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A Unique Insert of Leucyl-tRNA Synthetase is Required for Aminoacylation and Not Amino Acid Editing†

Michael T. Vu and **Susan A. Martinis***
Department of Biochemistry, University of Illinois at Urbana-Champaign, Roger Adams Laboratory, Box B4, 600 South Mathews Avenue, Urbana, IL 61801

Abstract

Leucyl-tRNA synthetase (LeuRS) is a class I enzyme, which houses its aminoacylation active site in a canonical core that is defined by a Rossmann nucleotide binding fold. In addition, many LeuRSs bear a unique polypeptide insert comprised of about 50 amino acids located just upstream of the conserved KMSKS sequence. The role of this leucine-specific domain (LS-domain) remains undefined. We hypothesized that this domain may be important for substrate recognition in aminoacylation and/or amino acid editing. We carried out a series of deletion mutations and chimeric swaps within the leucine-specific domain of *Escherichia coli*. Our results support that the leucine-specific domain is critical for aminoacylation, but not required for editing activity. Kinetic analysis determined that deletion of the LS-domain primarily impacts k_{cat}. Because of its proximity to the aminoacylation active site, we propose that this domain interacts with the tRNA during amino acid activation and/or tRNA aminoacylation. Although the leucine-specific domain does not appear to be important to the editing complex, it remains possible that it aids the dynamic translocation process that moves tRNA from the aminoacylation to the editing complex.

Aminoacylation is catalyzed by an ancient family of enzymes called the aminoacyl-tRNA synthetases (aaRS) (1,2). It is important to translation that the aaRS correctly link amino acid to the corresponding tRNA acceptors. Each of the aaRS is responsible for covalently attaching one of twenty standard amino acids to a set of cognate tRNA isoacceptors. The esterification of amino acid to tRNA occurs via a two-step reaction:

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

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

The first reversible step involves the activation of an amino acid concomitant with ATP hydrolysis to form an aminoacyl-adenylate intermediate. The amino acid is then transferred from the adenylate intermediate to the 3′ terminal adenosine moiety of the tRNA. The charged aminoacyl-tRNA binds to EF-Tu and is transported to the ribosome to extend the polypeptide chain.

Leucyl-tRNA synthetase (LeuRS) is a class I aaRS, and is responsible for accurately aminoacylating leucine to its cognate $tRNA^{Leu}$ isoacceptors (3). As is characteristic of all class I synthetases, the catalytic core is defined by a Rossmann nucleotide binding fold (4) that comprises the main body of the enzyme (5). This canonical class I core of LeuRS contains inserts and appendages (5-7). For example, the enzyme has an amino acid editing function that

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*To whom correspondence should be addressed: Department of Biochemistry, University of Illinois at Urbana-Champaign, Roger Adams Laboratory, Box B4, 600 South Mathews Avenue, Urbana, IL 61801. Phone: 217-244-2405. Fax: 217-244-5858. Email: martinis@life.uiuc.edu

resides within a large insert called the connective polypeptide 1 (CP1) (8). In addition, a novel C-terminal domain has been proposed to be important for tRNA interactions (9-12).

One unique small insert called the leucine-specific domain (LS-domain) is found in many bacterial LeuRSs (5). Its function remains undefined. This compact domain that is comprised of five β-strands and two short α-helices flanks the entrance of the aminoacylation active site (5). The LS-domain is inserted after the last β-strand of the Rossmann nucleotide binding fold and is linked to the conserved KMSKS sequence (Figure 1). Primary sequence alignments show that the LS-domain is not particularly conserved in sequence or length (Figure 1A). Indeed it is completely missing in LeuRSs of many organisms including *Bacillus subtilis, Helicobacter pylori*, and *Pyrococcus horikoshii* (data not shown).

A large ensemble of LeuRS co-crystal structures that include tRNA complexes in the aminoacylation and editing conformation have failed to suggest a specific role for the LS-domain (5,9,13-16). The co-crystal structure of *P. horikoshii* LeuRS with the tRNA in the aminoacylation conformation has been solved (13). But *P. horikoshii* LeuRS does not possess a LS-domain. However, comparison between the *apo T. thermophilus* LeuRS and its complex with the tRNA in the editing conformation shows that the LS-domain is dynamic and moves approximately 19° (9). In the editing conformation of *T. thermophilus* LeuRS, the LS-domain does not make any contact with the tRNA. We hypothesized that this movement of the LS-domain may influence tRNA interactions or even interact with the tRNA during its entry and/or exit. It is also possible that the LS-domain plays a role in tRNA translocation from the aminoacylation active site over 30 Å to the editing active site that is located in the CP1 domain.

To identify a function for the LS-domain, we carried out deletion and chimeric peptide replacement analysis. We tested each mutant LeuRS for alterations in aminoacylation and post-transfer editing activity. Our results support that the LS-domain is important to the overall aminoacylation reaction, but does not significantly impact post-transfer editing of mischarged $tRNA^{Leu}$.

Experimental Procedures

Materials and Resources

Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC) or Integrated DNA Technologies (Coralville, IA). Tritium-labeled amino acids and [^{32}P]-pyrophosphate were purchased from Perkin Elmer (Boston, MA). Cloned *Pfu* DNA polymerase, dNTPs and competent cells were obtained from Stratagene (La Jolla, CA). Structure predictions of mutant and chimeric proteins were generated using the DeepView/Swiss Pdb Viewer version 3.7 (17)

Mutagenesis and Purification of E. coli LeuRS

Each 50 μL polymerase chain reaction (PCR) contained 63 ng p15ec3-1 template, which encodes the *E. coli leuS* gene (18), 0.4 μM of each forward and reverse primer that contained the mutation, 0.4 mM of each dNTP, and 2.5 U *Pfu* DNA polymerase in commercially prepared buffer. The mutant plasmid was amplified, screened, and confirmed by DNA sequencing as described previously (19). Plasmids expressing mutant and wild-type LeuRS were used to transform *E. coli* BL21(DE3). Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hr and LeuRS was purified by affinity chromatography via a fused N-terminal six-histidine tag (19). The final concentration was determined using a Bradford Protein Assay as described in the commercial protocol (Bio-Rad, Hercules, CA).

Isolation of in vitro transcribed $tRNA^{Leu}$ and [^{3}H]-ile-$tRNA^{Leu}$

T7 RNA polymerase was purified (20) and $tRNA^{Leu}_{UAA}$ ($tRNA^{Leu}$) was transcribed via *in vitro* run-off transcription (21). The plasmid containing the gene for *E. coli* $tRNA^{Leu}$ ($ptDNA^{Leu}$) (22,23) was digested with 25 U *Bst* N1 (Promega) in a 1 mL reaction at 60 °C overnight and then used as template for *in vitro* transcription as described previously (24). The ethanol-precipitated RNA was separated on a 10 % acrylamide (19:1), 8 M urea gel. The $tRNA^{Leu}$ band was detected by UV shadowing, excised, and recovered (19). The concentration was determined based on the absorbance at 260 nm at 80 °C using the extinction coefficient 840,700 L M^{-1} cm^{-1}. Purified $tRNA^{Leu}$ was refolded by denaturing at 80 °C for 1 min, followed by addition of 1 mM $MgCl_2$, and quick-cooled on ice. The tRNA was stored at -20 °C.

Mischarged [^{3}H]-Ile-$tRNA^{Leu}$ was generated in a reaction containing 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 4 mM ATP, 22 μM [^{3}H]-isoleucine (150 μCi/ml), 4 μM $tRNA^{Leu}$ and 26 μM of an editing-defective LeuRS (19). The reaction was incubated at room temperature for 3 h and then quenched with 4 μL of 10 % acetic acid (25), followed by extraction using phenol/chloroform/isoamyl alcohol (25:24:1, pH 4.3). A one-half volume of 4.6 M ammonium acetate, pH 5.0, and 3 μL of 25 mg/ml glycogen was added followed by ethanol precipitation at -80 °C. The tRNA was resuspended in 61 μL of 50 mM KH_2PO_4 (pH 5.0) and stored at -20 °C.

Enzyme Activity Assays

Each aminoacylation reaction contained 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 22 μM [^{3}H]-leucine (150 μCi/ml), 4 μM $tRNA^{Leu}$ and 100 nM enzyme and was initiated with 4 mM ATP. Kinetic rate constants for aminoacylation were measured using six different concentrations of *in vitro* transcribed $tRNA^{Leu}$ ranging from 0.2 to 20 μM. The apparent rate constants were determined based on the average of three values. The post-transfer editing reaction consisted of 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, approximately 6.5 μM [^{3}H]-ile-$tRNA^{Leu}$ (150 μCi/ml) and was initiated with 100 nM enzyme. Aliquots for either aminoacylation or post-transfer editing were quenched at specific time points by transferring 10 μL onto a Whatman filter pad that was pre-soaked with 5 % trichloroacetic acid (TCA). The pads were washed and radioactivity quantitated as described previously (19).

Inorganic pyrophosphate (PP_i) exchange assays were carried out in 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid (HEPES), pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mM l-leucine, 1 mM [^{32}P]-PP_i (78 mCi/ml), and 500 nM of enzyme (26). The reaction was initiated with 1 mM ATP and aliquots were quenched by spotting 2 μL onto a polyethyleneimine thin-layer chromatography (TLC) plate (Scientific Adsorbents Inc., Atlanta) that had been pre-run in water. ATP and PP_i were separated using 750 mM KH_2PO_4 (pH 3.5) and 4 M urea. Radiolabeled products were analyzed via phosphorimaging using a FUJIFILM BAS-MS 2040 imaging plate (FUJIFILM Medical System USA, Stanford, CT).

RNA Binding Assays

Nitrocellulose filter binding assays to measure tRNA binding to wild type and mutant LeuRSs were carried out using 50 μL reactions that contained 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 20 μM l-leucine, 0.1 μM 5′ [^{32}P]-labeled tRNA, and varying concentrations of enzyme ranging from 0.1 to 2 μM. The reaction was incubated at 37 °C for 5 min and then quenched by spotting all 50 μL on a nitrocellulose membrane assembled on a MINIFOLD-1 Spot-Blot System (Schleicher & Schuell, Keene, N.H.). The membrane was washed with 200 μL binding buffer (60 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT) to remove unbound tRNA. Radiolabeled products were analyzed via phosphorimaging using a FUJIFILM BAS-MS 2040 imaging plate (FUJIFILM Medical System USA, Stanford, CT).

Results

Deletion of the LS-domain abolishes aminoacylation activity, but not post-transfer editing activity

The LS-domain is found in many bacterial LeuRSs, but not in the homologous IleRS and ValRS. We carried out a primary sequence alignment between LeuRS, IleRS, and ValRS to determine the end points of the LS-domain insert (Figure 1C). Based on structural and primary sequence alignments, we deleted the entire 49 amino acid insert, which spans Val 569 to Gly 618 in *E. coli* LeuRS. The LS-domain was replaced with either three or five alanines, which link the last β-strand of the Rossmann fold to the KMSKS conserved sequence. Based on the x-ray crystal structures of LeuRS, IleRS, and ValRS, we hypothesized that these alanine linker peptides would maintain the approximate distance between the two flanking β-strands that connect the LS-domain to the main body.

The LeuRS wild type and deletion mutants were purified by affinity chromatography. Aminoacylation activity of both mutant LeuRSs that lacked the LS-domain was abolished (Figure 2A). Interestingly however, post-transfer editing activity of mischarged ile-tRNA^{Leu} was largely unaffected and similar to the LeuRS wild type enzyme (Figure 2B). Thus, the LS-domain plays a significant role in aminoacylation, but is not required for amino acid editing.

Chimeric LeuRS and ValRS mutants restore limited aminoacylation activity

We hypothesized that the short three or five alanine replacements of the LS-domain may have constrained movement of the conserved mobile KMSKS motif that is critical for aminoacylation activity (27-29). Sequence alignments with the homologous class I IleRS and ValRS enzymes identified a short peptide sequence that correlated with the location of the LS-domain insert (Figure 1C). The orientation of the short peptide in the x-ray crystal structure of *T. thermophilus* ValRS (30,31) overlapped well with the *T. thermophilus* (5) and *E. coli* (32) LeuRS catalytic core. Thus, we introduced a seven residue sequence (VLDEKGQ) from *T. thermophilus* ValRS to replace the LeuRS LS-domain (ΔLSD-valRStt). Likewise, we also swapped IRDDEGQ from *E. coli* ValRS into LeuRS (ΔLSD-valRSec).

The chimeric LeuRS that contains the *T. thermophilus* ValRS peptide stimulated some aminoacylation, albeit significantly decreased compared to the LeuRS wild type (Figure 3). Kinetic analysis showed about a 30-fold decrease in the apparent k_{cat}/K_m compared to the LeuRS wild type enzyme (Table 1). In contrast, the *E. coli* ValRS peptide replacement failed to restore aminoacylation activity. Both chimeric mutants retained substantial post-transfer editing activity relative to that of wild type enzyme (Figure 3B). The K_D was measured for the ΔLSD-valRStt deletion mutant (0.4 ± 0.1 μM) using nitrocellulose filter binding assays and was similar to the wild-type protein (0.3 μM). Interesting, the K_D was not measurable for the inactive ΔLSD-valRSec deletion mutant suggesting that enzyme-tRNA interactions are very sensitive to the sequence of this peptide linker. This supports that the difference in leucylation activities may lie within the molecular details of the LS-domain rather than its overall global domain structure.

The two ValRS peptides from *T. thermophilus* and *E. coli* are highly similar. To identify specific amino acid determinants that might be important for aminoacylation activity, we systematically swapped different amino acids within this seven amino acid insert of the chimeric LeuRS mutants that contained the non-functional *E. coli* LeuRS peptide (ΔLSD-valRSec) (Figure 4A). In particular, we substituted an arginine for a leucine (R vec L) as well as a glutamic acid for a lysine (E vec K) in the *E. coli* ValRS peptide. Both single mutations stimulated aminoacylation activity of the ΔLSD-valRSec mutant (Figure 4B). As found for the

LS-domain deletion mutants above, swapping these peptides residue(s) had minimal, if any, effects on post-transfer editing (Figure 4C).

Interestingly, the R vec L, and E vec K single mutations had K_m values of 1.6 and 1.3 μM respectively, which were comparable to the wild type enzyme (12). However, a large decrease in the apparent k_{cat} of about 25-fold results in substantially diminished activity. Combination of these two mutations (RE vec LK) showed further enhancement of aminoacylation activity compared to the single chimeric mutants (Figure 4B). While the double LeuRS mutant and chimeric ΔLSD-valRStt had a five-fold increase in the apparent k_{cat}, compared to the single mutant, this enhanced activity was diminished somewhat by a corresponding increase in apparent K_m of approximately five-fold.

We also tested whether leucylation activity of the inactive ΔLSD-valRSec could be enhanced further by swapping a third residue. Using the RE vec LK LeuRS chimeric mutant as a starting point, we swapped two homologous residues. One aspartic acid is conserved, while the second aspartic acid is replaced by a homologous glutamic acid (Figure 4A). This latter aspartic acid in the *E. coli* ValRS peptide was swapped with glutamic acid (RED vec LKE). In addition, the isoleucine was replaced with valine to yield a triple mutant, REI vec LKV. Both of the triple mutations had little, if any, affect on post-transfer editing (Figure 4D). Leucylation activity was slightly increased for the REI vec LKV mutant relative to RED vec LKE (Figure 4B). The RE vec LK double mutant exhibited lower leucylation activity compared to both the two triple mutants (RED vec LKE and REI vec LKV) and ΔLSD-valRStt, which appeared to be primarily due to an increase in the apparent K_m to 5.4 μM for $tRNA^{Leu}$. All three mutants had comparable apparent k_{cat} parameters that were about six-fold decreased compared to wild type (Table 1). Since the LeuRS mutants RED vec LKE, REI vec LKV and ΔLSD-valRStt have similar apparent K_m and k_{cat} parameters, it appears that the key residues within the context of the ValRS peptide for stimulating leucylation activity are the conserved leucine and lysine residues.

Chimeric mutations selectively impact amino acid activation or transfer to tRNA

Amino acid-dependent pyrophosphate exchange assays were carried out to test activity of the first step of the aminoacylation reaction. All of the chimeric LeuRS mutants, even ΔLSD-valRSec, which was inactive in aminoacylation, activated amino acid (Figure 5). Interestingly however, the triple mutations (REI vec LKV and RED vec LKE) as well as ΔLSD-valRStt were significantly more active in pyrophosphate exchange than the double and single mutations. Although the leucine-dependent pyrophosphate exchange activity of the triple mutations were decreased compared to the wild type LeuRS, they were similar to the ΔLSD-valRStt. This suggests that the peptide from ValRS is optimized for amino acid activation, but homologous valine and glutamic acid substitutions may actually impede transfer of the amino acid to the tRNA. Likewise, we hypothesize that although the available x-ray crystal structures fail to detect direct interactions between the LS-domain and the $tRNA^{Leu}$, this domain that is idiosyncratic to LeuRS, influences tRNA aminoacylation during catalysis.

Discussion

The LS-domain is found in most bacteria and some eukaryotic LeuRSs. Although the x-ray crystal structure of *T. thermophilus* LeuRS (5) shows that the LS-domain folds into a discrete domain, it is not conserved in sequence or size (Figure 1). Thus, it may have evolved in a species-specific manner. Herein, we determined that the LS-domain is clearly important for aminoacylation, but has little, if any, impact on amino acid editing. Although the x-ray crystal structure did not indicate any interactions between the LS-domain and the tRNA or propose obvious roles for this idiosyncratic domain during catalysis, our investigation suggests that it significantly influences tRNA interactions with the enzyme. The LS-domain may interact

directly with $tRNA^{Leu}$ during catalysis at a transient step that is not captured by the ensemble of x-ray co-crystal structures. This might include substrate binding, translocation, and/or product release.

The LS-domain may also influence critical neighboring sequences in aminoacylation. The more conserved KMSKS sequence is located in a loop just downstream of the LS-domain. The role of the mobile KMSKS motif in the class I tryosyl-tRNA synthetase (TyrRS) has been extensively analyzed via structural and biochemical investigations at various stages of the reaction (33-36). Upon ATP binding, the KMSKS loop shifts to a "closed" conformation where the two lysines interact with the α- and β-phosphates of the ATP molecule respectively, moving it closer to the active site (36). The α-phosphate group of the ATP molecule is juxtaposed to the carbonyl oxygen of the bound tyrosine ligand to facilitate adenylate bond formation (36, 37). Interestingly however, the structure of the TyrRS-adenylate complex suggests that in the closed conformation, the KMSKS would clash with the 3′ adenosine of the tRNA acceptor end (37). Thus, Kobayashi *et al* have suggested that the flexible KMSKS loop moves to a "semi-open" conformation to facilitate entry of the tRNA acceptor stem (36). Once the 3′ CCA end of the tRNA is in the active site, the KMSKS loop could again adopt the "closed" conformation and interact with the phosphate backbone of the tRNA 3′ CCA end (36).

Deletion of the entire LS-domain may hinder movement of the KMSKS motif that is required for catalysis. Replacement of the LS-domain with a short chimeric sequence that is found in another class I enzyme could better facilitate the movement of the KMSKS loop to initiate activation, but hinders KMSKS flexibility to efficiently revert to the "semi-open" conformation that allows entry of the tRNA acceptor end to the active site. Thus, the ΔLSD-valRStt and the two triple chimeric LeuRS mutants productively activated amino acids, but failed to efficiently transfer the amino acid to tRNA during aminoacylation. In LeuRS, it is possible that both the LS-domain and the KMSKS loop work in tandem to sequentially and efficiently orchestrate substrate binding for catalysis and/or product release. Kinetic analysis showed that this peptide is exquisitely sensitive to changes in k_{cat} and K_m, but in an idiosyncratic way. It is also possible that this sensitivity is amplified by the adjacent KMSKS conserved sequences that is required for aminoacylation (27,29,38,39).

Structure predictions (17) of the ValRS peptides that were inserted into the two chimeric LeuRS mutants suggest that the inserted sequence of the chimeric LeuRS folds into a short α-helix. Interestingly, this correlates with the *P. horikoshii* TyrRS structure where the sequence just upstream of the KMSKS motif also forms a helix (34). This helix has been proposed to contribute to tRNA binding (34). Structures of TyrRS from *Archaeoglobus fulgidus, P. horikoshii*, and *Aeropyrum pernix* show that the KMSKS sequence has adopted different orientations among the three organisms (34). In the tyrosine bound form, the KMSKS loop is positioned in a "closed" conformation for *A. pernix*. In contrast, the conformation of the KMSKS loop of *P. horikoshii* TyrRS correlates to the "semi-closed" form. The homologous KMSSS loop of the *A. fulgidus* TyrRS is positioned further away from the active site (34). Thus, the mobility of the KMSKS sequence required for catalysis may differ among LeuRSs with and without the LS-domain insert. Among the LeuRSs that contain the LS-domain, the mobility of the KMSKS sequence might be altered to permit cooperative movement that would enhance catalysis.

It still remains unclear why LeuRS of some organisms evolved to incorporate this compact LS-domain and others do not. Sequence alignments of corresponding LeuRSs lacking the LS-domain show that the peptide sequence before the KMSKS motif is relatively homologous among the ValRSs and IleRSs. Perhaps, the LS-domain was inserted in some, but not all LeuRSs, to confer an evolutionary advantage to tRNA selection and/or catalysis by certain LeuRSs.

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Abbreviations are as follows

aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; LS-domain, leucine-specific domain; CP1, connective polypeptide 1.

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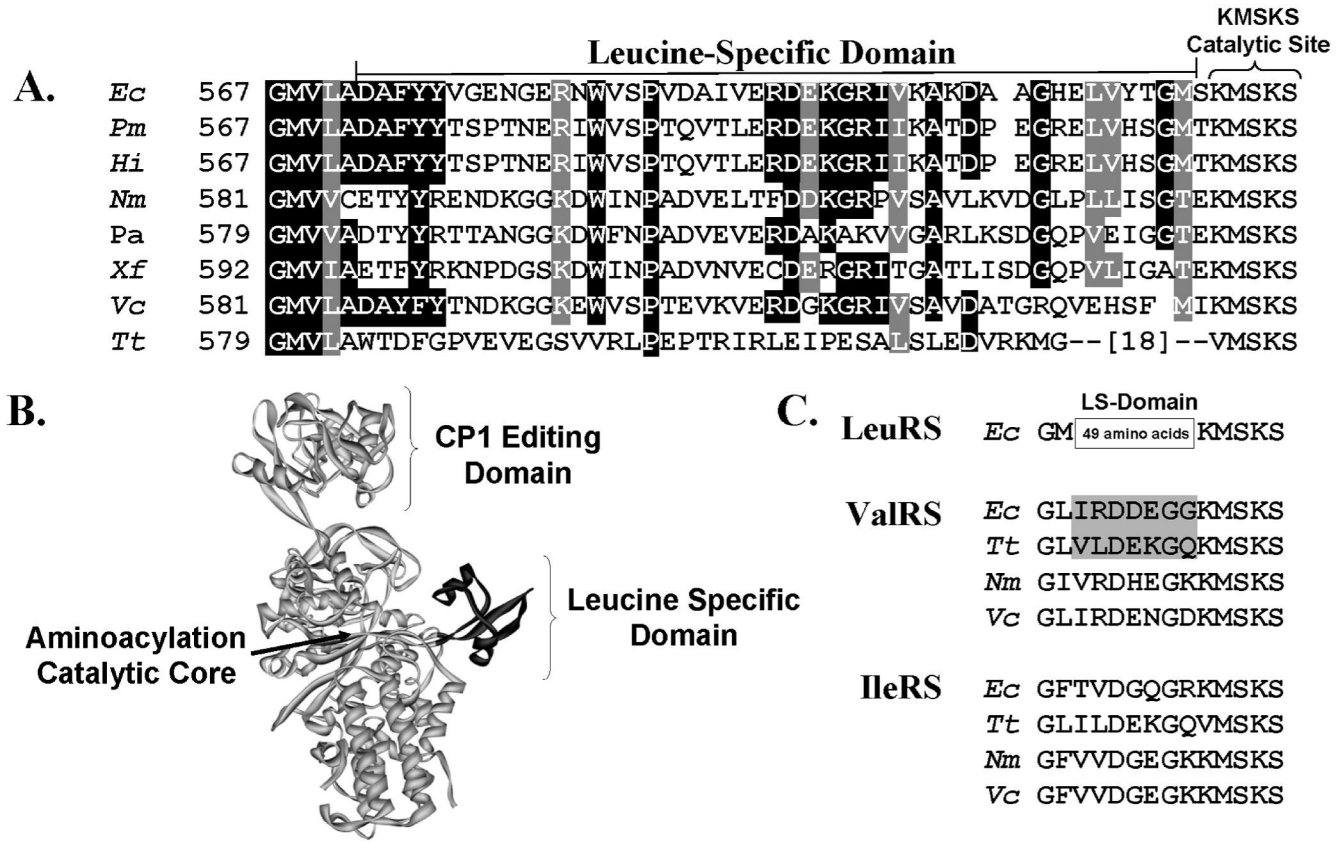

Figure 1.

Primary and Tertiary Structure of LeuRS Enzymes. (A) Primary sequence alignment of LeuRSs from various organisms. Conserved and homologous residues are highlighted in black and gray, respectively. The bar above the sequence indicates the LS-domain sequence of *E. coli* LeuRS. Abbreviations are as follows: Ec, E. coli; Pm, Pasteurella multoci; Hi, Haemophilus influenzae; Nm, Neisseria meningitidis; Pa, Pseudomonas aeruginosa; Xf, Xylella fastidiosa; Vc, Vibrio cholerae; Tt, Thermus thermophilus. (B) Homology model of *E. coli* LeuRS (32). The protein is shown as a ribbon diagram. The LS-domain is highlighted in black, while the canonical class I core and CP1 editing domain are colored in gray. The bold arrow points to the Rossmann nucleotide binding fold that comprises the aminoacylation active site. (C) Sequence alignment of LeuRS with regions of ValRS and IleRS that correlate to the insertion site of the LS-domain. The shaded box highlights the two ValRS peptides that were used to make chimeric replacements of the LS-domain in *E. coli* LeuRS.

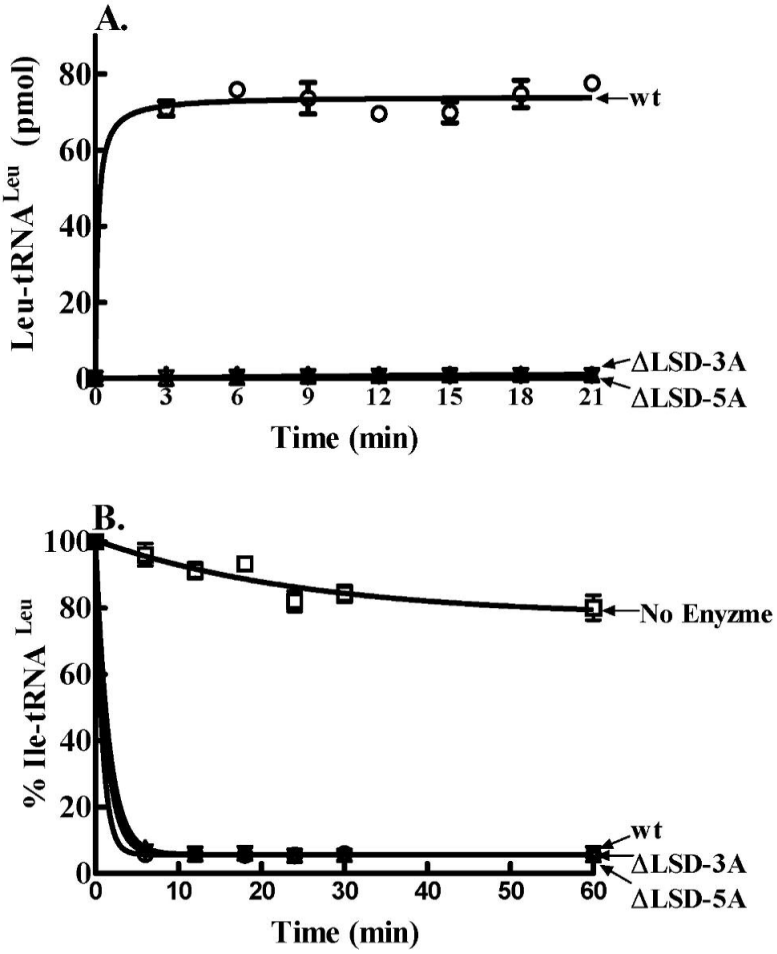

Figure 2.

Enzymatic activity of wild-type and LeuRS LS-domain deletion mutants. (A) Aminoacylation reactions were carried out using 4 μM *in vitro* transcribed tRNA^{Leu} and 100 nM enzyme. (B) Post-transfer editing reactions included approximately 6.5 μM [^{3}H]-Ile-tRNA^{Leu} and 100 nM enzyme. Symbols representing wild-type and mutant enzymes are as follows: no enzyme (□), wild-type (○), ΔLSD-5A (△), ΔLSD-3A (▽). Error bars represent the assay reproduced at least in triplicate and are presented for each point.

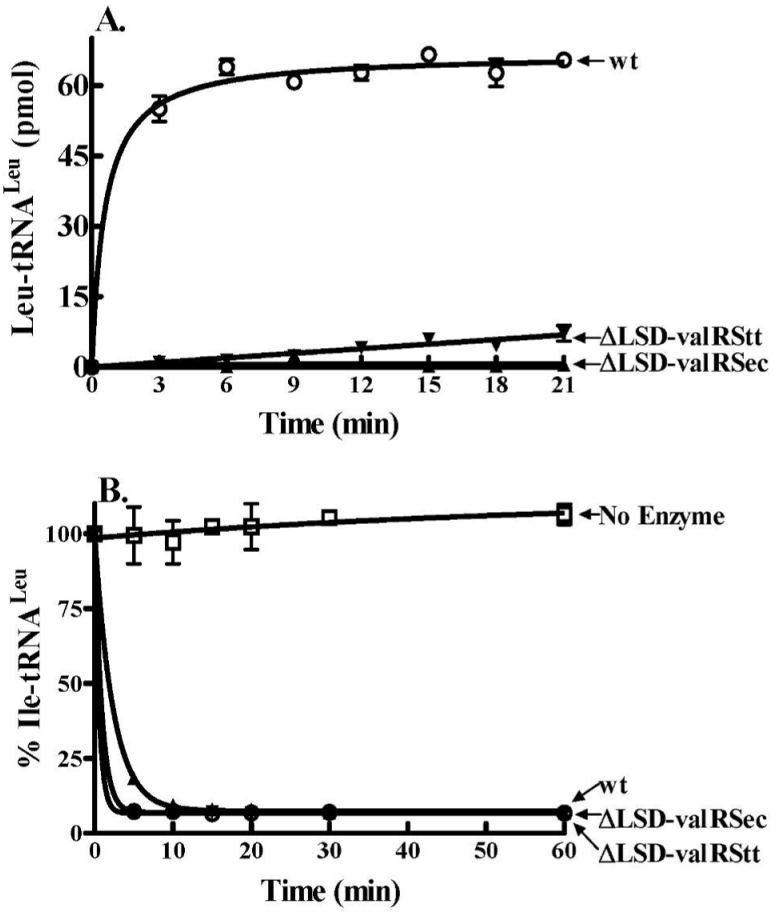

Figure 3.

Enzymatic activity of chimeric LeuRS mutants. (A) Aminoacylation reactions were carried out using 4 μM *in vitro* transcribed tRNA^{Leu} and 100 nM enzyme. (B) Post-transfer editing reactions included about 6.5 μM [^{3}H]-Ile-tRNA^{Leu} and 100 nM enzyme. Symbols representing wild-type and mutant enzymes are as follows: no enzyme (□), wild-type (○), ΔLSD-valRStt (▼), ΔLSD-valRSec (▲). Error bars represent the assay reproduced at least in triplicate and are presented for each point.

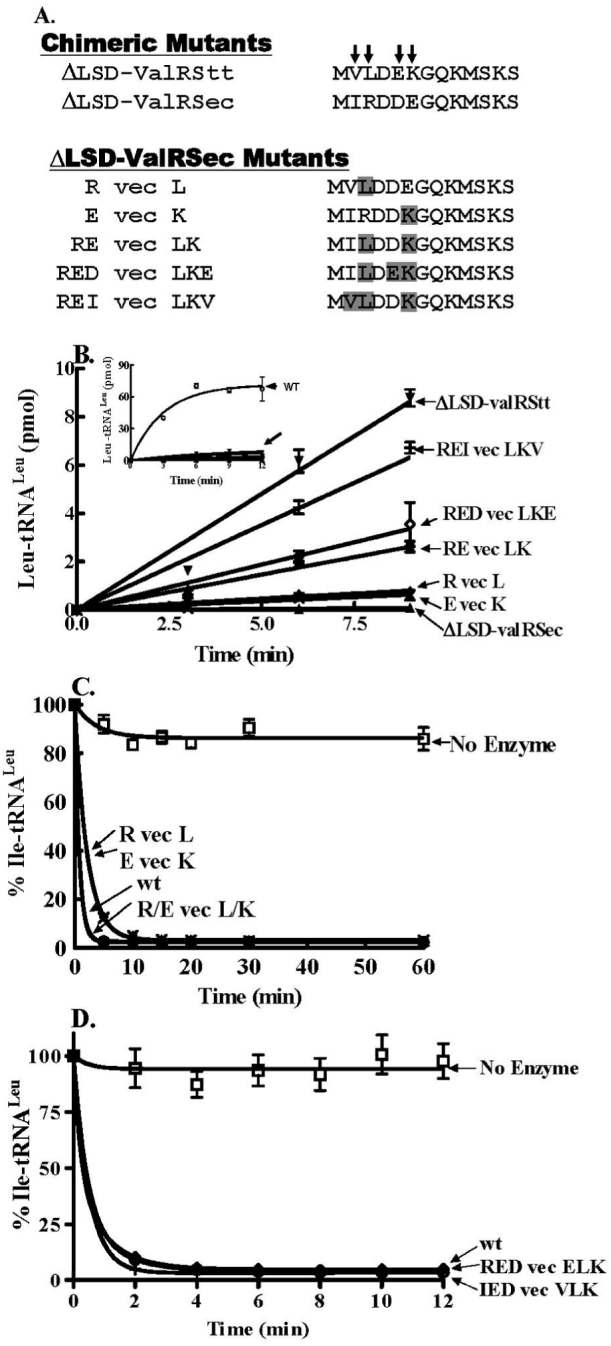

Figure 4.

Enzymatic activity of derivatives of ΔLSD-valRSec chimeric mutants. (A) Sequence of chimeric LeuRS mutants that contain a ValRS peptide insert and site-specific mutations within the inserted peptide of the ΔLSD-ValRSed chimeras. Sites that were targeted by specific substitution are indicated with arrows. Residues within the chimeric sequence ΔLSD-valRSec that were substituted by the correlating *T. thermophilus* LeuRS residue are shaded. (B) Aminoacylation activity of chimeric mutants. The reaction was carried out using 4 μM *in vitro* transcribed $tRNA^{Leu}$ and 100 nM enzyme. An insert illustrates the wild-type activity relative to the mutants. The bold arrow within the insert identifies the region of the graph that has been blown up. (C) Post-transfer editing activity of single and double mutations of ΔLSD-

valRSec. (D) Post-transfer editing activity of triple mutations of ΔLSD-valRSec. Post-transfer editing assays included about 6.5 μM [^{3}H]-Ile-tRNA^{Leu} and 100 nM enzyme. Symbols representing wild-type and mutant enzymes are as follows: no enzyme (□), wild-type (○), ΔLSD-valRStt (▼), ΔLSD-valRSec (▲), R vec L (◆), E vec K (✖), RE vec LK (✱), RED vec LKE (◇), and REI vec LKV (✚). Error bars represent the assay reproduced at least in triplicate and are presented for each point.

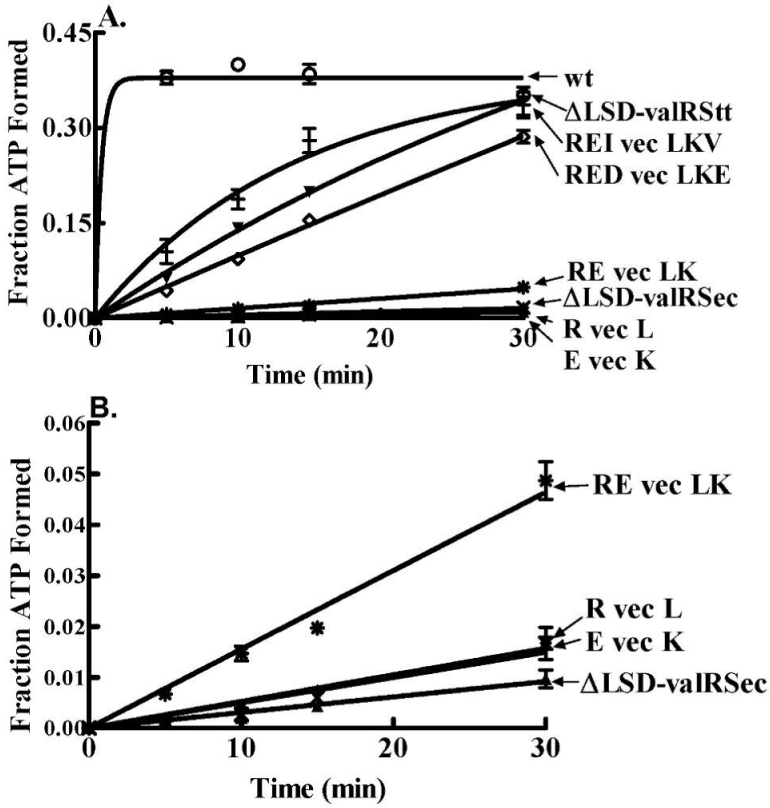

Figure 5.

Pyrophosphate exchange activity of chimeric mutants. (A) The amino acid-dependent pyrophosphate exchange reaction was carried out in the presence of 1 mM leucine, 1 mM ATP and 1 mM [^{32}P]-PP_i. (B) Weakly active mutants in A are blown up. Symbols representing wild-type and mutant enzymes are as follows: wild-type (○), ΔLSD-valRStt (▼), ΔLSD-valRSec (▲), R vec L (◆), E vec K (✖), RE vec LK (✱), RED vec LKE (◇), and REI vec LKV (✚). Error bars represent the assay reproduced at least in triplicate and are presented for each point.

Table 1

Apparent Kinetic Parameters for Aminoacylation[a]

enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1} s^{-1}$)	*relative*
Wild type LeuRS	0.73 ± 0.1	9.6 ± 2.7	13.0	1
ΔLSD-valRStt	2.0 ± 0.4	0.9 ± 0.1	0.45	0.03
R vec L	1.6 ± 0.2	0.43 ± 0.08	0.28	0.02
E vec K	1.3 ± 0.1	0.33 ± 0.05	0.25	0.02
RE vec LK	5.4 ± 0.6	2.0 ± 0.1	0.37	0.03
RED vec LKE	2.4 ± 0.4	1.4 ± 0.3	0.58	0.04
REI vec LKV	2.2 ± 0.7	1.4 ± 0.2	0.64	0.05

[a] Apparent rate constrants were determined using *in vitro* transcribed $tRNA^{Leu}$ ranging in concentration from 0.2 to 20 μM.