Modulation of Substrate Specificity within the Amino Acid Editing Site of Leucyl-tRNA Synthetase†

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ABSTRACT: The aminoacyl-tRNA synthetases covalently link transfer RNAs to their cognate amino acids. Some of the tRNA synthetases have evolved editing mechanisms to ensure fidelity in this first step of protein synthesis. The amino acid editing site for leucyl- (LeuRS) and isoleucyl- (IleRS) tRNA synthetases reside within homologous CP1 domains. In each case, a threonine-rich peptide and a second conserved GTG region that are separated by about 100 amino acids comprise parts of the hydrolytic editing site. While a number of sites are conserved between these two enzymes and likely confer a commonality to the mechanisms, some positions are idiosyncratic to LeuRS or IleRS. Herein, we provide evidence that a conserved arginine and threonine at respective sites in LeuRS and IleRS diverged to confer amino acid substrate recognition. This site complements other sites in the amino acid binding pocket of the editing active site of *Escherichia coli* LeuRS, including Thr^{252} and Val^{338}, which collectively fine-tune amino acid specificity to confer fidelity.

The fidelity of protein synthesis is dependent on the aminoacyl-tRNA synthetases (aaRSs[1]), which link amino acids to their cognate transfer RNAs (tRNAs) (*1*, *2*). There are typically up to twenty types of aaRSs per cell or organelle. Each corresponds to one of the standard amino acids that are incorporated into proteins during ribosome-based translation. The aaRSs catalyze the formation of aminoacyl-tRNA in a two-step reaction mechanism:

$$\text{amino acid (AA)} + \text{ATP} \rightleftarrows \text{AA-AMP} + \text{pyrophosphate (PP}_i\text{)} \quad (1)$$

$$\text{AA-AMP} + \text{tRNA} \rightarrow \text{AA-tRNA} + \text{AMP} \quad (2)$$

The charged tRNA is then bound by an elongation factor and transported to the ribosome where the amino acid is incorporated into the nascent peptide chain.

Because of structural similarities between some amino acids, a number of aaRSs have difficulty completely discriminating their cognate substrate from other similar amino acids during catalysis (*3*). To minimize the production of mischarged tRNAs, many aaRSs have developed amino acid editing activities. A "double-sieve" mechanism (*4*−*6*) introduces two separate amino acid binding sieves or pockets with different strategies for recognition to enhance fidelity. In the synthetases, the "coarse sieve" corresponds to the amino acid activation and aminoacylation active site. It binds cognate amino acid, as well as related analogues that can also fit in the amino acid binding pocket to form activated aminoacyl-adenylates and charged tRNAs. A second "fine sieve" can be correlated to the amino acid editing site. Its primary purpose is to clear mistakes created in the amino-acylation active site, but also must prevent the correct amino acid from being hydrolyzed from the aminoacyl-adenylate intermediate or charged tRNA.

The aminoacylation active sites of the class I editing leucyl- (LeuRS) (*7*), isoleucyl- (IleRS) (*8*), and valyl-tRNA synthetases (ValRS) (*9*) are located within the canonical class I Rossmann fold core of the synthetase. The editing sieves for LeuRS, IleRS, and ValRS reside in separate homologous domains called CP1 (*10*−*12*). The editing site is marked by a universally conserved aspartic acid that anchors the common amino moiety of the bound amino acid via a hydrogen bond (*13*, *14*). ValRSs and bacterial LeuRSs also have a highly conserved second aspartic acid that is three amino acids upstream and in close tertiary proximity to the universally conserved aspartic acid. In cocrystal structures of *Thermus thermophilus* LeuRS, this Asp^{344} interacts via a bridging water molecule in a network of hydrogen bonds to the universally conserved aspartic acid, the bound substrate's amino moiety, and also the oxygen atom of the ester linkage (*14*). In the "pre-transfer editing complex" of LeuRS (*14*) and a model for ValRS (*15*), this aspartic acid forms a hydrogen bond with the 3′ hydroxyl of the bound ribose.

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; CP1, connective peptide 1; DTT, D,L-dithiothreitol; Nva, norvaline; LeuRS, leucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; X###Y, X residue at position ### is substituted by Y amino acid.

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Each CP1 domain also shares a threonine-rich region that marks a portion of the editing site where catalysis occurs. Mutation of a conserved Thr^{252} residue to serine or alanine within the threonine-rich region of *Escherichia coli* LeuRS uncouples amino acid specificity such that correctly charged leucine is rapidly hydrolyzed from tRNA^{Leu} (*12*, *16*). Insertion of bulkier residues including tyrosine or phenylalanine blocks amino acid editing (*17*, *18*). The X-ray crystal structure of *T. thermophilus* LeuRS bound to either the pre- or post-transfer editing substrate analogue supports that the threonine side chain sterically blocks the γ-methyl group of the leucine side chain (*14*) and therefore prevents hydrolytic editing of the correctly charged Leu-tRNA^{Leu}.

The X-ray crystal structures (*14*) also suggested that a valine (Val^{340} in *T. thermophilus* LeuRS) within a second conserved CP1-based peptide might contribute to leucine specificity (Figure 1). In addition, conserved sites within these two LeuRS peptides that comprise the amino acid binding pocket of the editing site (including Thr^{247}, Thr^{248}, Thr^{252}, Ile^{337}, Pro^{341}, Ala^{342}, and His^{343}) are also highly conserved in IleRS. However, some neighboring sites are also conserved in LeuRS and IleRS, but have different amino acids at the corresponding sites in these respective enzymes (Figure 1D). We hypothesized that these positions might be important to amino acid recognition in the editing site that contributes significantly to the fidelity of protein synthesis. We mutationally analyzed these two peptides that line the amino acid binding pocket of LeuRS to characterize those that play a role in editing specificity.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes *Dpn*I and *Bst*NI were acquired from Promega (Madison, WI) and New England BioLabs Inc. (Beverly, MA) respectively. Cloned *Pfu* DNA polymerase and dNTP mix were purchased from Stratagene (La Jolla, CA). Tritium-labeled amino acids were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Crude *E. coli* tRNA^{Leu} was from Hoffmann-La Roche Ltd (Basel, Switzerland). For *in vitro* transcription, a total of 450 μg of plasmid ptDNAleu (*19*) containing the gene for *E. coli* tRNA^{Leu} was digested overnight with 25 U *Bst*N1 in a 1 mL reaction at 60 °C and then used as template (*20*). *E. coli* tRNA$^{Leu}_{UAA}$ (tRNA^{Leu}) was *in vitro* transcribed, purified, and aminoacylated using a published procedure (*21*). Oligonucleotide primer synthesis was performed by MWG Biotech (High Point, NC).

Mutagenesis and Purification of E. coli LeuRS. Wild type LeuRS was mutated to R249F, R249A, R249F/T252A, M336F, M336A, M336F/T252A, V338A, V338F, V338F/T252A, V338D, V338E, V338L, R249T, D251W, and R249T/D251W using polymerase chain reaction (PCR)-based reaction mutagenesis and the plasmid p15ec3-1 (*22*) as template as described previously (*21*). The mutant plasmids were selected in *E. coli* DH5α strain. The mutation in the isolated plasmid was confirmed by DNA sequencing at Lone Star Labs (Houston, TX).

Protein expression for mutant and wild type plasmids was carried out in *E. coli* BL21 strain (Stratagene, La Jolla, CA) as described previously (*21*). Each protein contained an N-terminal fused six-histidine tag and was purified to homogeneity via affinity chromatography using HIS-Select

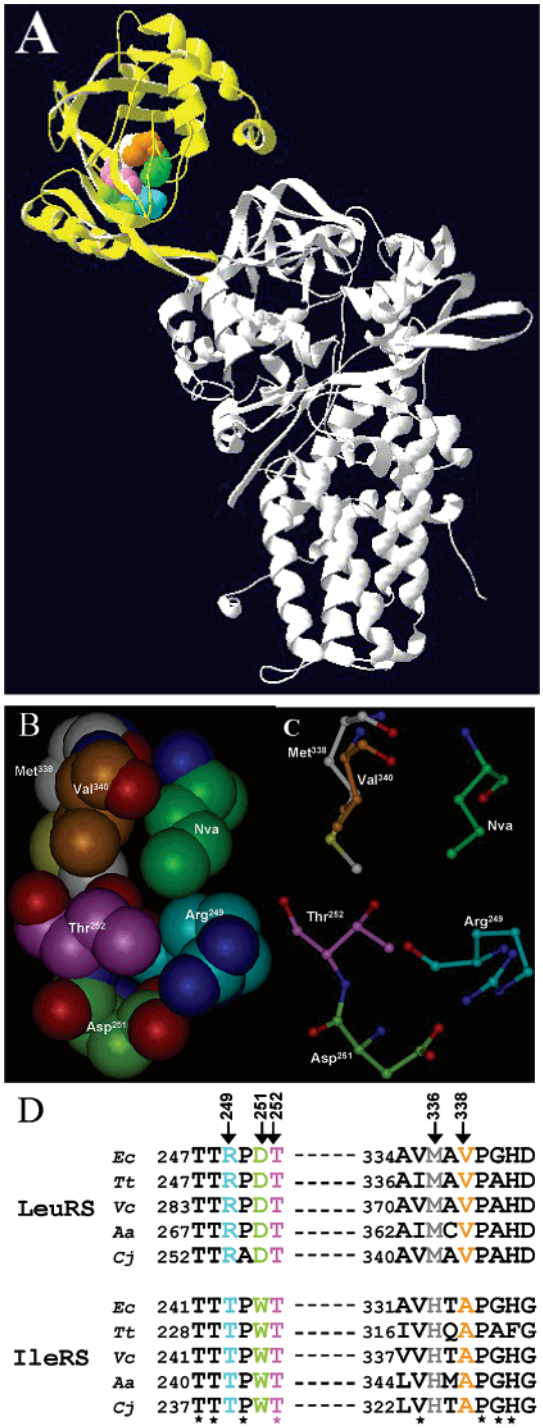

FIGURE 1: Tertiary and primary structure analysis of the amino acid editing site. (A) X-ray crystal structure of *T. thermophilus* LeuRS (*14*). The main body of the enzyme is colored in white, and the CP1 domain is yellow. The amino acid binding pocket of *T. thermophilus* LeuRS with norvaline (dark green) of the Nva2AA post-transfer editing analogue bound (*14*) is highlighted as a space-filling (B) and ball and stick model (C). The atoms are colored as follows: oxygen, red; sulfur, yellow; and nitrogen, blue; Arg^{249} carbons, light blue; Asp^{251} carbons, light green; Thr^{252} carbons, pink; Met^{338} (equivalent to Met^{336} in *E. coli* LeuRS) carbons, gray; and Val^{340} (equivalent to Val^{338} in *E. coli* LeuRS) carbons, orange. (D) Primary sequence alignment of LeuRS and IleRS amino acid editing site. The Arg^{249}, Asp^{251}, Thr^{252}, Met^{336}, and Val^{338} residues are colored in light blue, green, pink, gray, and orange respectively. Asterisks indicate residues that are highly conserved or homologous across both the LeuRS and IleRS enzymes. Abbreviations are as follows: Ec, *Escherichia coli*; Tt, *Thermus thermophilus*; Vc, *Vibrio cholerae*; Aa, *Aquifex aeolicus*; and Cj, *Campylobacter jejuni*.

HF Nickel Affinity Gel (Sigma, St. Louis, MO) (*21*). The final stock concentrations of proteins that were used in enzymatic assays were determined by a Bio-Rad Protein Assay as described in the commercial protocol.

Enzyme Assays. Aminoacylation reactions contained 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 4 mg/mL crude *E. coli* $tRNA^{Leu}$. The enzyme and amino acid concentrations are indicated in the appropriate figure legend. The reactions were initiated by adding 4 mM ATP. Aliquots for each reaction were quenched at specific time points and processed as previously described (*21*).

Post-transfer editing assays were carried out using *in vitro* transcribed *E. coli* $tRNA^{Leu}$ that was aminoacylated with [^{3}H]-isoleucine or [^{3}H]-leucine as described previously (*21*). Each reaction contained 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 0.8 μM Leu- or Ile-$tRNA^{Leu}$. Concentrations of Leu-$tRNA^{Leu}$ and Ile-$tRNA^{Leu}$ were varied from 0.1 to 1.0 μM to determine kinetic parameters. Enzyme concentrations were optimized for kinetic analysis of Ile-$tRNA^{Leu}$ hydrolysis as follows: WT, 5 nM; V338D, 10 nM; V338E, 15 nM; V338F, 1 μM; V338A, 10 nM; and V338L, 15 nM. In the case of Leu-$tRNA^{Leu}$ editing kinetic measurements, the enzyme concentrations were 25 nM wild type and 15 nM V338A mutant LeuRS: The apparent values for K_m and k_{cat} and their standard deviations were determined using the Enzyme Kinetics Module 1.1 of SigmaPlot software.

RESULTS

Mutational Analysis of the Editing Site's Amino Acid Binding Pocket. The X-ray crystal structures have been solved for LeuRS complexed to pre- and post-transfer editing analogues (*14*) as well as the tRNA bound in the editing complex (*23*, *24*). A universally conserved aspartic acid (Asp^{345} in *E. coli* LeuRS) as well as a highly conserved aspartic acid (Asp^{342} in *E. coli* LeuRS) that is three amino acids upstream interact and anchor the backbone of the amino acid and the ribose hydroxyl groups (*14*). In addition, two conserved threonine residues (Thr^{247} and Thr^{248} in *E. coli* LeuRS) also interact with the ribose hydroxyl groups and the covalent link to the amino acid (*21*). The side chains of Arg^{249}, Thr^{252}, Met^{336}, and Val^{338} line the amino acid binding pocket of the editing site and were hypothesized to confer specificity (*14*) (Figure 1). Previously, we determined that the Thr^{252} residue in *E. coli* LeuRS is a critical discriminator that blocks leucine from the editing site (*12*). Introduction of an alanine or serine uncouples specificity and allows hydrolysis of the Leu-$tRNA^{Leu}$ (*12*, *16*). Bulky mutations have also been substituted at the Thr^{252} site to fill in the amino acid binding pocket and block editing activity (*17*, *18*).

We combined the T252A mutation that uncouples specificity and scanned for effects on editing with bulky phenylalanine substitutions at Arg^{249}, Met^{336}, and Val^{338}. We hypothesized that introduction of phenylalanine at sites where the side chains actually lined the amino acid binding pocket would block substrate binding and impede overall editing activity to rescue the T252A phenotype. As shown in Figure 2, introduction of the large aromatic residue at Arg^{249} or Val^{338} rescued leucylation activity of the T252A mutation, suggesting that editing activity of Leu-$tRNA^{Leu}$ is decreased or abolished by R249F and V338F such that the robust

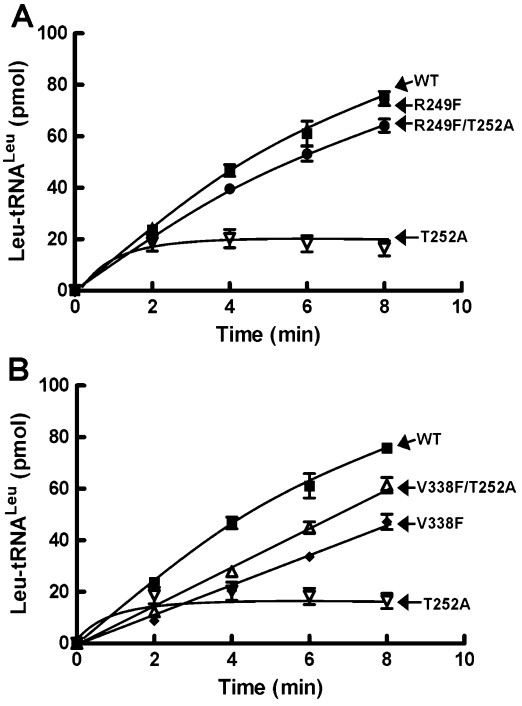

FIGURE 2: Leucylation of $tRNA^{Leu}$ by wild type and mutant enzymes. The Arg^{249} (A) and Val^{338} (B) single and double mutations with T252A are compared to the wild type enzyme and T252A LeuRS mutant. Each aminoacylation reaction was carried out in the presence of 4 mg/mL crude *E. coli* tRNA, 25.5 μM leucine (150 μCi/mL), and 500 nM enzyme. Symbols represent the following wild type and mutant LeuRSs: wild type (WT), ■; R249F,▲; V338F, ◆; R249F/T252A, ●; V338F/T252A, △; T252A, ▽. Error bars are the result of three reactions and indicated for each time point.

leucylation activity could now be detected. Substitution of Arg^{249} with an alanine shows little difference in leucine (*12*) or isoleucine specificity (data not shown). Similar to bulky mutations at the Thr^{252} position (*17*, *18*), we propose that the second phenylalanine mutation at Arg^{249} or Val^{338} fills in the amino acid binding pocket and prevents leucine and other amino acids from binding to the more open T252A amino acid binding pocket within the editing site and bars editing of Leu-$tRNA^{Leu}$.

The M336F/T252A double LeuRS mutant exhibited only slightly increased leucylation activity relative to the T252A single mutation (data not shown). A single M336A mutant enzyme did show a small increase in leucine editing activity, suggesting that it can enlarge the amino acid binding pocket to accommodate the correctly charged leucine. These combined results suggest that the conserved Met^{336} plays a relatively subtle role in amino acid discrimination compared to Thr^{252}, Arg^{249}, and Val^{338}.

Val^{338} Is Secondary to Thr^{252} as a Fine Discriminator of Leucine. Val^{338} has been proposed to aid Thr^{252} in blocking leucine from the editing site by directly clashing with one of the γ-methyl groups of leucine (*14*). Computational modeling also suggested that Val^{338} might play a role in maintaining the geometry of the amino acid editing site to confer specificity and activity (*16*). We further analyzed Val^{338} by introducing a series of single mutations that included V338D, V338E, V338F, V338A, and V338L and tested each of these mutants for aminoacylation activity. Only the V338A mutant LeuRS appeared to affect leucylation

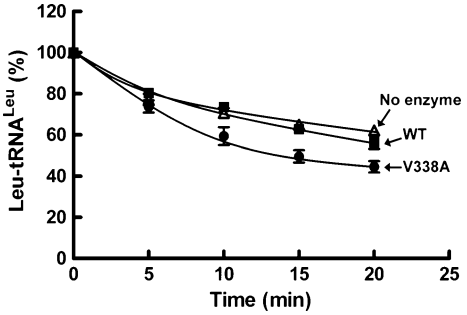

FIGURE 3: Wild type and V338 mutant LeuRS editing activity of Leu-tRNA^{Leu}. Each editing reaction was carried out in the presence of about 0.2 μM crude Leu-tRNA^{Leu} and 10 nM enzyme. Symbols are represented as follows: WT, ■; V338A,●; and no enzyme, △. Error bars are the result of three reactions and indicated for each time point.

Table 1: Apparent Kinetic Constants of Wild Type and Mutant Enzymes for Post-Transfer Editing Activity of Leu-tRNA^{Leu} [a]

constants	WT	V338A
K_M (μM)	0.7 ± 0.4	0.9 ± 0.3
k_{cat} (s^{-1})	0.12 ± 0.04	0.4 ± 0.07
k_{cat}/K_M (μM^{-1} s^{-1})	0.2	0.4

[a] These kinetic measurements are apparent values.

activity of crude tRNA^{Leu} (see Supporting Information), although it did not decrease yields of Leu-tRNA^{Leu} to the level generated by the T252A mutant LeuRS. We measured the editing activity of Leu-tRNA^{Leu} of the V338A LeuRS mutant and determined that there was only a very small 2-fold increase of hydrolysis of the correctly charged Leu-tRNA^{Leu}, compared to wild type LeuRS (Figure 3). Previously, we have shown that a much greater threshold of editing must occur to correct mischarging activity (*17*). The apparent k_{cat}/K_M of the wild type and V338A LeuRSs using Leu-tRNA^{Leu} as a substrate were 0.2 μM^{-1} s^{-1} and 0.4 μM^{-1} s^{-1} respectively (Table 1). Compared to a 24-fold increase in hydrolytic activity for the T252A mutant LeuRS (*16*), we propose that the significance of the Val^{338} site's role in blocking leucine from the editing active site is much less, if at all, significant.

Each of the Val^{338} single mutant LeuRSs was also tested for mischarging activity. As found for the V338F/T252A double mutant, the single introduction of the bulky phenylalanine residue nearly abolished post-transfer editing activity and facilitated mischarging of both isoleucine and valine to tRNA^{Leu} (Figure 4). The apparent k_{cat}/K_M of the V338F mutant LeuRS was reduced more than 2000-fold to 0.001 μM^{-1} s^{-1} (Table 2), while the apparent k_{cat}/K_M of the T252F LeuRS mutant was reduced only 20-fold to 0.1 μM^{-1} s^{-1} (*16*). Our combined results with the V338A and T252A mutant enzymes showed a 2- (Table 1) and 24-fold (*16*) decrease respectively in the apparent k_{cat}/K_M of Leu-tRNA^{Leu} hydrolysis in the editing site. Thus, we hypothesize that the primary role of Thr^{252} is to block cognate leucine from binding in the editing site to prevent hydrolysis (*12*), while Val^{338} is more important in accommodating the side chains of the targeted amino acids, in general, for editing. Similar to the Thr^{252} site (*16*), other mutations at Val^{338} can serve as adequate substitutes. However, the high conservation at both of these positions strongly suggests that threonine at the 252 position and valine at the 338 position are biologically optimal.

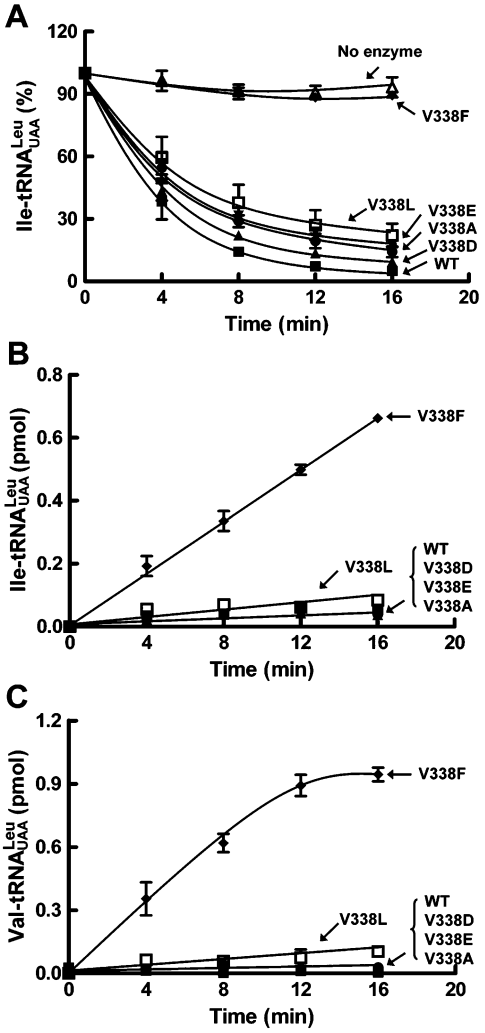

FIGURE 4: Wild type and V338 mutant LeuRS editing and mischarging activity. (A) Editing of Ile-tRNA^{Leu}. Each editing reaction was carried out in the presence of about 0.2 μM *in vitro* transcribed [^{3}H]-Ile-tRNA^{Leu} and 10 nM enzyme. (B) Mischarging of isoleucine to *in vitro* transcribed tRNA^{Leu}. Each reaction was carried out in the presence of 4 μM *in vitro* transcribed tRNA^{Leu}, 20 μM isoleucine (150 μCi/mL), and 0.7 μM enzyme. (C) Mischarging of valine to tRNA^{Leu}. Each reaction was carried out in the presence of 4 μM *in vitro* transcribed tRNA^{Leu}, 20 μM valine (150 μCi/mL), and 5 μM enzyme. Symbols are represented as follows: WT, ■; V338D, ▲; V338E, ▼; V338F, ◆; V338A, ●; and V338L, □.

Adaptation of Amino Acid Specificity in Coevolved Editing Sites. The conserved Arg^{249} is located within the threonine-rich region (T^{247}TRP(D/E)T) of the editing site of *E. coli* LeuRS. Comparison to the *E. coli* IleRS threonine-rich region (T^{241}TTPWT) shows that this site has diverged in an idiosyncratic way and is conserved as Thr^{243} (Figure 1D). A neighboring site also exhibits enzyme-specific divergence where an acidic residue in LeuRS is conserved as a tryptophan in IleRS. In the *T. thermophilus* crystal structure, the Asp^{251} and Arg^{249} are located within 6.3 Å and 3.5 Å respectively of the bound amino acid side chain (Figure 5). We hypothesized that this divergence might be important to conferring substrate specificity within the editing sites of LeuRS and IleRS.

Table 2: Apparent Kinetic Constants of Wild Type and Mutant Enzymes for Post-Transfer Editing Activity of Ile-tRNA^{Leu} [a]

constants	WT	V338D	V338E	V338L	V338A	V338F
K_M (μM)	0.13 ± 0.06	0.2 ± 0.08	0.2 ± 0.03	0.09 ± 0.01	0.2 ± 0.02	0.09 ± 0.001
k_{cat} (s^{-1})	0.4 ± 0.07	0.3 ± 0.06	0.2 ± 0.01	0.09 ± 0.003	0.5 ± 0.02	$1.19 \times 10^{-4} \pm 0.04 \times 10^{-4}$
k_{cat}/K_M (μM^{-1} s^{-1})	3.0	1.4	1.0	1.0	2.5	0.001

[a] These measured kinetic parameters are apparent values.

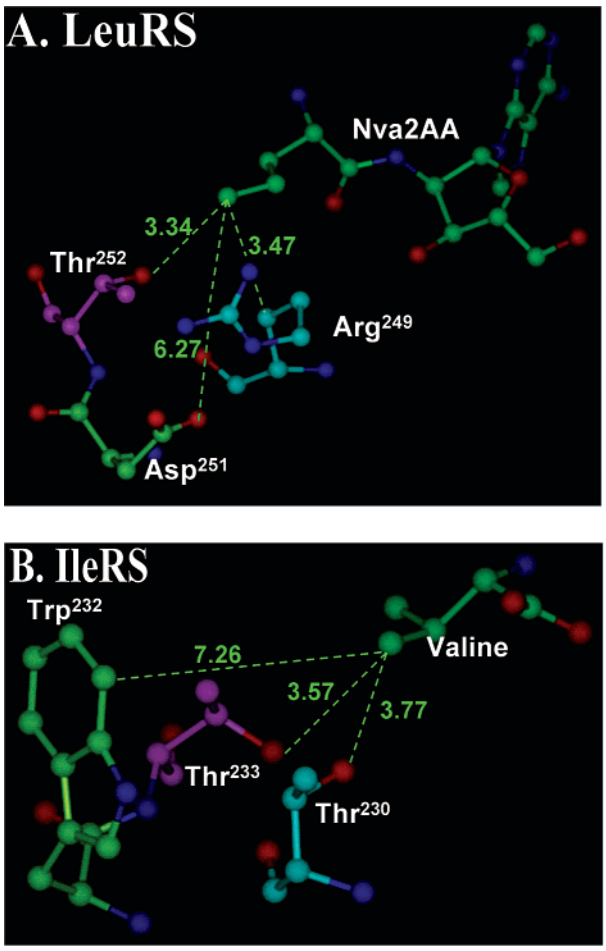

FIGURE 5: Structural analysis of the threonine-rich region within the LeuRS and IleRS CP1 domain editing site. (A) Editing site of *T. thermophilus* LeuRS. The carbon atoms are colored as follows: Arg^{249}, light blue; Asp^{251}, green; Thr^{252}, pink; and Nva2AA, green. Oxygen and nitrogen atoms are colored in red and blue respectively. The shortest distance between each residue and the norvaline ligand is marked in a dashed green line measured in angstroms. (B) Editing site of *T. thermophilus* IleRS. The carbon atoms are colored as follows: valine substrate, green; Thr^{230}, light blue; Trp^{232}, yellow green; and Thr^{233}, pink. Oxygen and nitrogen atoms are colored in red and blue respectively. The shortest distance (Å) between each residue and the valine ligand is marked by a dashed green line. This model was based on the X-ray crystal structure published by Nureki et al. (8).

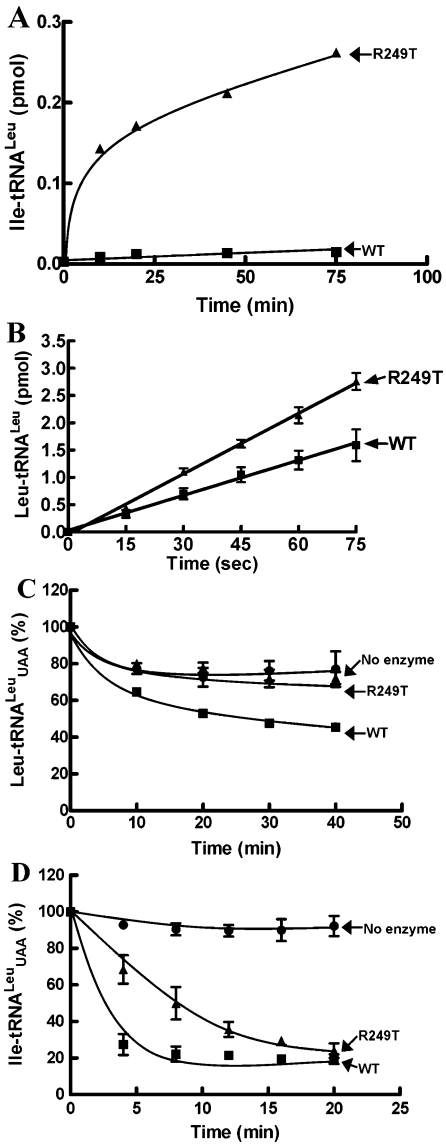

FIGURE 6: Aminoacylation and editing activities of wild type and the R249T mutant LeuRS. (A) Isoleucine misaminoacylation. Reactions are carried out in the presence of 1.7 μM [^{3}H]-isoleucine (150 μCi/mL), 4 mg/mL crude tRNA^{Leu}, and 5 μM enzyme at room temperature. (B) Initial leucylation velocities. Reactions are carried out in the presence of 21.8 μM [^{3}H]-leucine (166 μCi/mL), 2 mg/mL crude *E. coli* tRNA^{Leu}, and 10 nM enzyme at 30 °C. (C) Hydrolytic editing of *in vitro* transcribed Leu-tRNA^{Leu}. Reactions are carried out in the presence of 0.8 μM *in vitro* transcribed [^{3}H]-Leu-tRNA^{Leu} and 10 nM enzyme at room temperature. (D) Hydrolytic editing of *in vitro* transcribed Ile-tRNA^{Leu}. Reactions are carried out in the presence of 0.8 μM *in vitro* transcribed [^{3}H]-Ile-tRNA^{Leu} and 10 nM enzyme at room temperature. The enzymes are represented by symbols as follows: wild type, ■; R249T, ▲; no enzyme, ●. The error bars are based on reactions that were repeated at least in triplicate and are present for each point, although nominal in some cases.

In *E. coli* LeuRS, Arg^{249} corresponds to a threonine in IleRS and Asp^{251} is a tryptophan in IleRS. We swapped Arg^{249} and Asp^{251} in LeuRS with threonine (R249T) and tryptophan (D251W) respectively. We also created a double mutant (R249T/D251W) that incorporated both mutations into LeuRS. Mischarging activity for isoleucine was tested to identify mutation-dependent editing defects. The R249T mutant LeuRS (Figure 6A) and the double mutant (data not shown) displayed higher mischarging activities for isoleucine, while the D251W mutant LeuRS retained its fidelity similar to wild type LeuRS (data not shown). The leucylation activities were similar for each of the mutants (See Sup-

porting Information.) The initial velocities for the mischarging R249T mutant LeuRS are shown in Figure 6B. These results suggest that the substituted threonine in LeuRS blocks isoleucine from the editing site. It is possible that this site in IleRS plays a role in blocking cognate isoleucine from being hydrolyzed from correctly charged Ile-tRNA^{Ile}, similar to the role of Thr^{252} in LeuRS editing specificity.

Charged and mischarged *in vitro* transcribed tRNA^{Leu}, which was aminoacylated respectively with cognate leucine and noncognate isoleucine, was isolated and used to test the wild type and mutant LeuRSs' post-transfer editing activities. Over a long incubation period, none of the mutants hydrolyzed correctly charged Leu-tRNA^{Leu} at levels greater than the wild type LeuRS (Figure 6C and data not shown). As would be expected, the low-fidelity R249T single mutant (Figure 6D) and R249T/D251W double mutant LeuRSs showed decreased hydrolysis of mischarged Ile-tRNA^{Leu} compared to the wild type enzyme. This weakened editing activity facilitates stable formation of mischarged Ile-tRNA^{Leu}.

DISCUSSION

The homologous CP1 domains of LeuRS, IleRS, and ValRS are proposed to have arisen from the same common ancestor (*25*). As protein synthesis became more complex and required higher fidelity, evolutionary pressures added hydrolytic editing activities and successively crafted unique specificities for LeuRS, IleRS, and ValRS. Thus, remnants of the ancestral active site were likely retained while idiosyncratic features were introduced to confer or enhance specificity. Since these three aaRSs share similar editing mechanisms, residues that are involved in catalysis or bind the common part of the editing substrate would be expected to be conserved across LeuRS, IleRS, and ValRS. This includes, for example, a universally conserved aspartic acid that anchors the common amino moiety of the amino acid of the bound editing substrate (*13*, *14*). In addition, a second aspartic acid that is shared by ValRS and bacterial LeuRSs interacts with the central region that includes the ribose and amino acid backbone of the bound editing substrate (*14*, *15*). However, because of differences in the side chains of leucine, isoleucine, and valine as well as the idiosyncratic challenges produced by different sets of targeted noncognate amino acids, the respective amino acid binding pockets would require divergence to provide appropriate substrate specificities within each of the LeuRS, IleRS, and ValRS editing sites.

Our mutational work shows that swapping the conserved arginine residue from LeuRS to a conserved threonine that is found at the corresponding position of IleRS altered specificity. The R249T LeuRS mutant's decrease in editing activity of Ile-tRNA^{Leu} suggests that the binding of isoleucine might be partially blocked. The X-ray crystal structure of LeuRS bound to the unbranched norvalyl-based editing substrate analogues (*14*) shows that the β-methylene group of Arg^{249} is 3.47 Å (Figure 5A) from the γ-methyl group of norvaline in the amino acid binding pocket of the editing site. This β-position of arginine in LeuRS would correspond with the branched site of the conserved threonine side chain in IleRS. The cocrystal structure of *T. thermophilus* IleRS CP1 domain with a valine ligand (*26*) also shows that the distance between the hydroxyl group of Thr^{230} (equivalent to Thr^{243} of *E. coli* IleRS) and one of the branched methyl groups of the valine's side chain is 3.57 Å (Figure 5B). Thus, we hypothesized that the conserved threonine in the IleRS editing site helps block isoleucine to prevent hydrolysis of the correctly charged Ile-tRNA^{Ile} during the editing reaction, which primarily targets misactivated and mischarged valine. Indeed, it has been published recently that the *T. thermophilus* IleRS Thr^{230} blocks isoleucine from binding to the editing site (*27*). An alanine substitution at this site leads to rapid hydrolysis of the cognate Ile-tRNA^{Ile}. This suggests that the LeuRS and IleRS editing sites have diverged at this particular residue to confer specificity of their respective substrates.

Interestingly, a reverse mutation in *E. coli* IleRS at this site (T243R), which substitutes an arginine found at that site in LeuRS for the conserved threonine, also disrupts editing to a level that causes growth defects (*28*). *In vitro* analysis showed that valine was mischarged to tRNA^{Ile} due to the decrease in the dominant pre-transfer editing mechanism. Since the pre- and post-transfer editing activities appear to share an amino acid binding pocket in the editing site, it is likely that the arginine side chain blocks amino acid binding of the misactivated adenylate in the editing site pocket to decrease fidelity.

Mutation of the second idiosyncratic residue, LeuRS Asp^{251}, failed to significantly alter the enzyme's editing activities. The X-ray cocrystal structure of *T. thermophilus* LeuRS with a post-transfer editing substrate analogue (*14*) shows that the side chain oxygens of Asp^{251} are distant from the γ-methyl group of norvaline (6.27 Å and 7.14 Å respectively) and thus would likely not interact directly with the amino acid side chain (Figure 5). Likewise, the corresponding Trp^{232} of *T. thermophilus* IleRS (equivalent to Trp^{245} of *E. coli* IleRS) is 7.26 Å from the side chain of the bound valine (*26*). Interestingly, a W245E mutant in *E. coli* IleRS abolished editing activity (*29*). This is consistent with an earlier structure (*8*), in which the conserved Trp^{232} and Tyr^{386} formed an editing pocket that was proposed to be sufficient for valine binding, but too small for isoleucine. Changing the tryptophan to a charged residue was proposed to disrupt the hydrophobic pocket resulting in a loss of editing.

A nearly completely conserved threonine (Thr^{252} in *E. coli* LeuRS) is important to specificity in LeuRS (*12*, *16*, *17*). The side chain clashes with the γ-methyl branch of the leucine side chain to block it from binding (*14*). Mutation to an alanine uncouples specificity and facilitates hydrolysis of the correctly charged Leu-tRNA^{Leu}. The corresponding mutant in IleRS, where the completely conserved threonine is changed to alanine, did not show any alteration in editing activity (*29*). This would be expected because the isoleucine and valine side chains lack a γ-methyl branch. Thus, the conserved threonine residue in IleRS might represent a remnant of the divergence of IleRS and LeuRS. It also may have functionally diverged to play a more specific structural role in IleRS.

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SUPPORTING INFORMATION AVAILABLE

Figures depicting leucylation activity and leucine aminoacylation of $tRNA^{Leu}$ by wild type and mutant LeuRSs. This material is available free of charge via the Internet at http://pubs.acs.org.

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