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A Paradigm Shift for the Amino Acid Editing Mechanism of Human Cytoplasmic Leucyl-tRNA Synthetase†

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Abstract

Leucyl-tRNA synthetase (LeuRS) has been identified as a target for a novel class of boron-containing small molecules that bind to its editing active site. When the 3′ end of $tRNA^{Leu}$ binds to the editing active site, the boron crosslinks to the *cis* diols of its terminal ribose. The crosslinked RNA-protein complex blocks the overall aminoacylation activity of the enzyme. Similar to other LeuRSs, the human cytoplasmic enzyme (hscLeuRS) editing active site resides in a discrete domain called the connective polypeptide 1 domain (CP1), where mischarged tRNA binds for hydrolysis of the noncognate amino acid. The editing site of hscLeuRS includes a highly conserved threonine discriminator and universally conserved aspartic acid that were mutationally characterized. Substitution of the threonine residue to alanine uncoupled specificity similar to other LeuRSs. However, the introduction of bulky residues in the amino acid binding pocket failed to block deacylation of tRNA, indicating that the architecture of the amino acid binding pocket is different compared to other characterized LeuRSs. In addition, mutation of the universally conserved aspartic acid abolished $tRNA^{Leu}$ deacylation. Surprisingly though, this editing-defective hscLeuRS maintained fidelity. It is possible that an alternate editing mechanism may have been activated upon failure of the post-transfer editing active site.

Keywords

Pre-transfer editing; CP1 domain

Aminoacyl-tRNA synthetases (aaRSs) establish the genetic code by accurately linking the correct tRNA to its cognate amino acid in the first step of translation (1,2). In a two-step reaction mechanism, each aaRS ensures that the tRNA is correctly charged.

$$\text{ATP+Amino Acid (AA)} \leftrightarrows \text{AA} - \text{AMP+PP}_i \quad [1]$$

$$\text{AA} - \text{AMP+tRNA}^{AA} \rightarrow \text{AA} - \text{tRNA}^{AA}\text{+AMP} \quad [2]$$

The charged tRNAs are then shuttled to the ribosome via elongation factors. In order to protect against statistical errors that could be incorporated into proteins during translation, about half of the family of aaRSs have evolved proofreading mechanisms (2,3). These

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fidelity mechanisms are critical to the cell. Defects in aaRS editing result in cell death (4–6) and neurological disease in mammals (7).

Leucyl-tRNA synthetase (LeuRS) must accurately discriminate leucine from a number of standard and non-standard amino acids (5). The canonical core of LeuRS contains an aminoacylation active site that activates cognate amino acids as well as structurally similar non-cognate amino acids that include isoleucine and valine. A polypeptide called the connective polypeptide 1 (CP1) domain (8) is inserted into the core via two β-strand linkers (9). The LeuRS CP1 domain functions as a second "fine sieve" (10) that excludes correctly charged amino acids, but hydrolyzes misaminoacylated tRNA (11–14). This bi-domain architecture that provides a double sieve to enhance fidelity is reminiscent of all editing aaRSs. Synthetic sites and CP1 editing domains that are homologous to LeuRS are also specifically found in isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS) (15).

The editing active site for the CP1 domains of IleRS, ValRS and LeuRS are comprised of conserved peptides that are only partially differentiated to confer specificity (16). Each contains a universally conserved aspartic acid that is essential for hydrogen bonding to the amino group of the bound amino acid (Figure 1). Hydrolytic editing activity is abolished when the aspartate is substituted by an alanine in LeuRS (17), ValRS (18), and IleRS (19).

In addition, a threonine-rich region is important for catalysis and substrate specificity. One of these threonines in LeuRS functions specifically as a specificity determinant. Substitution of this conserved threonine with an alanine abolishes editing specificity by removing a steric hindrance in the editing pocket to facilitate leucine binding (14). Thus, correctly charged Leu-tRNA^{Leu} is hydrolyzed in the second sieve before undergoing product release. Substitution of this conserved threonine with a bulky residue in *Escherichia coli* LeuRS blocks amino acid editing resulting in a mischarging phenotype (11,20).

In many higher organisms, and even some archae and yeast, aaRS operates as a large or small complex. For example, in human cells, the aaRS macromolecular complex contains nine aaRSs including the lysyl-, aspartyl-, methionyl-, glutamyl-prolyl-, isoleucyl-, leucyl-, arginyl-, and glutaminyl-tRNA synthetases (21). We wondered if viable editing mechanisms that typically reside in separate domains such as the CP1 editing module might be maintained in human aaRSs that are confined to the complex. The primary structure of human cytoplasmic LeuRS (hscLeuRS) retains key amino acids in the editing active site that are important to bacterial LeuRS editing activities, although other sites within the region have diverged (Figure 1). For example, recent crystal structure information of the hscLeuRS editing domain shows that two residues have diverged to bulkier residues, leading to a smaller hydrophobic pocket within the CP1 domain of hscLeuRS (22). Eukaryotic cytosolic LeuRS has also been found to contain an I4 insertion in the CP1 domain that was hypothesized to function as a lid by closing over the editing pocket. This would result in a capped tighter hydrophobic editing pocket for hscLeuRS compared to the more spacious bacterial LeuRS pocket that is open to the environment (22).

We cloned the hscLeuRS gene and expressed it in *E. coli* independent of the multi-aaRS complex. Despite maintaining key features in the editing active site that are critical to its hydrolytic activity, mutational analysis of the human enzyme suggests that it has functionally differentiated to maintain fidelity.

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EXPERIMENTAL PROCEDURES

Materials

Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). Restriction enzymes *Dpn*I and *Bst*NI were obtained from Promega (Madison, WI) and New England BioLabs Inc. (Beverly, MA) respectively. Cloned *Pfu* DNA polymerase and dNTP mix were acquired from Stratagene (La Jolla, CA). Crude calf liver tRNA was purchased from Novagen (San Diego, CA). Tritium-labeled amino acids, as well as [^{32}P]-ATP and [^{32}P]-PP_i, were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The plasmid pHSLARS1 was a gift from Weimin Mao and MRK Alley (Anacor Pharmaceuticals; Palo Alto, CA).

RNA preparation

The CCA 3′ end of crude calf liver tRNA was added via a nucleotidyl transferase reaction. The plasmid pCCA encoding the nucleotide transferase enzyme was a gift from Dr. P. Schimmel (The Scripps Research Institute; La Jolla, CA). The enzyme was expressed in BL21 for 3 h at 37 °C with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then purified over a nickel affinity column (Sigma, St. Louis, MO). Each nucleotidyl transferase reaction contained 100 mM glycine, pH 9.0, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)), 100 μM ATP, 100 μM CTP, 10 mg/mL crude calf liver tRNA, 5 μM *E. coli* CCA nucleotidyl transferase and was incubated in a 70 °C heat block for 15 min. Protein was removed from the mixture by extraction with phenol/chloroform/isoamyl alcohol (125:24:1; Fisher Biotech, Fair Lawn, NJ) that was pre-equilibrated at pH 4.3. The tRNA was then ethanol precipitated and resuspended in nuclease-free water (Ambion, Austin, TX).

E. coli leucyl−$tRNA^{Leu}_{UAA}$ ($tRNA^{Leu}$) was transcribed from the plasmid ptDNAleu (23) that encoded the gene for *E. coli* $tRNA^{Leu}$. A total of 450 μg of plasmid ptDNAleu was first digested overnight with 25 U *Bst*NI at 60 °C in a total volume of 1 mL and then used as a template for run-off transcription (24). T7 RNA polymerase was expressed for three hours at 37 °C with 1 mM IPTG and then purified over a nickel column. Each transcription mixture contained 40 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 30 mM $MgCl_2$, 5 mM DTT, 0.01% Triton X-100, 50 μg/mL bovine serum albumin (BSA), 4 mM of each NTP, 80 mg/mL PEG8000, 0.02 unit/μL RNase inhibitor (Eppendorf, Hamburg, Germany), 2 mM spermidine, 0.01 mg/mL pyrophosphatase (Sigma, St. Louis, MO), and 0.8 μM T7 RNA polymerase. The reaction mixtures were incubated at 42 °C for 3 h followed by a second addition of 0.8 μM of T7 RNA polymerase, and then incubated for an additional 3 h period.

The reactions were precipitated with twice the volume of 100% ethanol and with 167 μg/mL glycogen as a carrier at −80 °C for at least 30 min. The RNA products were purified by electrophoresis on a 10% polyacrylamide gel that contained 8 M urea. The $tRNA^{Leu}$ band was visualized by UV shadowing and excised from the gel. The excised gel was crushed and soaked in 0.5 M NH_4OAC and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 and rocked gently at 37 °C to extract the tRNA. The supernatant was collected and the gel extracted two more times. Butanol extraction was used to concentrate the tRNA, which was then ethanol precipitated and resuspended in nuclease-free water (Ambion, Austin, TX). The concentration of the tRNA was calculated based on its extinction coefficient of 840,700 liters/mol·cm (25).

Mutagenesis

Site directed mutagenesis was performed via the polymerase chain reaction (PCR) using the template plasmid pHSLARS1 that encoded the wild-type *homo sapiens* cytoplasmic LeuRS (hscLeuRS) gene. This plasmid was utilized to make mutants including T298A (pJPT298A)

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using primers T298A-Fwd (5'-CAG ACC TGA GGC CAT GTT TGG GC-3') and T298A-Rev (5'-GCC CAA ACA TGG CCT CAG GTC TG-3'), T298Y (pJPT298Y) using primers T298Y-Fwd (5'-CAG ACC TGA GTA CAT GTT TGG GC-3') and T298Y-Rev (5'-GCC CAA ACA TGT ACT CAG GTC TG-3'), and D399A (pJPD399A) using primers D399A-Fwd (5'-CTC CCC TGA TGC TAT TGC TGC CC-3') and D399A-Rev (5'-GGG CAG CAA TAG CAT CAG GGG AG-3'). Each 50 μL PCR mixture contained 50 ng of DNA template, 100 ng each of forward and reverse primer, 200 μM dNTPs, and 2.5 U of *Pfu* DNA polymerase in commercial buffer. Each PCR reaction was carried out at 95°C for 30 sec; 55°C for 30 sec; and 68°C for 30 min for one cycle, and then 16 cycles at 95°C for 30 sec, 58°C for 30 sec and 68°C for 22 min. The reaction was completed with a final incubation at 68°C for 22 min. A restriction digestion of each PCR mixture with 20 units of *Dpn*I was carried out for 4 h at 37 °C and then used for transformation of *E. coli* DH5α. The mutant sequences of the LeuRS genes were confirmed by DNA sequencing (UIUC Core Sequencing Facility, Urbana, IL).

Expression and Purification of hscLeuRS

Mutant and wild-type plasmids were used to transform *E. coli* strain BL21 (DE3) codon PLUS (Stratagene). A single colony was used to inoculate a 3 mL culture of LB with 100 μg/mL ampicillin and grown overnight at 37 °C. This 3 mL aliquot was then transferred to 1 L of LB containing 100 μg/mL ampicillin and grown at 37 °C until the OD_{600} was between 0.6 and 1.0. Protein expression was induced overnight at room temperature by the addition of 1 mM IPTG.

The next day, cells were harvested by centrifugation at 6000 rpm for 15 min in an Avanti J-E centrifuge (Beckman Coulter, Fullerton, CA). The cell pellet was resuspended in 7 mL of HA-1 buffer (20 mM NaP_i, 10 mM Tris, pH 8.0, 300 mM NaCl, 5 mM imidazole, and 5% glycerol) and then sonicated once at 50% power using a Vibra Cell sonicator (Sonic, Newtown, CT). The lysate was spun at 12,000 rpm for 30 min at 4 °C. The supernatant was collected and the pellet discarded.

The supernatant containing hscLeuRS was combined with a HIS-Select HF Nickel Affinity (Sigma, St, Louis, MO) resin that had been pre-equilibrated with HA-1 buffer. The supernatant was rocked gently with the nickel resin for 2 h at 4 °C to allow for the N-terminal fused six-histidine tag on each protein to bind to the resin. The resin was then washed with a total of 100 mL HA-2 buffer (20 mM NaP_i, 10 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 5% glycerol) and the protein was eluted with 10 mL 100 mM imidazole in HA-1 buffer. The protein solution was dialyzed against a 100 mM KP_i, pH 6.8, 10 mM β-mercaptoethanol, 50% glycerol buffer and stored at −20 °C. The concentration of protein was determined on the basis of its absorbance at 280 nm and calculated using an estimated extinction coefficient of 178,080 liters/mol·cm computed by the ExPASy Protparam tool (http://ca.expasy.org/tools/protparam.html).

Aminoacylation assays

Each aminoacylation reaction contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (HEPES), pH 7.6, 30 mM KCl, 30 mM $MgCl_2$, 0.02% BSA, 1 mM DTT, 4 mg/mL calf liver tRNA, 22 μM [^{3}H]-leucine (158 Ci/mmole) and 500 nM enzyme. Misaminoacylation assays were carried out in the same manner except with 1 μM enzyme, 20 μM *E. coli* transcribed $tRNA^{Leu}$ and 22 μM [^{3}H]-isoleucine (93 Ci/mmole). Reactions were initiated with 4 mM ATP and carried out in a 30 °C heat block. Aliquots of 20 μL were taken at different time points and quenched on Whatman filter pads that were pre-soaked with 5% trichloroacetic acid (TCA). The pads were washed three times for 10 min each with cold 5% TCA, once with cold 70% ethanol, and once with anhydrous ether.

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The washed pads were then dried under a heat lamp. Radioactivity was quantified in a Beckman LS 6000IC scintillation counter (Beckman Coulter, Fullerton, CA).

Deacylation assays

Transcribed *E. coli* $tRNA^{Leu}$ at a final concentration of 8 μM was aminoacylated with 1 μM wild-type *E. coli* LeuRS or misaminoacylated with 1 μM editing-defective *E. coli* LeuRS in a reaction containing 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 4 mM ATP. The reactions were carried out at room temperature for either 60 min (aminoacylation) or 180 min (misaminoacylation) and then quenched with 0.18% acetic acid (26). Protein was removed from the mixture by a phenol/chloroform/isoamyl alcohol mixture (125:24:1; Fisher Biotech, Fair Lawn, NJ) at pH 4.3. The charged tRNA was then ethanol precipitated and resuspended in 50 mM KP_i, pH 5.0.

Deacylation reactions each contained 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 300 mM KCl, and approximately 4 μM of $[^3H]$-Ile-$tRNA^{Leu}$ or $[^3H]$-Leu-$tRNA^{Leu}$. Reactions were conducted in a 30 °C heat block and initiated with 500 nM enzyme. Aliquots of 10 μL were taken at different time points and quenched on Whatman filter pads that were pre-soaked with 5% TCA. The pads were washed and analyzed as described above.

Inorganic pyrophosphate (PP_i) exchange assays

Reactions containing 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 300 mM KCl, 1 mM DTT, 1 mM $[^{32}P]$-PP_i (78 mCi/mL; Amersham Pharmacia Biotech), 1 mM leucine, and 1 μM enzyme were initiated with 1 mM ATP. Aliquots of 2 μL were taken at different time points and spotted on a cellulose polyethyleneimine (PEI) thin-layer chromatography (TLC) plate (Scientific Adsorbents Inc., Atlanta, GA). The reaction components were separated by TLC in 750 mM KH_2PO_4 (pH 3.5) and 4 mM urea (27). The separated radiolabeled bands were visualized by phosphorimaging using a FUJIX BAS 1000 film (FUJI-FILM Medical Systems U.S.A., Stanford, CT) and quantified with a Storm 840 molecular dynamics imager (GE Healthcare, Piscataway, NJ).

ATPase assays

Each reaction mixture contained 100 mM Tris, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 5 mM amino acid, 36.2 μM α-$[^{32}P]$-ATP (3000 Ci/mmole), and 10 μM *E. coli* transcribed tRNA. The reactions were initiated with 1 μM enzyme. Aliquots of 2 μL were taken at different time points and spotted on a cellulose PEI TLC plate (Scientific Adsorbents Inc., Atlanta, GA). The reaction components were separated by TLC in 750 mM KH_2PO_4 (pH 3.5). The separated radiolabeled bands were visualized by phosphorimaging using a FUJIX BAS 1000 film (FUJI-FILM Medical Systems U.S.A., Stanford, CT) and quantified with a Storm 840 molecular dynamics imager (GE Healthcare, Piscataway, NJ).

RESULTS

The editing active site within the CP1 domain of LeuRS is marked by conserved peptide motifs. A highly conserved threonine within a threonine-rich region acts as a specificity determinant (Figure 1A) and blocks leucine from binding for hydrolysis of correctly charged Leu-$tRNA^{Leu}$. Substitution of this key threonine with a bulky residue such as tyrosine fills up the amino acid binding pocket in *E. coli* LeuRS to prevent editing (11, 20). In a separate motif, a universally conserved aspartic acid (Figure 1A) that is also found in IleRS and ValRS anchors the amino acid via a hydrogen bond with its amino moiety in the active site. Substitution of the aspartic acid by alanine abolishes editing activity in LeuRS and IleRS (17–19).

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The threonine specificity determinant and universal aspartic acid are also found in hscLeuRS amongst many other conserved features of the editing active site. This suggests that LeuRS from a higher eukaryote maintains fidelity using an editing active site architecture that is common to lower organisms. We tested this hypothesis by substituting the conserved threonine at position 298 of hscLeuRS (Thr^{252} in *E. coli* LeuRS) with an alanine (T298A) and also with a bulky tyrosine (T298Y). The universal aspartate at position 399 in hscLeuRS was also substituted with an alanine. The mutant and wild-type proteins were expressed in *E. coli* and purified by affinity chromatography with a six-histidine tag.

The enzymatic activities for each of the mutants were characterized and compared to wild-type hscLeuRS. Both the T298Y and D399A mutant hscLeuRSs exhibited robust aminoacylation of crude calf liver tRNA, albeit the latter activity was reduced compared to wild-type (Figure 2A). As would be expected based on analysis of other LeuRSs (14), the T298A LeuRS mutant failed to accumulate Leu-$tRNA^{Leu}$ (Figure 2A). The T298A hscLeuRS mutant also uncouples specificity in the editing active site and hydrolyzes Leu-$tRNA^{Leu}$ (Figure 2B).

We wondered if the relatively reduced aminoacylation activities of the D399A LeuRS mutant enzyme might be due in part to changes in the amino acid activation step [1] even though the mutation is located in the CP1 domain, which is completely separate from the canonical aminoacylation core. Pyrophosphate exchange assays determined that the D399A LeuRS mutant activated leucine at reduced rates (Figure 3). The decrease in leucine-dependent pyrophosphate exchange activity by the distal D399A mutation was largely due to a K_M effect (Table 1). The K_M was about 40-fold higher for leucine activation by the D399A LeuRS mutant (0.9 ± 0.2 mM) as compared to the human wild-type enzyme (0.024 ± 0.009 mM). The k_{cat} for leucine activation of the wild type and mutant enzymes were 2.1 ± 0.4 s^{-1} and 5.6 ± 0.1 s^{-1}, respectively. This reduction in amino acid activation activity is consistent with the lower levels of leucylation activity for the D399A LeuRS mutant compared to the wild-type enzyme. These results suggest that a long-distance inter-domain communication mechanism for the protein exists between the aminoacylation and amino acid editing active site that is at least partially tRNA-independent.

Mischarged *E. coli* Ile-$tRNA^{Leu}$ was isolated to test the wild-type and mutant LeuRSs directly for tRNA deacylation activity. The D399A mutant of LeuRS eliminated Ile-$tRNA^{Leu}$ deacylation activity (Figure 4), which is consistent with all other alanine substitutions at this key site in LeuRS (17), IleRS (19) and ValRS (18). In contrast though, substitution of the conserved threonine with a bulky tyrosine residue (T298Y) in the hscLeuRS maintained Ile-$tRNA^{Leu}$ deacylation activity. This suggests that even though the threonine specificity determinant is maintained, the architecture around the amino acid binding pocket has changed for the human enzyme. A recent crystal structure for the CP1 domain of hscLeuRS and the related yeast cytoplasmic enzyme showed that the eukaryotic LeuRS CP1 domain has acquired insertions (22). In particular, the I4 insertion (shown in blue on Figure 1B) is juxtaposed to the editing active site and has been hypothesized to act as a lid to sequester substrate in the hydrolytic pocket (22). It is possible that closing the I4 insertion lid might promote an induced fit for the mischarged $tRNA^{Leu}$ to be edited, even in the presence of a bulky residue substitution in the human LeuRS editing site.

In the absence of deacylation activity, we expected that the mutant D399A hscLeuRS would mischarge $tRNA^{Leu}$ similar to other editing-defective LeuRSs (17–19). Crude calf liver tRNA was mischarged by the D399A LeuRS mutant, but only at low levels (data not shown) despite a robust aminoacylation activity (Figure 2A). By comparison, crude *E. coli* tRNA was mischarged at higher levels (Figure 5A) with a k_{cat} of 0.0018 ± 0.0005 s^{-1} by the D399A hscLeuRS mutant, albeit more weakly than LeuRS mutants from other origins that

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had ablated the tRNA deacylation activity. The D399A LeuRS mutant failed to charge isoleucine to *in vitro* transcribed *E. coli* $tRNA^{Leu}$, even at high concentrations of 1 μM enzyme (Figure 5B). This suggests that the low levels of mischarging activity are dependent on tRNA modifications. It is possible that these RNA modifications stabilize tertiary structure to facilitate RNA-protein interactions for aminoacylation. As would be expected, wild-type hscLeuRS maintained fidelity and did not mischarge crude or transcribed *E. coli* tRNA.

We hypothesized that, similar to the long-distance effect by the D399A mutation on leucine activation (Figure 3), isoleucine activation might also be significantly reduced, resulting in only low mischarging activities for the editing-defective enzyme. However, the D399A mutation had a much less pronounced effect on the K_M and k_{cat} for isoleucine-dependent pyrophosphate exchange activity with k_{cat}/k_M values that were nearly equivalent for the wild type and mutant enzymes (Table 1). Thus, these long distance inter-domain effects between the editing and aminoacylation active sites are sensitive to the cognate amino acid. It is also possible that low misaminoacylation activity by the D399A hscLeuRS could be due to a redundant fidelity mechanism that originates in the aminoacylation active site. In the absence of tRNA, isoleucine stimulated the D399A mutant hscLeuRS to form AMP with a k_{cat} of 0.06 ± 0.02 s^{-1} and K_M of 1.8 ± 0.5 mM in ATPase assays. If this represents a case of a putative pre-transfer editing pathway, then the mechanism would be independent of the D399A mutation in hscLeuRS since the activated isoleucyl-adenylates would be directly cleaved in or expelled from the aminoacylation active site.

DISCUSSION

Representative of all LeuRSs, the hscLeuRS contains specific active sites for aminoacylation and editing that are located in separate domains. X-ray crystal structures for LeuRSs from all three kingdoms (22,28,29) have shown that within the CP1 editing domain, the LeuRS editing active site is marked by a threonine-rich peptide. In the human enzymes, it includes the highly conserved Thr^{298} specificity determinant (Thr^{252} in *E. coli* LeuRS) as well as Thr^{293} (Thr^{247} in *E. coli* LeuRS) that has been shown to be important in orientating the editing substrate in the editing active site for catalysis by LeuRS (30). It also has the “GTG adenine-binding region” as well as a universally conserved aspartic acid that interacts with the amino group of the bound amino acid (17). However, four peripheral peptide insertions distinguish the hscLeuRS and other eukaryotic CP1 domains from their bacterial counterparts. X-ray crystallography structures for the hscLeuRS and yeast cytoplasmic LeuRS show that although the integrity of the editing core is clearly maintained, these peripheral insertions appear to induce some local structural rearrangements (22).

The I4 peptide insertion in the LeuRS CP1 domain is hypothesized to cap the editing active site when mischarged tRNA is bound (22). Another insertion, called I2, contacts the bound editing substrate analogue. In the yeast cytoplasmic LeuRS CP1 domain structure, the I2 insert also interacts with a network of water molecules. It is possible then that displacement of these water molecules could provide some plasticity for the editing active site to facilitate an induced fit mechanism. In the case of our results, water molecules could be displaced by the introduction of the bulky tyrosine residue at the Thr 298 specificity site in the hscLeuRS CP1 domain. Based on the structure of the hscLeuRS CP1 domain, we hypothesize that the tyrosine residue could flip into vacant space over the neighboring Val 497 (Figure 1B) to allow binding of the editing substrate. Indeed, closure of the I4 lid might facilitate an induced fit mechanism for the hscLeuRS editing site that adequately includes the bulky Tyr 298 mutation.

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The universal aspartic acid in the editing active site of the hscLeuRS is critical to tRNA deacylation as found for all other enzymes where it has been tested. Here, we demonstrated that an alanine substitution at the conserved site blocks deacylation activity *in vitro*. This is consistent with *in vivo* experiments showing that the introduction of a bulky positively charged lysine residue for the aspartic acid failed to protect yeast knock out cells from high concentrations of norvaline (31).

Surprisingly, the universally conserved aspartic acid in the CP1 domain was not only important to editing active site substrate interactions, but also interactions with the amino acid in the synthetic active site. Substitution of the aspartic acid resulted in a long distance K_M effect on the amino acid activation step, which would be expected to be governed primarily by the aminoacylation active site in the canonical core. Significantly, this long distance effect was selective for the cognate leucine amino acid suggesting that the CP1 domain might also distally influence amino acid discrimination within the aminoacylation core.

Since aminoacylation activity is also decreased for the D399A hscLeuRS mutant, it is possible that tRNA mediates this distal amino acid discrimination effect in the synthetic site. In this case, tRNA would be expected to bind in the so-called “exit/entry” position where its 3’ end is near the editing active site (32,33). Translocation of the tRNA could be slowed as it sweeps through the altered editing active site enroute to the aminoacylation active site. However, these long-distance effects were also detected in the absence of tRNA during pyrophosphate exchange assays. Thus, subtle changes in the editing active site must be transmitted via nodes within the protein itself to influence amino acid activation (34).

The D399A hscLeuRS mutant only exhibited weak mischarging activity relative to other corresponding LeuRS, IleRS, and ValRS mutants, where post-transfer editing activity was completely abolished. Since the kinetic parameters for isoleucine activation were similar to the wild type and mutant LeuRS, we hypothesize that the tRNA plays a significant role in maintaining fidelity. Indeed, mischarging activity seemed particularly sensitive to the presence of tRNA modifications and the origin of the tRNA. Ironically, *E. coli* crude tRNA yielded greater levels of mischarged tRNA. However, this could be because the *E. coli* tRNA was missing important elements that were embodied in the mammalian calf liver tRNA, which minimized mischarging of isoleucine by LeuRS. If this were the case, we hypothesize that the human enzyme relies, at least in part, on pre-transfer editing that originates from the aminoacylation active site. A redundant mechanism for amino acid editing that originates in the aminoacylation active site would allow fidelity to be rescued in the absence of post-transfer editing activity in the CP1 domain.

The hscLeuRS is part of a large macromolecular complex that includes nine aaRSs and three small auxiliary proteins (35,36). A cryo-EM structure for the multi-aaRS complex has been solved at 30 Å and shows that this large complex is approximately 19 × 16 × 10 nm (21,37). Although the 3.25 Å X-ray crystal structure of the human cytoplasmic LeuRS CP1 domain has also been solved (22), it is unclear how this editing domain and the remainder of the human LeuRS fit into the macromolecular complex. In the context of the multi-aaRS complex, it is possible that domain motions such as the CP1 domain might be constrained. In this case, redundant fidelity pathways may also serve as a more efficient amino acid editing mechanism that doesn’t require translocation of the mischarged tRNA to another editing domain. Recently, we showed that in the absence of the CP1 domain for *E. coli* and yeast mitochondrial LeuRS, the enzyme maintained fidelity by targeting the activated adenylate intermediate (38). In this case, isoleucyl adenylate could be hydrolyzed within the aminoacylation active site (39) or selectively released for hydrolysis in the cellular milieu (40). Redundant mechanisms in amino acid editing could be essential to maintain a level of

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protein synthesis fidelity, particularly in mammals that would guard against neurological disease (7).

We hypothesize that some of the architectural and mechanistic differences that are specific to hscLeuRS may have been acquired in part because of its association with the multi-aaRS complex. These differences have likely facilitated discovery of a novel class of benzoxaborole anti-fungal compounds, which are in development for the treatment of onchomycosis. In particular, AN3018 inhibits yeast cytoplasmic LeuRS by forming a covalent adduct with the 3′ adenosine of the $tRNA^{Leu}$ to trap it in the editing site (32). Significantly though, complementation experiments using yeast knock-out tester strains have indicated that the hscLeuRS is susceptible to AN2690 (31). Furthermore, the D399K hscLeuRS editing-deficient mutant is resistant to AN2690 in complemented yeast cells (31), indicating the importance of the Asp 399 residue in hscLeuRS for binding within the editing site. The AN2690 inhibitor appears to bind to the human enzyme in these yeast complementation assays, in spite of crystal structure analysis of the hscLeuRS CP1 domain that suggests that the size of the hydrophobic editing pocket is smaller than that of the yeast enzyme (22). Nevertheless, a network of water molecules and an induced fit mechanism in the editing site could provide plasticity, especially in a version of hscLeuRS that is freed from its multi-aaRS macromolecular complex. It is also important to note that native residence within the multi-aaRS macromolecular complex might hinder inhibitor interactions with the hscLeuRS CP1 domain, particularly in a mechanism that requires cross-linking the large tRNA substrate that spans two protein domains.

ABBREVIATIONS

aaRSs	aminoacyl-tRNA synthetases
tRNA	transfer RNA
hscLeuRS	human cytoplasmic leucyl-tRNA synthetase
IleRS	isoleucyl-tRNA synthetase
ValRS	valyl-tRNA synthetase
CP1	connective polypeptide 1
I4	insertion 4

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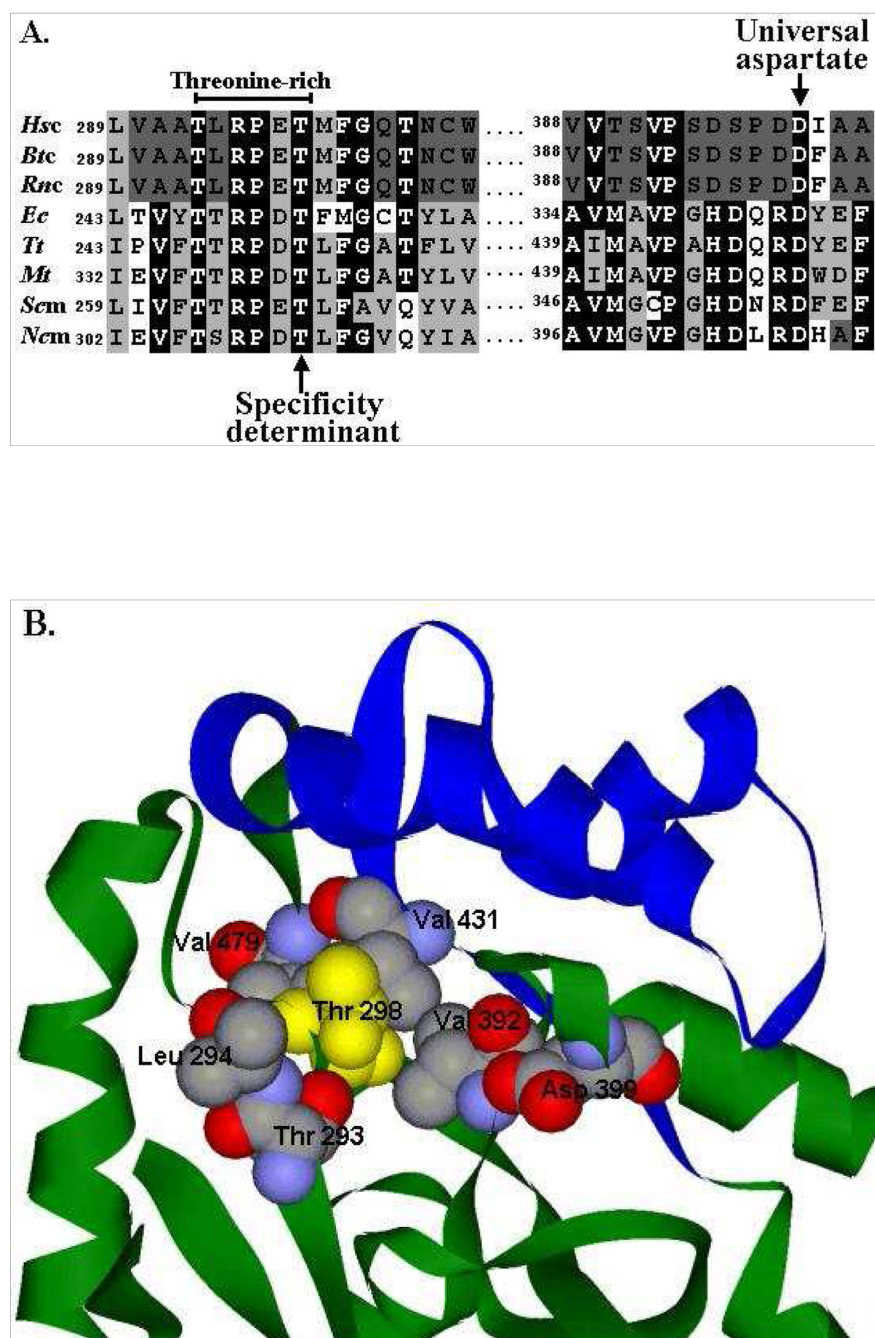

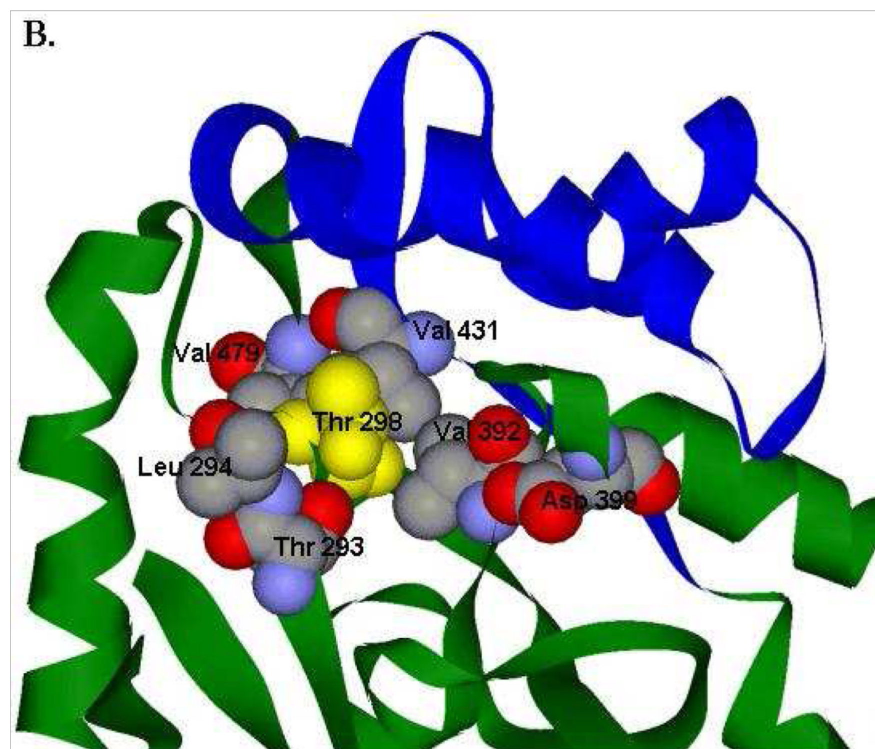

Figure 1.
Multiple sequence alignment of the LeuRS editing pocket and crystal structure of hscLeuRS CP1 domain. A. Sequence alignment of conserved regions within the LeuRS editing pocket. The threonine specificity determinant (T298) and universally conserved aspartate (D399) in hscLeuRS are indicated by arrows. Highly conserved residues are highlighted in black. Two shades of gray distinguish homologous amino acid sites. Abbreviations are as follows: *Hs, Homo sapiens; Bt, Bos taurus; Rn, Rattus norvegicus; Ec, E. coli; Tt, Thermus thermophilus; Mt, Mycobacterium tuberculosis; Sc, Saccharomyces cerevisiae; Nc, Neurospora crassa;* c, cytoplasmic; and m, mitochondrial. B. Crystal structure of the hscLeuRS CP1 domain (PDB entry 2WFD) showing the editing pocket containing the

threonine-rich region and the universally conserved aspartate. Residues Thr^{293}, Thr^{294}, Thr^{298}, Val^{392}, Asp^{399}, Val^{431} and Val^{479} are shown in space-filling form. The atoms are colored as follows: oxygen, red; nitrogen, light blue; carbons, gray. Thr^{298} is highlighted completely in yellow. The I4 insertion is highlighted in blue.

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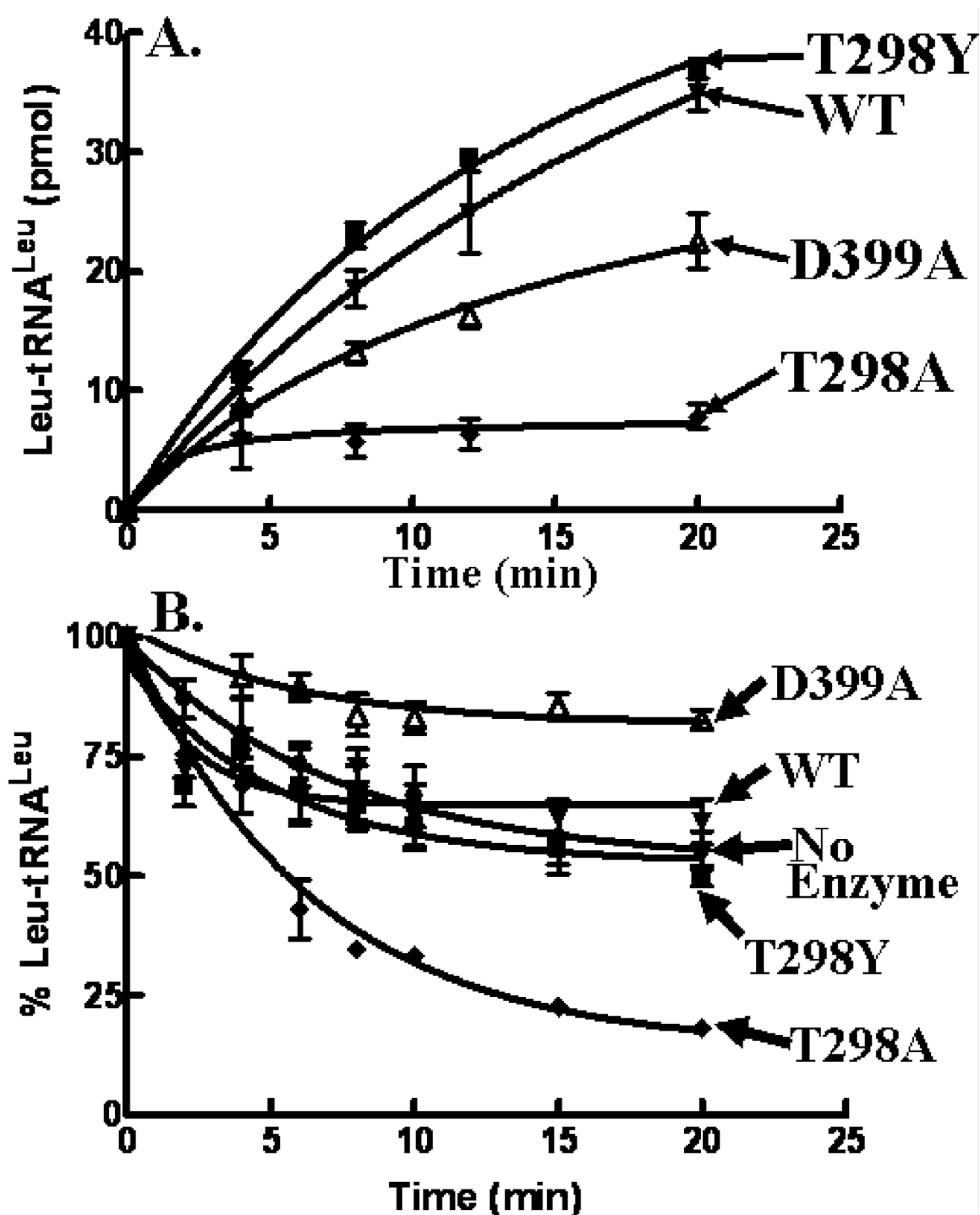

Figure 2.

Leucine aminoacylation and deacylation by wild-type and mutant hscLeuRS. A. Leucylation activity was measured with 500 nM enzyme and 4 mg/ml of crude calf liver tRNA (Novagen, Gibbstown, NJ). B. Post-transfer editing reactions were carried out with 500 nM enzyme and ~4 μM *E. coli* Leu-tRNA^{Leu}. All enzyme reactions were incubated at 30 °C. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (▼); T298A, (♦); D399A, (△); T298Y, (■); and no enzyme, (●). Error bars are based on the results of three reactions each and indicated for each point.

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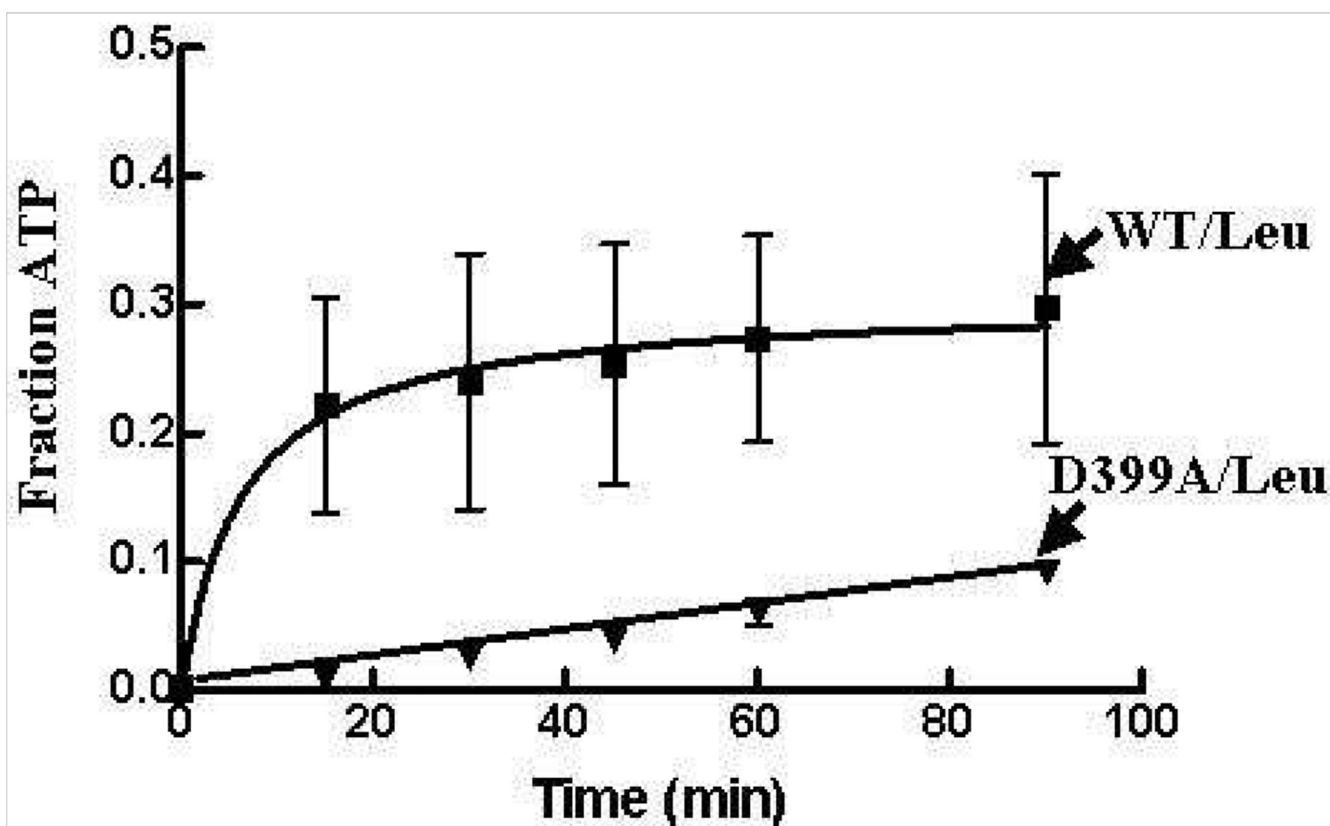

Figure 3.
Pyrophosphate (PP_i) exchange activity of wild type and D399A hscLeuRS. Leucine-dependent PP_i exchange assays were carried out with 1 μM hscLeuRS and 10 mM leucine. Symbols are as follows: WT with leucine, (■); and D399A with leucine, (▼). Error bars are based on the results of three reactions each and indicated for each point.

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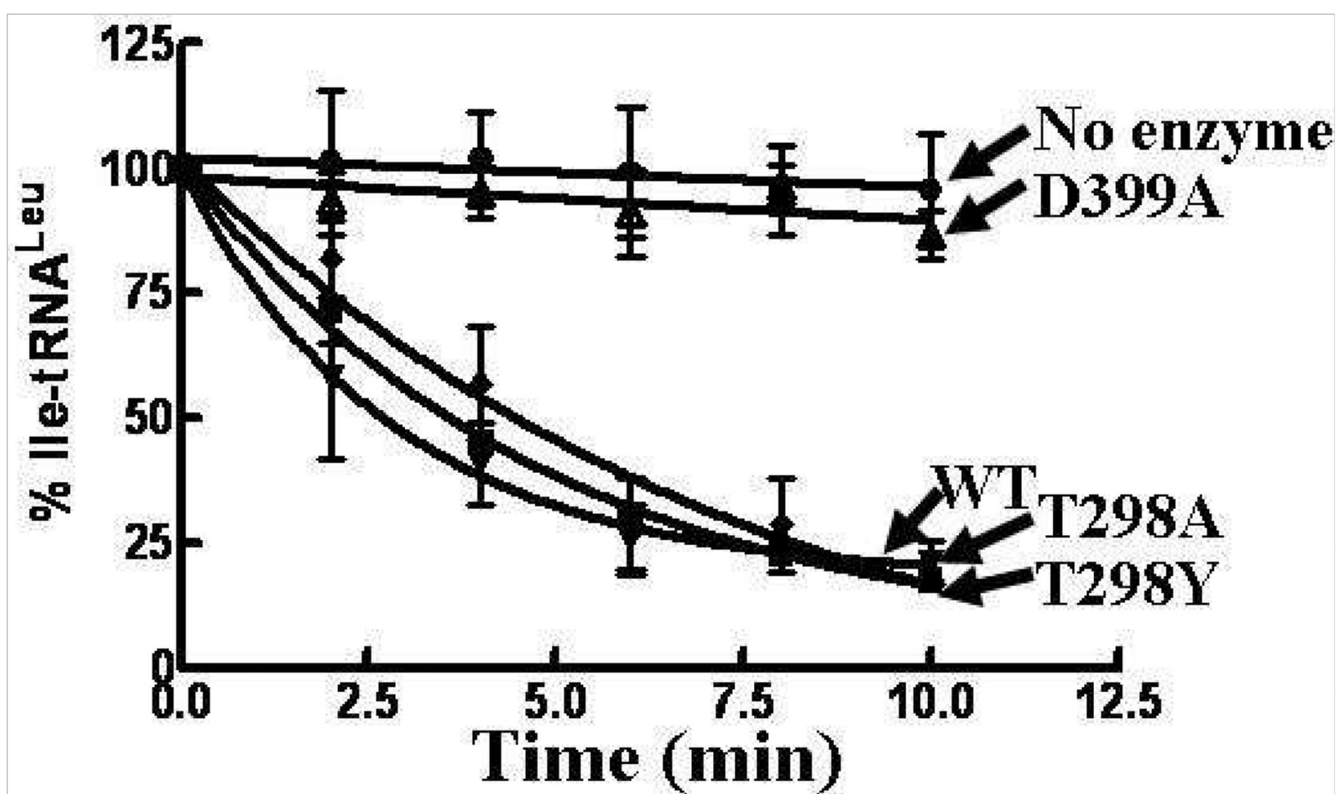

Figure 4.
Deacylation activity of wild-type and mutant hscLeuRS. Post-transfer editing assays were carried out with 500 nM enzyme and ~4 μM *E. coli* Leu-$tRNA^{Leu}$ at 30 °C. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (▼); T298A, (♦); D399A, (△); T298Y, (■); and no enzyme, (●). Error bars are based on the results of three reactions each and indicated for each point.

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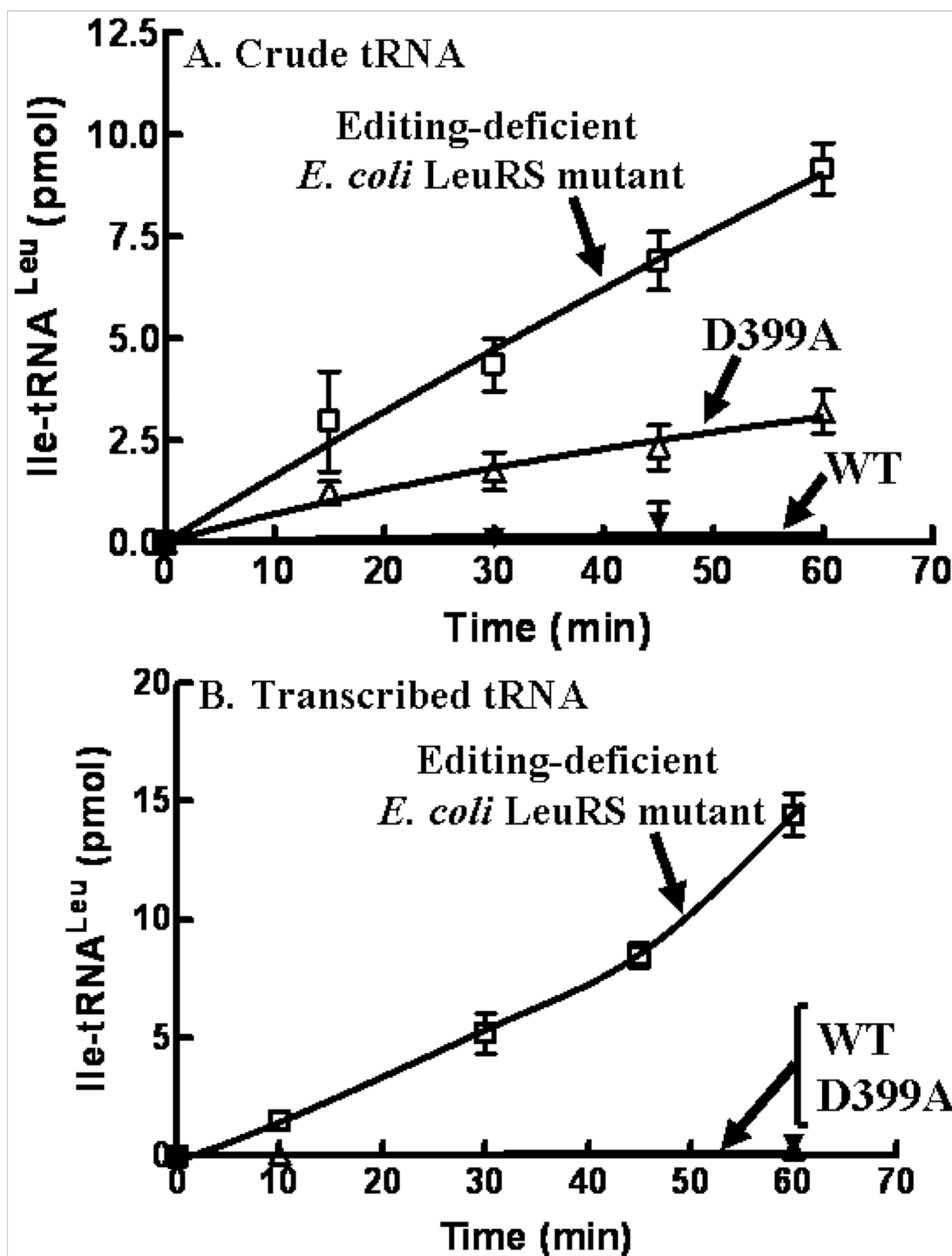

Figure 5.

Mischarging activity of wild type and mutant hscLeuRS with transcribed and crude *E. coli* tRNA. A. Misaminoacylation reactions were carried out with 1 μM enzyme and 20 μM of *in vitro* transcribed *E. coli* tRNA. B. Misaminoacylation reactions were carried out with 1 μM enzyme and 4 mg/ml of crude *E. coli* tRNA. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (▼); D399A, (△) and an editing-deficient *E. coli* LeuRS mutant, (□). Error bars are based on the results of three reactions each and indicated for each point.

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Table 1

Apparent Kinetic Parameters for Amino Acid Activation

	K_M (mM)		k_{cat} (S^{-1})		k_{cat}/K_M ($S^{-1}mM^{-1}$)	
Enzyme	**Leu**	**IIe**	**Leu**	**IIe**	**Leu**	**IIe**
Hsc WT	0.024 ± 0.0089	2.04 ± 0.80	2.1 ± 0.43	0.34 ± 0.17	94.3 ± 23.4	0.16 ± 0.035
Hsc D399A	0.90 ± 0.19	3.3 ± 1.2	5.6 ± 0.067	0.51 ± 0.044	5.6 ± 0.40	0.17 ± 0.043