A Conserved Threonine within *Escherichia coli* Leucyl-tRNA Synthetase Prevents Hydrolytic Editing of Leucyl-tRNA^{Leu} †

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ABSTRACT: Aminoacyl-tRNA synthetases ensure the fidelity of protein synthesis by accurately selecting and activating cognate amino acids for aminoacylation of the correct tRNA. Some tRNA synthetases have evolved an editing active site that is separate from the amino acid activation site providing two steps or "sieves" for amino acid selection. These two sieves rely on different strategies for amino acid recognition to significantly enhance the accuracy of aminoacylation. We have performed alanine scanning mutagenesis in a conserved threonine-rich region of the *Escherichia coli* leucyl-tRNA synthetase's CP1 domain that is hypothesized to contain a putative editing active site. Characterization of purified mutant proteins led to the identification of a single conserved threonine that prevents the cognate leucine amino acid from being hydrolyzed after aminoacylation of the tRNA. Mutation of this threonine to an alanine eliminates discrimination of the cognate amino acid in the editing active site. This provides a molecular example of an amino acid discrimination mechanism in the tRNA synthetase's editing active site.

The fidelity of protein synthesis is highly dependent upon the accurate aminoacylation of an amino acid to the correct tRNA (*1*, *2*). Each of the 20 standard amino acids is specifically recognized and activated by a single member of a family of enzymes called the aminoacyl-tRNA synthetases (aaRSs).[1] The aaRSs form an adenylate intermediate and covalently link or "charge" the amino acid to its cognate tRNA in the following two-step reaction:

$$\text{amino acid (AA)} + \text{ATP} \underset{\text{synthetase}}{\overset{\text{tRNA}}{\rightleftharpoons}} \text{AA-AMP} + \text{PP}_i \quad (1)$$

$$\text{AA-AMP} + \text{tRNA} \xrightarrow[\text{synthetase}]{\text{tRNA}} \text{tRNA-AA} + \text{AMP} \quad (2)$$

The barriers of enzyme recognition for isosteric amino acid substrates, such as isoleucine and valine that differ by only a single methyl moiety, were first noted by Pauling (*3*). Theoretical calculations predicted that valine would be misincorporated for 1 out of 5 isoleucine sites within a protein. However, actual misincorporation was measured to be about 1 in 3000 (*4*). Subsequently, it was shown that isoleucyl-tRNA synthetase (IleRS) misactivates valine but also has an editing mechanism to hydrolyze incorrectly activated and/or charged noncognate amino acids (*5*, *6*).

IleRS and other aaRSs operate by a "double sieve" model that contains separate amino acid activation and editing sites (*7*, *8*). These distinct sieves or active sites have varied specificities. The first "coarse" sieve activates cognate amino acids and also, to a lesser extent, isosteric noncognate amino acids that are typically smaller and fit into the cognate amino acid binding pocket. The second "fine" sieve excludes the cognate amino acid but hydrolyzes or edits misactivated amino acids and/or mischarged tRNAs.

Indeed, separate aminoacyl and editing sites have been identified for IleRS (*9*−*13*) and valyl-tRNA synthetase (ValRS) (*13*, *14*). The synthetic active site is located in an ATP-binding Rossmann fold that is common to all class I aaRSs (*15*). The second sieve lies within a large inserted domain (∼200 amino acids) called connective polypeptide 1 (CP1) that interrupts the Rossmann fold (*9*, *10*, *12*−*14*, *16*). The CP1 domains of IleRS and ValRS have been isolated and determined to specifically and respectively deacylate mischarged Val-tRNA^{Ile} and Thr-tRNA^{Val} (*13*).

The CP1 domain of leucyl-tRNA synthetase (LeuRS) shares extensive homology with the CP1 domains of IleRS and ValRS (Figure 1). Likewise, LeuRS has also been shown to misactivate and edit a series of amino acid derivatives (*17*−*20*). In *Escherichia coli* LeuRS, insertion duplication of a 40 amino acid region within the CP1 domain yielded mischarged Ile- and Met-tRNA^{Leu} (*17*). This insertion presumably disrupted a CP1-based editing active site in LeuRS.

Using alanine scanning mutagenesis, we targeted a conserved threonine-rich region, T_{247}TRPDT$_{252}$ (Figure 1), within the CP1 domain of *E. coli* LeuRS to probe amino acid recognition and editing activity of its CP1-based editing active site. A single mutation was identified in the editing active site that altered amino acid discrimination. Specifically, mutation of threonine 252 to an alanine (T252A)[2] eliminated discrimination of Leu-tRNA^{Leu}, resulting in hydrolysis of its correctly charged cognate product. We

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; CP1, connective polypeptide 1; PP_i, inorganic pyrophosphate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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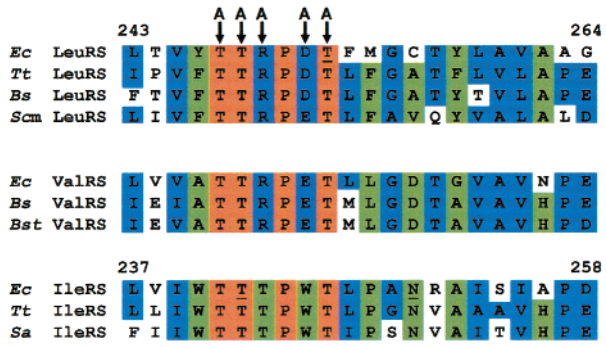

FIGURE 1: Primary sequence alignment of the conserved editing region within the CP1 domain of LeuRS, ValRS, and IleRS. Ten representative sequences are shown. Residues highlighted in red are completely conserved within LeuRS and with one exception (R249) with ValRS and IleRS. Those shaded in blue are homologous within LeuRS and in most cases with ValRS and IleRS. Amino acids highlighted in green are homologous but only to a particular aaRS. Arrows identify sites in *E. coli* LeuRS that were mutated via alanine scanning mutagenesis. Underlined letters in the sequences of *E. coli* IleRS and *E. coli* LeuRS refer to residues that affect editing activity when mutated. Abbreviations: Ec, *E. coli*; Tt, *T. thermophilus*; Bs, *B. subtilis*; Scm, *S. cerevisiae* (mitochondrial); Sa, *S. aureus*; Bst, *B. stearothermophilus*. Sequence alignments were generated using the Baylor College of Medicine Search Launcher ClustalW 1.8 Global progressive alignment program (*29*).

hypothesize that this conserved threonine residue blocks leucine from entering and/or binding efficiently to the amino acid binding pocket of the editing active site.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were purchased from Sigma-Genosys (Woodlands, TX). Radiolabeled amino acids and ^{32}P-labeled compounds were acquired respectively from Amersham Pharmacia Biotech (Piscataway, NJ) and NEN Life Science Products, Inc. (Boston, MA). Restriction enzymes were purchased from Promega (Madison, WI) except for *Bst*NI, which was from New England BioLabs, Inc. (Beverly, MA).

Protein Preparation. The *E. coli leu*S gene cloned into a pET-15 vector (Novagen, Madison, WI) was overexpressed and purified as described previously (*19*). Purified protein concentrations were determined via the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Mutagenesis of the LeuRS gene was carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing.

Preparation of tRNA^{Leu} and [^{3}H]-Leu-tRNA^{Leu}. The ptDNA^{leu} plasmid (*21*) was provided by the laboratory of Dr. J. Abelson (California Institute of Technology, Pasadena). *E. coli* tRNA$^{Leu}_{UAA}$ was synthesized by T7 RNA polymerase runoff transcription using *Bst*NI-digested ptDNA^{leu} (*22*). The tRNA was purified on a 16% polyacrylamide (19:1), 8 M urea denaturing gel via electrophoresis.

Purified tRNA^{Leu} was denatured at 70 °C for 1 min, followed by addition of 1 mM $MgCl_2$ and quick-cooling on ice. Folded tRNA^{Leu} was aminoacylated with [^{3}H]-leucine in a reaction containing 60 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 10 mM $MgCl_2$, 4 mM ATP, 1 mM dithiothreitol (DTT), 7 μM tRNA^{Leu}, 20.5 μM [^{3}H]-leucine (200 μCi/mL), and 40 nM LeuRS for 30 min at 37 °C. The pH was adjusted to 5.0 using acetic acid (0.001% v/v) (*23*) and the charged RNA immediately extracted using phenol/chloroform/isoamyl alcohol (25:24:1) (pH 5.1) (Fisher Biotech, Fair Lawn, NJ), followed by ethanol precipitation, resuspension in 50 mM KH_2PO_4 (pH 5.0), and storage at −20 °C.

Aminoacylation Activity Assay. The aminoacylation activities of wild-type and mutant LeuRSs were measured at 25 °C similar to that described previously and above except 15 nM enzyme and 4 μM tRNA were incorporated (*19*). The reactions were initiated by the addition of ATP. To test for methionine or isoleucine mischarging, 100 nM enzyme was introduced as well as 20−80 μM [^{35}S]-methionine (370 μCi/mL) or 20 μM [^{3}H]-isoleucine (200 μCi/mL).

Hydrolytic Editing Assay. The hydrolytic activity of *E. coli* LeuRS was measured using a reaction mixture consisting of 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, and [^{3}H]-Leu-tRNA^{Leu} (∼100 μCi/mL) (*12*, *23*). The reaction was initiated by the addition of 100 nM enzyme. At selected time intervals, reaction aliquots were quenched on pads prewetted with 5% trichloroacetic acid, washed, and quantitated by scintillation counting as described previously (*19*).

Inorganic Pyrophosphate (PP$_i$) Exchange Assay. The reaction mixture consisted of 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mM [^{32}P]-PP$_i$ (2.5 μCi/ml), and 1 mM leucine and was initiated by the addition of 50 nM enzyme (*20*). Aliquots were quenched on polyethylenimine thin-layer chromatography (TLC) plates (Scientific Adsorbents Inc., Atlanta) that had been prerun in water. The TLC plate was developed in 750 mM KH_2PO_4, pH 3.5, and 4 M urea, at 25 °C. Separated radiolabeled bands were detected and quantified by phosphorimaging using a FUJIX BAS 1000 (FUJIFILM Medical Systems U.S.A., Inc.).

RESULTS

Mutational Analysis of the E. coli LeuRS Editing Active Site. The hydrolytic amino acid editing site of LeuRS, IleRS, and ValRS is located in the inserted CP1 domain that splits the Rossmann fold in half (*9*, *10*, *12*−*14*, *16*, *17*, *24*, *25*). Within the CP1 domain, a threonine-rich region is conserved among all three synthetases (Figure 1) and has been shown to be important to editing activity in IleRS (*9*, *12*). Crystal structures of *Thermus thermophilus* LeuRS, *T. thermophilus* ValRS, *T. thermophilus* IleRS, and *Staphylococcus aureus* IleRS exhibit structural homology within this region (*9*, *10*, *14*, *25*), which suggests an overlap in hydrolytic editing mechanisms (*12*). To identify key editing determinants, we performed alanine scanning mutagenesis within this threonine-rich region of *E. coli* LeuRS.

Five *E. coli* LeuRS mutants,[2] T247A, T248A, R249A, D251A, and T252A (shown via arrows in Figure 1), were expressed with a fused N-terminal six-histidine tag and purified by affinity chromatography. Each mutant was tested for alterations in either or both steps of the aminoacylation reaction (eqs 1 and 2). Leucylation activities of LeuRS mutants T247A, T248A, and R249A were similar to that measured for the wild-type enzyme (Figure 2). The T252A

[2] Mutations in which the X residue at position ### is substituted by a Y amino acid are labeled X###Y. For example, T252A contains an alanine substituted for the wild-type threonine residue at position 252.

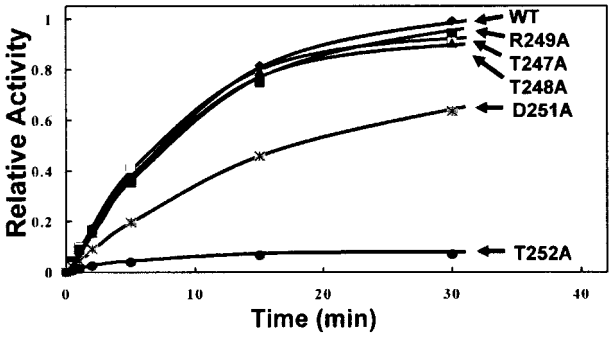

FIGURE 2: Aminoacylation of $tRNA^{Leu}$ with leucine. Each reaction was repeated at least three times and normalized relative to the wild-type enzyme's activity. Symbols represent aminoacylation activity by wild-type and mutant LeuRSs as follows: wild-type (WT), closed diamond; R249A, closed square; T247A, open square; T248A, closed triangle; D251A, shaded asterisk; T252A, closed circle. Activities for different preparations of the D251A mutant LeuRS varied as described in the text but were consistently decreased by up to 70% compared to wild-type LeuRS. One representative activity assay for the D251A mutant protein is shown.

LeuRS mutant was also expressed stably but appeared to be totally defective in charging tRNA.

Multiple preparations of the D251A mutant LeuRS resulted in varied, but generally lower yields of purified protein, suggesting that this mutant protein may be unstable. Its aminoacylation activity was decreased and ranged depending on the mutant protein preparation from about 30% to 60% of wild-type charging activity. The D251A mutant LeuRS's charging activity also reached a plateau for $tRNA^{Leu}$ aminoacylation that was decreased by 35% compared to the wild-type enzyme (data not shown). It is possible that the D251A mutant's instability resulted in lower enzymatic activity; however, the precise reason for its decreased aminoacylation rates remains unclear.

Characterization of the LeuRS T252A Defect. The T252A LeuRS mutant was further investigated to determine if leucine is activated in the first step of the aminoacylation reaction (eq 1). We incorporated [^{32}P]-PP_i into the reversible adenylation reaction and monitored amino acid-dependent PP_i exchange activity by the wild-type and mutant LeuRSs. Reactants and products labeled with ^{32}P were separated on TLC plates as described in Experimental Procedures and are shown in Figure 3A. The T252A LeuRS mutant exhibited ~68% PP_i exchange activity compared to the wild-type enzyme. Thus, the T252A LeuRS mutant that appears to be deficient in aminoacylation activity activates leucine. It is possible that the T252A LeuRS cannot transfer the activated amino acid to $tRNA^{Leu}$. Alternatively, since the T252A mutation is near the putative editing site, we hypothesized that the cognate amino acid is transferred to the tRNA and, upon translocation to the enzyme's editing active site, is subsequently hydrolyzed. While it is also plausible that the leucyladenylate intermediate could be translocated to the editing site, previous work suggested that *E. coli* LeuRS lacks a pretransfer editing activity (*18*).

To test our hypothesis that the mutant was editing tRNA charged correctly with cognate leucine, [^{3}H]-Leu-$tRNA^{Leu}$ was incubated with both wild-type LeuRS and the T252A LeuRS mutant. In the presence of wild-type LeuRS, Leu-$tRNA^{Leu}$ was hydrolyzed at low levels that are similar to a control reaction without enzyme present (Figure 4). In

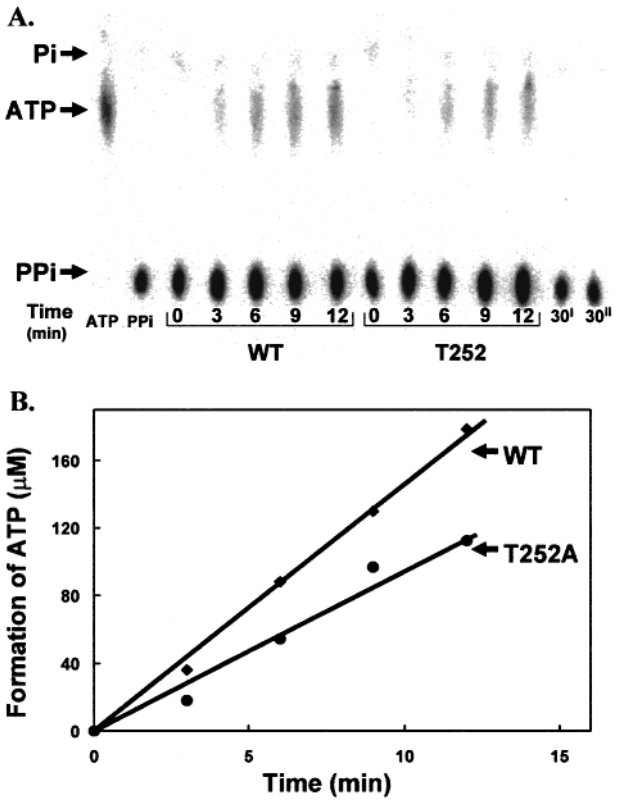

FIGURE 3: Leucine-dependent PP_i exchange activity for wild-type and T252A LeuRS. (A) Aliquots of 2 μL were quenched directly on the TLC plate at specific time points as indicated. ATP was chromatographically separated from PP_i [and background phosphate (P_i)] and analyzed by phosphorimaging (*19*). The two lanes on the right are 30 min control reactions in which amino acid was omitted from the reaction mixture (i, wild-type; ii, T252A). (B) Phosphorimaged data were converted quantitatively to determine ATP formation. Symbols: wild-type (WT) LeuRS, closed diamond; T252A LeuRS, closed circle.

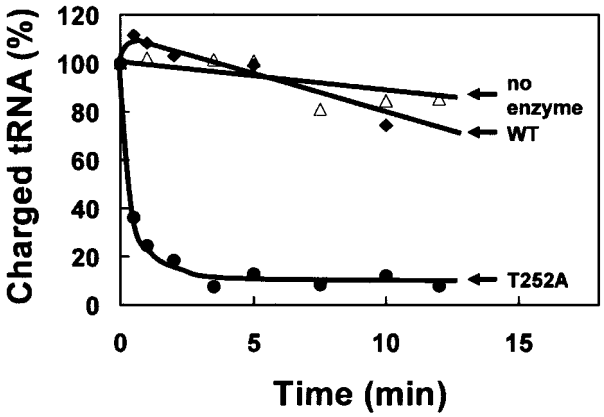

FIGURE 4: Deacylation of Leu-$tRNA^{Leu}$ by wild-type and T252A LeuRS. Symbols: wild-type (WT) LeuRS, closed diamond; T252A LeuRS, closed circle; no enzyme, open triangle.

contrast, rapid deacylation of the cognate amino acid from $tRNA^{Leu}$ was induced by the T252A LeuRS mutant. T247A, T248A, R249A, and D251A LeuRS mutant enzymes were also tested and have tRNA hydrolysis rates comparable to that of the wild-type enzyme (data not shown).

Previously, we and others determined that LeuRS misactivates a wide range of noncognate amino acids (*17*−*20*). We tested the T252A LeuRS mutant to determine if it could stably aminoacylate [^{3}H]-isoleucine and/or [^{35}S]-methionine

to $tRNA^{Leu}$. Despite repeated attempts, we were unable to detect significant levels of mischarged $tRNA^{Leu}$, suggesting that the editing active site targeting noncognate amino acids remained intact.

These results clearly show that the T252A mutation alters amino acid discrimination in the *E. coli* LeuRS editing active site. We propose that the conserved T252 residue is a critical determinant of the LeuRS editing active site that blocks charged cognate amino acid from binding and subsequent hydrolysis from the charged tRNA. Thus, the conserved threonine-rich region within *E. coli* LeuRS contains molecular discriminants that are important to recognition by the second fine sieve of the aaRS double sieve mechanism of fidelity (*7*, *8*).

DISCUSSION

The exquisite ability of some aaRSs to accurately discriminate closely related amino acids is dependent on substrate recognition by a double sieve model (*7*, *8*). Each of the two sieves function by different but complementary amino acid recognition mechanisms to confer high specificity. The first coarse sieve is the canonical tRNA synthetase active site and is responsible for activating and aminoacylating cognate amino acids. However, noncognate isosteric amino acids that fit into the synthetic amino acid binding pocket may also be misactivated. The hydrolytic active site is comprised of a fine sieve that is largely designed to block binding of the cognate amino acid. Concurrently, this editing active site targets misactivated and/or mischarged amino acids. Thus the first sieve is conformed to specifically bind the cognate amino acid, but the second fine sieve must be highly evolved to exclude the cognate amino acid.

The role of amino acid discrimination within the editing active sites of IleRS and ValRS is clear. The editing activity of IleRS targets misactivated or mischarged valine, which differs from isoleucine by a single methyl group (*5*). ValRS hydrolyzes mischarged threonine that has a hydroxyl moiety that is isosteric with the branched methyl group in valine (*26*, *27*). In each of these examples the editing domain has likely evolved to bind a single very specific noncognate amino acid that is highly isosteric to the cognate amino acid.

LeuRS also exhibits an editing activity that has been localized to the conserved CP1 domain (*17*). However, it is not clear which noncognate amino acid threatens the fidelity of LeuRS activity since none of the standard amino acids differ from leucine, for example, by as little as an isosteric methyl or hydroxyl group. Since LeuRS misactivates numerous noncognate amino acids (*17*−*20*, *28*), we hypothesize that the editing active site of LeuRS may broadly interact with different amino acids, for example, those containing straight-chained side chains as well as smaller branched and unbranched amino acids. These include methionine and isoleucine that are mischarged to $tRNA^{Leu}$ when the CP1 domain is dramatically disrupted by insertion mutagenesis (*17*). Therefore, the LeuRS editing active site requires flexibility to accommodate an array of mischarged amino acids but, of course, must impede leucine binding.

We have identified a single mutation within a conserved threonine-rich editing region that uncouples the fine amino acid discrimination of *E. coli* LeuRS. Substitution of threonine at position 252 by an alanine (T252A) yields a LeuRS mutant that appears to hydrolyze the correctly charged $tRNA^{Leu}$ in a futile cycle.

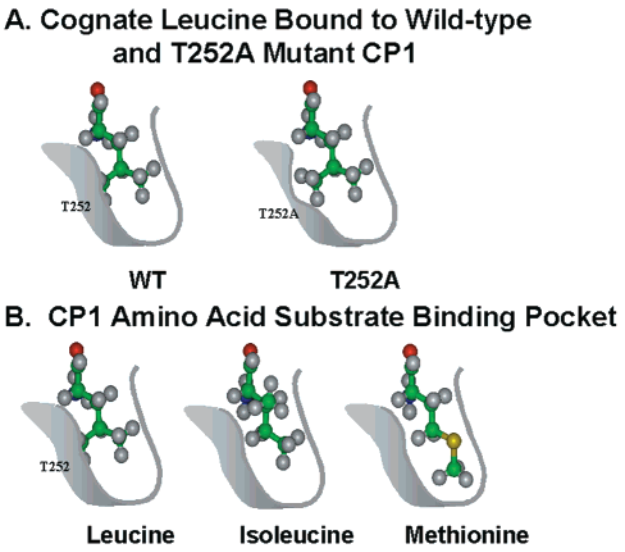

FIGURE 5: Cartoon representation of the LeuRS editing active site. The T252 residue is proposed to comprise a critical structural component of the amino acid binding pocket. (A) T252 sterically blocks leucine binding (left) but when substituted by an alanine allows leucine to bind competently (right). (B) As discussed in the main text, the straight-chained methionine and β-branched isoleucine are unimpeded by the T252 barrier.

The *T. thermophilus* LeuRS crystal structure shows that the side chain of T252 lines a pocket in the CP1 domain (*25*). We hypothesize that this pocket is the fine editing sieve that binds noncognate amino acids mischarged to $tRNA^{Leu}$ but, significantly, excludes leucine that is correctly aminoacylated. Figure 5 suggests that the T252 residue in *E. coli* LeuRS resides near the bottom of the putative amino acid binding pocket and that the conserved threonine side chain sterically blocks the γ-branched methyl of leucine. Alternatively, the threonine residue may not directly contact the amino acid substrate but indirectly induce a structural or conformational barrier that impedes leucine binding. For either a direct or indirect role, substitution of the T252 with an alanine opens the pocket, allowing leucine to bind competently for hydrolysis (Figure 5A).

As discussed above, disruption of the LeuRS editing domain yields mischarged Ile- and Met-$tRNA^{Leu}$ (*17*). Our model in Figure 5B indicates how a straight-chained methionine side chain could slide past the T252 barrier. Likewise, the β-branched methyl of isoleucine would be located above the T252 barrier within the binding pocket. Since we were unable to isolate mischarged methionine or isoleucine, the T252A mutation did not appear to affect catalysis or editing active site recognition of these two noncognate amino acids. Thus, although the integrity of the CP1-based editing active site is maintained, this single mutation has altered the fine amino acid discrimination mechanism of the second active site sieve. We propose that the conserved T252 residue plays a critical role in *E. coli* LeuRS as a discriminant to exclude cognate charged Leu-$tRNA^{Leu}$ from being bound and subsequently hydrolyzed by the second fine sieve.

We have also made the analogous mutation of this conserved threonine residue in yeast cytoplasmic LeuRS (ycLeuRS). In contrast, the corresponding mutant did not hydrolyze leucyl-tRNA^{Leu} (T. L. Lincecum and S. A. Martinis, unpublished data). It is possible that LeuRS editing active sites may vary and rely on other or combinations of multiple amino acid determinants within the substrate binding pocket to block cognate leucine from hydrolysis. Alternatively, it is also significant that the ycLeuRS functions solely by a pretransfer mechanism that hydrolyzes misactivated adenylate compounds (*18*). This is distinct from the *E. coli* LeuRS posttransfer editing mechanism that requires transfer of the amino acid to the tRNA prior to editing, and therefore, the yeast enzyme may require different mechanistic determinants for editing.

X-ray crystallography structures and docking models for ValRS and IleRS suggest that the CP1-based pre- and posttransfer editing active sites rely on the same adenosyl binding sites for interactions with either editing substrate (*14*). Significantly however, these editing active sites have different aminoacyl binding pockets to accommodate the quite chemically distinct aminoacyl adenylate and charged tRNA substrates that are respectively targeted for pre- and posttransfer editing. If the amino acid binding pockets and other features of the pre- and posttransfer editing active sites are distinct, two independent mutation sites would be required to selectively alter the single exclusive editing active sites that are respectively responsible for ycLeuRS pretransfer editing activity and *E. coli* LeuRS posttransfer editing activity.

The threonine-rich region in LeuRS overlaps with a valine-specific cleft in the IleRS CP1 domain that was identified in the *T. thermophilus* enzyme by Nureki et al. and marked by conserved tryptophan and threonine residues (*9*; W245 and T243 in *E. coli* IleRS; Figure 1). A 47 amino acid deletion that included the threonine-rich region resulted in a stable *E. coli* IleRS mutant that retained aminoacylation activity but disrupted the editing site and abolished Val-tRNA^{Ile} editing activity (*9*). An alanine substitution (T242A) in the threonine-rich region also decreased hydrolysis of a misactivated valyladenylate complex while a T242A/N250A double mutant as well as a T242P single mutant increased Val-tRNA^{Ile} production (*9*, *12*). These mutations support that the threonine-rich regions in IleRS and LeuRS play critical roles in each enzyme's respective editing activity.

The key T252 editing residue in LeuRS is also conserved in IleRS and ValRS (Figure 1). Extensive overlaps in the primary and tertiary structures within this subfamily of editing enzymes (IleRS, LeuRS, and ValRS) suggest that the CP1 domain was added in evolution prior to their divergence. If the common ancestor aminoacylated amino acids with aliphatic side chains in general, then the role of the shared CP1 editing domain is not clear. However, upon divergence of these class I aaRSs to attain amino acid specificity, coevolution of both the synthetic and editing active sites may have occurred or even been required for high fidelity. Thus, as the pocket "filled in" to accommodate smaller amino acids for editing by IleRS and ValRS, it is possible that the T252 residue was recruited for a critical structural rather than specificity role. Interestingly, conserved residues within the threonine-rich region that are specific only to IleRS (T243 and W245 in *E. coli*) have been suggested to interact with a valine molecule (*9*).

Translocation of the mischarged noncognate amino acid to the editing site would span approximately 30 Å (*9*, *10*, *14*, *25*). On the basis of the cocrystal structure of *S. aureus* IleRS complexed with tRNA^{Ile}, Silvian and Steitz have hypothesized that the 3′ tRNA end may shuttle the mischarged amino acid between the synthetic and editing sites in a mechanism reminiscent of DNA polymerase I (*10*). In contrast to the DNA polymerase, where editing is nonspecific and controlled by kinetic mechanisms, editing of cognate and noncognate amino acids by LeuRS and other aaRSs may be more closely dictated by specificity and discrimination within the editing active site. This raises the possibility that aaRSs that exhibit editing mechanisms may sample charged tRNA that is linked to both noncognate and cognate amino acids prior to product release.

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