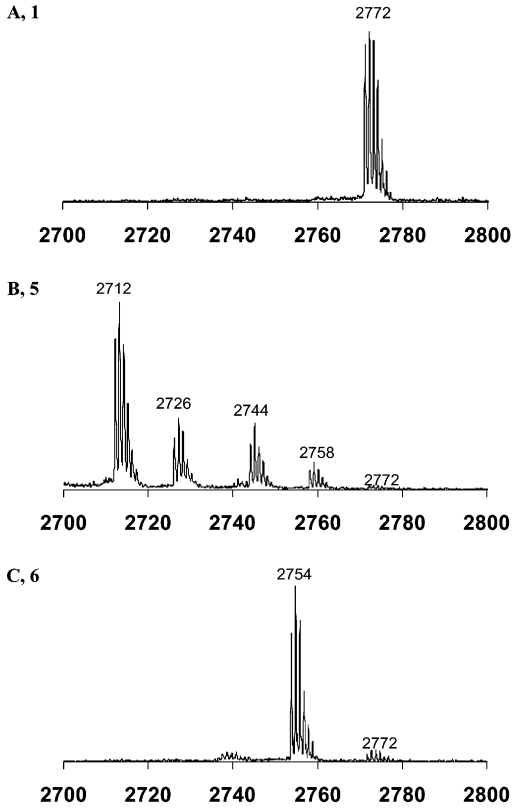


FIGURE 6: MALDI-MS of tryptic peptide fragment 1 containing (A) **1**, (B) **5**, and (C) **6**. The sequence of this fragment is GSHHHHHHGS***M***ASGD**L**ENEVAQ**L**ER; the wild-type mass is 2772 (A). For **5** and **6**, the observed decreases in mass are due to the substitution at the lone methionine position, as well as at the two leucine sites. The patterns that were observed agree well with the results of amino acid analysis (Table 2).

Incorporation of Other Analogues. The incorporation of **5** and **6** expands the repertoire of aliphatic amino acids available for protein biosynthesis. However, the inert hydrocarbon side chains of these analogues are inaccessible to chemoselective protein modification. We therefore investigated analogues **7**−**12** as potential leucine surrogates. Each of these analogues contains an unsaturated moiety, which should be subject to metal-mediated metathesis (*42*, *43*) and other coupling reactions. Analogues **7**−**9** are isosteric to the three-carbon side chain analogue **5**; analogues **10**−**12** are isosteric to the four-carbon side chain analogue **6**. None of the analogues supported synthesis of A1 when wild-type LeuRS was overexpressed (not shown); Figure 4C shows the results when the T252Y mutant was overexpressed. All of the analogues except **8** and **9** (lanes 4 and 5) supported protein expression at levels higher than the background [**8** is not incorporated despite the higher intensity of the protein band; composition analysis shows that methionine is incorporated in place of leucine in media supplemented with **8** (see below)].

Proteins expressed in media supplemented with analogues **7**−**12** were analyzed by tryptic mass spectrometry as before. The spectra of tryptic fragment 2 are shown in Figure 7. The analogues incorporated into these fragments can be clearly identified: **7** (mass difference per substitution of −16, Figure 7A), **10** (mass difference of −2, Figure 7D), **11** (mass difference of −4, Figure 7E), and **12** (mass difference of −4, Figure 7F). Instead of the expected decrease of 18 mass

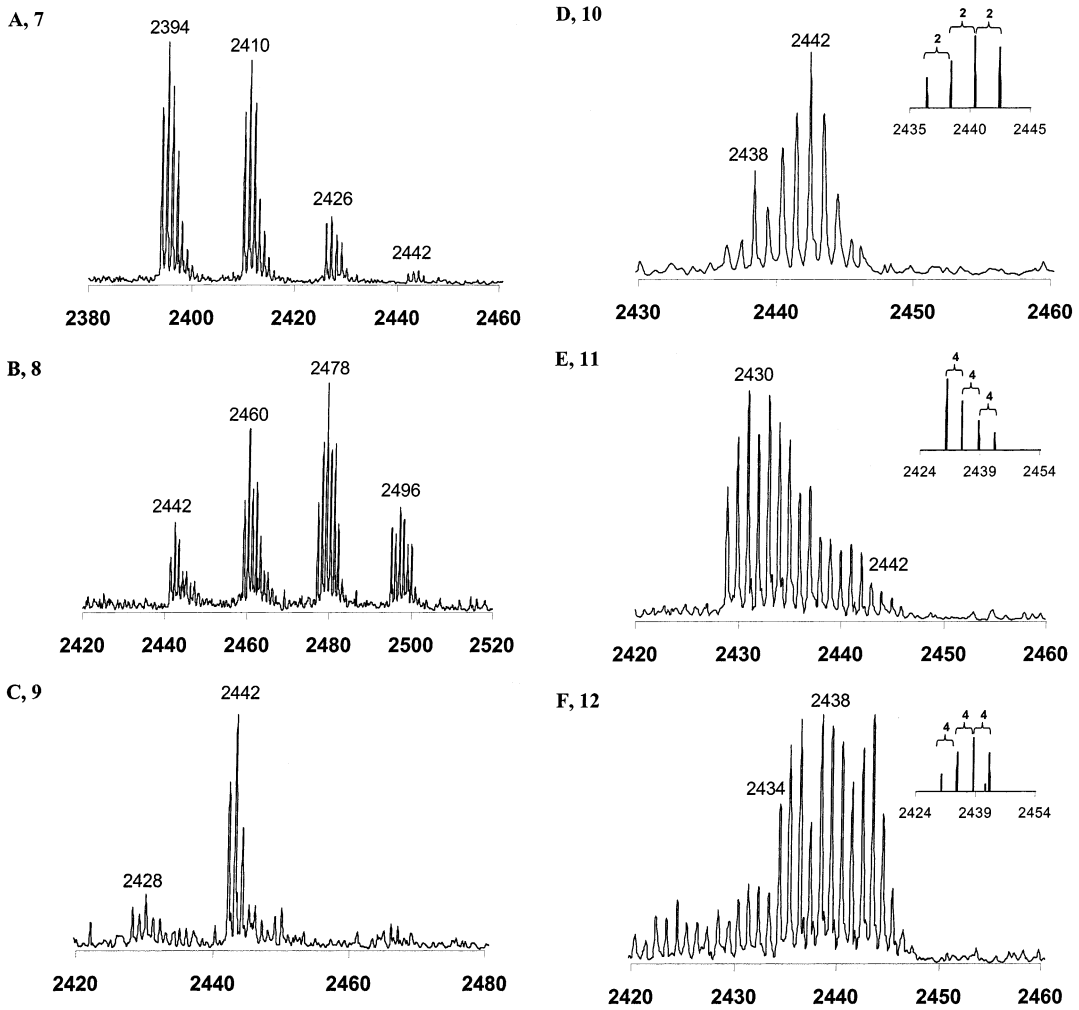


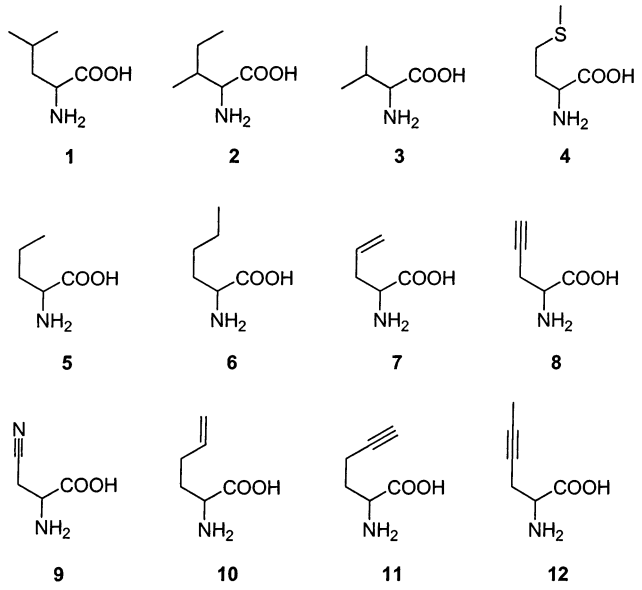
FIGURE 7: MALDI-MS of tryptic peptide fragment 2 containing unsaturated amino acid analogues (A) **7**, (B) **8**, (C) **9**, (D) **10**, (E) **11**, and (F) **12**. Analogue **8** is not incorporated. The unexpected increase of 18 mass units in panel B is attributed to the incorporation of methionine (see the text). Analogue **9** is not incorporated, as seen from SDS−PAGE and MS (C). The insets in panels D−F are isotope-filtered for clarity.

units upon substitution of leucine with **8**, we detected species with incremental *increases* in mass of 18 units, a pattern similar to that observed with methionine. Propargylglycine is known to inhibit γ-cystathionase, which is involved in the conversion of homocysteine to cysteine (*44*, *45*). When this biosynthetic pathway is blocked, homocysteine is shuttled through the methionine biosynthetic pathway, leading to the intracellular accumulation of methionine. Hence, it is likely that while **8** is not incorporated into protein under our conditions, the augmentation of the cellular pool of methionine was sufficient to allow substantial protein synthesis (Figure 4C, lane 4). We also examined the composition of the trace amounts of protein recovered from cells treated with **9** (Figure 7C). A single peak corresponding to the wild-type mass was observed, rather than peaks separated by 16 mass units as expected for incorporation of **9**, confirming the previous observation that **9** is not a good substrate for LeuRS. Analogues **7** and **10**−**12** were all previously shown to be methionine analogues (*30*). As a result, tryptic fragments 1 containing **7** or any of the analogues **10**−**12** all show mass distributions characteristic of incorporation of the analogue at both leucine and methionine sites, as discussed above for fragments containing **5** and **6**.

If an equal probability of incorporating an analogue at all leucine positions in all copies of A1 is assumed, the relative intensities of the tryptic fragments in Figures 5 and 7 can be described by the terms of the polynomial $[(1 - p) + p]^n$, where p is the fraction of leucine sites occupied by an analogue and n is the number of leucine sites in the fragment.

We assessed the validity of this approach by comparing the values of p obtained from a least-squares fit of the spectra in Figure 5 with those determined by amino acid analysis. For fragment 2, the intensities of the peaks corresponding to 0, 1, 2, and 3 sites of substitution are $(1 - p)^3$, $3(1 - p)^2p$, $3(1 - p)p^2$, and p^3, respectively. For samples containing valine, methionine, and **5**, the values of p obtained from fitting the spectra are 41, 66, and 82%, respectively, as compared to values of 44, 54, and 79%, respectively, obtained from amino acid analysis. One should not expect better agreement. The MALDI method is not rigorously quantitative; assumption of a single p may not be valid, and the cellular amino acid pools change over the course of the experiment. Nevertheless, the MALDI spectra provide a rough estimate of the level of substitution. The values obtained in this way were 76, 37, 74, and 37% for analogues **7** and **10**−**12**, respectively.

Scheme 2



Isoleucine and Valine Analogues. We also examined the isoleucine analogues 5,5,5-trifluoroisoleucine, 2-amino-3-methyl-5-pentenoic acid, and 2-amino-3-methyl-5-pentynoic acid. None of these analogues supported measurable levels of protein synthesis. For these substrates, the observed lack of translational activity is likely to be due to slow activation by the synthetic site. Similar results were obtained for the valine derivatives 4,4,4-trifluorovaline and αbu. The five-carbon side chain analogue 2-aminoheptanoic acid also displayed no translational activity as an analogue of leucine. We attribute the lack of incorporation to weak binding at the synthetic active site of LeuRS. The back wall of the leucine binding pocket is formed in part by a histidine residue [position 537 in *E. coli* LeuRS and position 545 in *Thermus thermophilus* LeuRS (*12*)], which limits the maximum tolerable number of carbon atoms on the side chain to four. Amino acids with longer side chains must adopt entropically unfavorable conformations to be accommodated in the active site. Mutation of this histidine residue to a smaller amino acid to create an enlarged and more flexible pocket, along with the editing domain mutation T252Y, might allow the incorporation of amino acids with longer side chains.

The motivation for this study was to identify a suitable host strain for introducing the noncanonical amino acids shown in Scheme 2 into recombinant proteins. Our laboratory and others (*46*−*50*) have established a variety of techniques for introducing new chemical functionality into cellular proteins, including overexpression of wild-type synthetases (*25*, *26*) and introduction of active site mutations (*28*, *29*). The results reported here, along with recent work on ValRS (*17*), show that modulation of the editing cavity geometry is a general and powerful tool for the incorporation of novel amino acids in vivo. Amino acids such as **7** and **10**−**12**, which contain unsaturated side chains, provide attractive orthogonal sites for protein modification. We are currently investigating chemoselective protein derivatization methods for recombinant proteins produced in the host described here.

ACKNOWLEDGMENT

We thank Dr. Mona Shahgholi and Dr. Gary Hathaway for assistance with typtic digest mass spectrometry. We are grateful to Dr. Anthony Bishop from Prof. Schimmel's group for his advice on aminoacylation assays. We also thank Prof. Stephen Cusack for providing coordinates of the *T. thermophilus* LeuRS crystal structure coordinates and P. Wang, I. Carrico, and K. Kirshenbaum for fruitful discussions.

NOTE ADDED IN PROOF

While this paper was under review, Mursinna and Martinis (*34*) demonstrated that the T252Y mutant is defective in posttransfer editing of Ile-tRNA^{Leu} in vitro.

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BI026130X