Modulation of Aminoacylation and Editing Properties of Leucyl-tRNA Synthetase by a Conserved Structural Module*

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Background: A structural module following the KMSKS catalytic loop is conserved in most class I synthetases.

Results: This module contributes to aminoacylation and editing of leucyl-tRNA synthetases (LeuRS).

Conclusion: This module affects the activities of LeuRS in both a structure- and sequence-dependent manner.

Significance: This work further extends the function of stem-contact fold in LeuRS.

A conserved structural module following the KMSKS catalytic loop exhibits α-α-β-α topology in class Ia and Ib aminoacyl-tRNA synthetases. However, the function of this domain has received little attention. Here, we describe the effect this module has on the aminoacylation and editing capacities of leucyl-tRNA synthetases (LeuRSs) by characterizing the key residues from various species. Mutation of highly conserved basic residues on the third α-helix of this domain impairs the affinity of LeuRS for the anticodon stem of tRNA\textsuperscript{Leu}, which decreases both aminoacylation and editing activities. Two glycine residues on this α-helix contribute to flexibility, leucine activation, and editing of LeuRS from Escherichia coli (EcLeuRS). Acidic residues on the β-strand enhance the editing activity of EcLeuRS and sense the size of the tRNA\textsuperscript{Leu}\textsubscript{D}-loop. Incorporation of these residues stimulates the tRNA-dependent editing activity of the chimeric minimalist enzyme Mycoplasma mobile LeuRS fused to the connective polypeptide 1 editing domain and leucine-specific domain from EcLeuRS. Together, these results reveal the stem contact-fold to be a functional as well as a structural linker between the catalytic site and the tRNA binding domain. Sequence comparison of the EcLeuRS stem contact-fold domain with editing-deficient enzymes suggests that key residues of this module have evolved an adaptive strategy to follow the editing functions of LeuRS.

Aminoacyl-tRNA synthetases (aaRSs)\textsuperscript{2} are a large and diverse family of enzymes that catalyze the attachment of amino acids to their cognate tRNAs in a two-step aminoacylation reaction as follows: 1) amino acid activation by ATP hydrolysis to form an aminoacyl-adenylate intermediate, and 2) transfer of the aminoacyl moiety from the intermediate to the cognate tRNA isoacceptor to form aminoacyl-tRNA (aa-tRNA) (1–3). Based on sequence homology and the structures of the catalytic active sites, aaRSs are divided into two classes of 10 members each. Class I synthetases are further divided into three subclasses, a, b, and c, according to sequence homology (4–6). Leucyl-tRNA synthetase (LeuRS) belongs to class I aaRSs that include a typical Rossmann dinucleotide-binding fold active site architecture with the signature sequence modules HIGH and KMSKS (6). According to evolutionary models, the primitive catalytic core is extended by the insertion and/or fusion of additional domains (also called modules) in LeuRSs (7), most of which have inserted a large connective polypeptide 1 (CP1) domain that is responsible for amino acid editing. To ensure translation accuracy, LeuRSs have evolved a mechanism to remove aminoacyl AMP (aa-AMP; pre-transfer editing) and aa-tRNA (post-transfer editing) (8). Although post-transfer editing is carried out by the CP1 domain in most LeuRSs, this domain has been naturally deleted in LeuRS from Mycoplasma mobile (MmLeuRS) (9), and MmLeuRS is therefore unable to maintain its catalytic fidelity in post-transfer editing functions. However, once the CP1 domain of LeuRS from Escherichia coli (EcLeuRS) is inserted into MmLeuRS, the engineered enzyme functions like a typical prokaryotic LeuRS with tRNA-dependent editing activity (10).

Additionally, LeuRSs recruit the tRNA anticodon binding domain to bind tRNA\textsuperscript{Leu} isoacceptors (11, 12). The refined crystal structure of the EcLeuRS and tRNA\textsuperscript{Leu} complex revealed a tRNA anticodon binding domain composed of a cylindrical α-helical bundle domain of five helices that is common to all class Ia synthetases. EcLeuRS establishes nonspecific backbone contacts between tRNA\textsuperscript{Leu} nucleotides 12, 13, 22–26, and 42 with amino acid residues 667–686 and 749–760 of enzyme (PDB entry 4AQ7 and 4ARC, see Ref. 11). Interestingly, a highly conserved structural module following the KMSKS loop exhibits its characteristic α-α-β-α topology in most class Ia and Ib aaRSs (Fig. 1A). Both the KMSKS loop and this structural mod-

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2 The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; CP1, connective peptide 1; LeuRS, leucyl-tRNA synthetase; LSD, leucine-specific domain; Nva, norvaline; SC-fold, stem contact-fold; PhLeuRS, P. horikoshii LeuRS; hLeuRS, human cytoplasm LeuRS; MmLeuRS, M. mobile LeuRS; EcLeuR, E. coli LeuRS; hMLeuRS, human mitochondrial LeuRS; PDB, Protein Data Bank.
ule constitute the stem contact (SC)-fold (13–18). The last α-helix of the SC-fold is almost coaxial to the first α-helix of the helix bundle domain but is separated by a short linker segment in most class I synthetases (13). However, the SC-fold of LeuRS lacks this segment, and its last α-helix instead overlaps with the first α-helix of the helix bundle domain (Fig. 1A). A previous study suggested that the effect of this class I/a/b SC-fold domain on tRNA recognition is structure-dependent rather than sequence-dependent, because sequence is much less conserved than structure in this case (17). Furthermore, the SC-fold is likely to dock against the inner side of the L-shaped tRNA, thereby positioning the anticodon stem (13).

LeuRSs also employ other appended domains, such as the C-terminal domain and leucine-specific domain (LSD), to facilitate tRNALeu binding during the tRNALeu-charging reaction (7, 20, 21). Meanwhile, elements embedded in the sequence of each tRNA promote specific interactions with its cognate aaRS (22); for example, the so-called discriminator nucleotide A73 in tRNALeu is critical for both the aminocoylation and editing activities of LeuRS (23–25). The tertiary interactions between the D- and T-loops that form the elbow region of L-shaped tRNALeu are critical elements for leucine identification (26), and the nucleotide G42 in the anticodon arms of tRNALeu from Saccharomyces cerevisiae is crucial during the editing process (27, 28). All these results were consistent with the seminal studies performed on isoleucyl-tRNA synthetase showing that the editing activity was influenced by the D-loop size of tRNAlle (29).

Here, we systematically investigated the key residues of the conserved SC-fold structural module in prokaryotic and eukaryotic LeuRSs (Fig. 1B). Crucial basic and Gly residues in the third α-helix (α3) of the SC-fold were found to modulate the aminocoylation and editing activities of LeuRS. Additionally, several key residues of helix α3 interact with the tRNALeu anticodon stem, whereas acidic residues of the β-strand control the tRNA-dependent editing activity by sensing the size of the tRNALeu D-loop. These findings were further confirmed by experiments performed on the chimeric MmLeuRS-CP1/LSD enzyme, a minimal LeuRS enzyme fused with the E. coli CP1 domain and LSD (21). The role of the β-strand in enhancing the editing activity of the chimera was further confirmed by substituting the crucial residues for the E. coli enzyme. Together, these results support an influential role for the SC-fold module in both aminocoylation and editing functions of LeuRSs.

EXPERIMENTAL PROCEDURES

Expression and Purification of LeuRSs and Their Mutants from Different Species—LeuRSs from human cytoplasm (hcLeuRS), Aquifex aeolicus (AaLeuRS), Pyrococcus horikoshii (PhLeuRS), E. coli (EcLeuRS), and M. mobile (MmLeuRS) along with their mutants were obtained and purified as reported previously by our laboratory (28, 30, 31). Final concentrations were determined using Bradford protein assays according to the manufacturer’s protocol (Bio-Rad). Mutations were engineered using the KOD Plus mutagenesis kit and confirmed by DNA sequencing (BioSun Bioscience).

Preparation of RNA Substrates—E. coli tRNALeuGAG (EctRNAGLuGA), A. aeolicus tRNALeuGAG (AarRNAleuGA), and human cytoplasmic tRNALeuGAG (hctRNAleuGA) with accepting capabilities between 1400 and 1600 pmol/A260 units were prepared from over-producing strains constructed in our laboratory (30–33). In vitro transcription of P. horikoshii tRNAGAG (PhtRNAleuGA), M. mobile tRNAGAG (MmRNAleuGA), M. mobile tRNAGAG (MmRNAleuGA), and their mutated derivatives were prepared using T7 RNA polymerase as described previously (20, 27). The MmRNAleuGA transcript and its mutated derivatives (–G12U, –G13U, –G24U, –C25U, –G39U, –C40U, –C41U, –C42U, –C41U/C42U) and the MmRNAleuGA transcript and mutant (+C17a) all had accepting activities between 1200 and 1500 pmol/A260 units. [3H]Ile–EctRNAleuGA was obtained using the editing-deficient EcLeuRS-Y330D mutant as described previously (34).

AMP Formation—Because the net effect of the editing reaction is the consumption of ATP, editing can be measured by monitoring AMP formation in the presence of a noncognate amino acid. AMP formation rates for MmLeuRS-CP1/LSD, EcLeuRS, hcLeuRS, AaLeuRS, and their mutants were measured as described previously (20, 26, 28). MmRNAleuGA and its mutants were used to study the editing ability of the chimeric MmLeuRS-CP1/LSD. The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 12 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 4 mM ATP, 10 μM tRNAleuGAG, 40 μM [3H]Ile (11 Ci/mmol), and enzyme (5 mM EcLeuRS or 20 mM MmLeuRS and their mutants). Aminocoylation activities of AaLeuRS (20 nM), PhLeuRS (100 nM), hcLeuRS (20 nM), and their mutants in the presence of their cognate tRNAs were measured as reported previously (30–33). All LeuRSs and derivatives were assayed at 37 °C with the exception of MmLeuRS (30 °C) and AaLeuRS (65 °C) and their mutants. Cognate tRNALeu concentrations ranging from 0.5 to 30 μM were used to determine the Kₘ value of enzymes for their cognate tRNALeu. For MmLeuRS, MmLeuRS-CP1/LSD and their mutants, MmRNAleuGA were used. The deacylation reaction of EcLeuRS and its mutants was measured by determining hydrolytic rates at 37 °C in 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 12 mM MgCl₂, 0.5 mM DTT, and 1 μM [3H]Ile–EctRNAleuGA (300 μCi/μmol). Reactions were initiated with enzyme diluted to 20 nm. Because radioactive Nva is commercially unavailable, [3H]Ile was used as a source to prepare mischarged tRNALeu.
and [32P]ATP. The plates were visualized by phosphorimaging and analyzed using MultiGauge version 3.0 software (Fujifilm). [32P]AMP spot gray densities were compared with those of known [32P]ATP concentrations, and rate constants (k_{obs}) were obtained by plotting [32P]AMP formation versus time.

Measurement of tRNA Equilibrium Dissociation Constants by Filter Binding Assays—EcLeuRS-[32P]tRNAGAG

EcLeuRS-[32P]tRNAGAG complex formations were monitored using the classical filter binding method of Berg and co-workers (35, 36). Radiolabeled M. mobile or E. coli [32P]tRNAGAG (60 nM, 60,000 cpm) and human cytoplasmic [32P]tRNAGAG (30 nM, 30,000 cpm) were incubated in 50 μl of binding buffer (50 mM HEPES-KOH (pH 6.8), 30 mM KCl, 12 mM MgCl2) in the presence of increasing concentrations of LeuRS proteins for 15 min at 4 °C. Samples were filtered through nitrocellulose membranes (Millipore, 0.22 μm) previously equilibrated in washing buffer consisting of 50 mM potassium phosphate (pH 5.5) and 50 mM MgCl2. Filters were then washed twice with 0.3 ml of the washing buffer and air-dried before radioactive quantification. All data were analyzed using GraphPad PRISM software.

RESULTS

Crucial Basic Residues of the Connecting Structural Module Are Essential for the Aminoacylation Activity of LeuRSs—The refined structure of EcLeuRS revealed that the third α-helix (Ser661 to Lys683; helix α3; PDB entry 4ARC and 4AQ7; Fig. 1, B and C) is the only region of the SC-fold that interacts directly with tRNA in both aminoacylation and editing conformations (11, 12), indicating that this α-helix may be critical for both processes. To investigate the effect of the α-helix on enzymatic activity, we constructed a deletion mutant lacking the third α-helix. Despite the 22-amino acid deletion, CD spectroscopy data also indicated the deletion mutant was well expressed in a soluble form and had a definite stable secondary structure (Fig. 2A). However, this construct completely lacked both aminoacylation and editing activities, supporting the hypothesis that this helix is critical for these functions (Fig. 2B and Tables 1 and 2).

Considering the interaction between tRNA and aaRS is predominantly mediated by crucial basic residues (37), in EcLeuRS, we investigated the function of residues Arg668, Lys671, and Arg672 in the third α-helix of the SC-fold by site-directed mutagenesis (PDB codes 4ARC and 4AQ7; Fig. 1, B and C). EcLeuRS-K671A did not affect the catalytic efficiency (k_{cat}/K_m) of wild-type (WT) EcLeuRS (Table 1). The k_{cat}/K_m values of the double mutant EcLeuRS-R668A/R672A were only 6.4% of that of the native enzyme, and the catalytic efficiency of the EcLeuRS-R668E/R672E double-site mutant was severely diminished, with only 1.4% of tRNA aminoacylation activity remaining (Table 1). Although mutations at Arg668 or Arg672 decreased the aminoacylation activity to different extents, all of these mutants, including the almost inactive EcLeuRS-R668E/R672E, exhibited intact Leu activation activity comparable with the WT enzyme (data not shown), suggesting these Arg residues are not involved in the amino acid activation step but rather the second tRNA transfer step. These results also suggest that the enzyme mutations alter interactions with the tRNA^{Leu}...
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that are critical for the stabilization of the transition state of the aminoacylation reaction but not for the affinity of enzyme for tRNA.

We next investigated the two crucial basic residues of helix α3 of the SC-fold of LeuRS from A. aeolicus (AaLeuRS) and M. mobile (MmLeuRS). AaLeuRS forms a unique heterodimer with its SC-fold located in the β-subunit. The counterparts of EcLeuRS-Arg⁶⁶⁸ and EcLeuRS-Arg⁶⁷² in AaLeuRS are βArg⁹⁴ and βArg⁹⁸, respectively, and mutating these residues in AaLeuRS had a similar effect on aminoacylation; AaLeuRS-βR98A did not alter the catalytic efficiency, whereas mutating βArg⁹⁴ to either Ala or Glu decreased \( k_{cat}/K_m \) values to 34 and 22% of that of WT AaLeuRS (Table 1). When the Ala mutants were combined in βR94A/R98A, the aminoacylation activity dropped to only 17% of that of WT AaLeuRS, and the double Glu mutant βR94E/R98E exhibited an even lower \( k_{cat}/K_m \) value (only 4.4% of that of WT AaLeuRS; Table 1).

In MmLeuRS, Lys⁴⁵² and Arg⁴⁵⁶ correspond to the Arg⁶⁶⁸ and Arg⁶⁷² residues of EcLeuRS, respectively. Substitution of Lys⁴⁵² with either Ala or Glu had only a minimal effect on aminoacylation activity. However the tRNA catalytic efficiency of these mutants was significantly decreased to 25% for MmLeuRS-R456A and to 21% for MmLeuRS-R456E of that of the WT MmLeuRS (Table 1). Notably, the \( K_m \) values for the above MmLeuRS mutants were not significantly altered. These results suggest that helix α3 in the SC-fold of MmLeuRS interacts with tRNA in a different manner to that of EcLeuRS and AaLeuRS.

The crucial basic residues of helix α3 in the archaeal P. horikoshii LeuRS (PhLeuRS) (PDB entry 1WZ2) and eukaryotic human cytoplasmic LeuRS (hcLeuRS) enzymes were investigated. PhLeuRS contains five positively charged residues (Lys⁶⁹², Lys⁶⁹⁶, Arg⁶⁹⁸, Lys⁶⁹⁹, and Arg⁷⁰³) in helix α3, and alanine-scanning mutagenesis revealed that the \( K_m \) value for tRNA of PhLeuRS-K696A was 0.65 mM, an ~3-fold increase compared with the WT value (0.20 μM). The \( k_{cat} \) of PhLeuRS-R703A (0.0055 s⁻¹) was much lower than that of native PhLeuRS (0.020 s⁻¹; Table 1). The other PhLeuRS mutants had no effect on tRNA charging activity.

Arg⁷⁶⁶ is the only basic residue in helix α3 of hcLeuRS SC-fold (Fig. 1C). The mutant hcLeuRS-R766A decreased the \( k_{cat}/K_m \) value to less than 10% that of the native hcLeuRS (Table 1). These results demonstrate that basic residues on helix α3 of the SC-fold are critical for catalytic efficiency, which may explain their high sequence conservation in both prokaryotic and eukaryotic LeuRSs.

Crucial Basic Residues Contribute to the Fidelity of LeuRSs—TLC assays were performed to check whether the mutations of the basic residues affected the editing activity of LeuRS against Nva. AMP formation rates (\( k_{obs} \)) of EcLeuRS-R668A/R672A and -R668E/R672E were ~11 and 9.1% that of the WT EcLeuRS in the presence of tRNA\(^{\text{Leu}}\)GAG and Nva (Table 2). The \( k_{obs} \) values of the single-site mutants EcLeuRS-R668A and EcLeuRS-R672E were 67 and 59% that of wild-type EcLeuRS in the presence of tRNA\(^{\text{Leu}}\)GAG and Nva (Table 2 and Fig. 3). Remarkably, mutating each basic residue to the acidic Glu severely impacted the AMP formation of LeuRS. The \( k_{obs} \) values of EcLeuRS-R672E and EcLeuRS-R668E were decreased significantly to 20 and 12% that of WT EcLeuRS for tRNA\(^{\text{Leu}}\)GAG in the presence of Nva (Table 2 and Fig. 3). These results suggested that the basic residues contribute to both the AMP formation and catalytic efficiency of EcLeuRS.

To identify which editing pathway was affected by these residues, we measured the \( k_{obs} \) value of the mutants in the presence of Nva but in the absence of tRNA. The \( k_{obs} \) value of the editing-defective EcLeuRS-R672A mutant was 1.6-fold that of the native enzyme in the absence of tRNA (Table 3). In contrast, \( k_{obs} \) values without tRNA for the other mutants were similar to the WT values (Table 3). These results reveal that mutation of the basic residues largely impacts the tRNA-dependent transfer editing capacity of EcLeuRS. Further decylation results indicated the impaired tRNA-dependent transfer editing of the mutants resulted from their decreased hydrolysis ability (Fig. 2B).

Finally, we probed the basic residues of AaLeuRS and hcLeuRS. Mutations of either Arg⁹⁴ or Arg⁹⁸ of AaLeuRS to Ala or Glu markedly decreased the editing activity of the enzyme, suggesting that both residues are essential for the editing process. The \( k_{obs} \) values were ~0.20 s⁻¹ in the presence of AatRNA\(^{\text{GAG}}\) and Nva, which was around 10% that of WT AaLeuRS, and the double mutant βR94E/R98E was even lower.
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TABLE 1
Kinetic constants of LeuRSs and their mutants at basic residues from various species for their cognate tRNAs in aminocacylation reaction

Kinetic constants were determined using the tRNA charging assay described under “Experimental Procedures.” All parameters are the average of three repeats with the standard deviations indicated. NM means not measurable (too low to be measured).

| Enzyme  | Mutant | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | Relative value |
|---------|--------|------------|----------------------|----------------------------------|---------------|
| EcLeuRS | WT$^a$  | 2.2 ± 0.1  | 4.9 ± 0.3            | 2.2                              | 1.0           |
|         | -R668A | 2.8 ± 0.2  | 1.4 ± 0.1            | 0.50                             | 0.23          |
|         | -R668E | 5.4 ± 0.5  | 0.59 ± 0.05          | 0.11                             | 0.050         |
|         | -R672A | 2.4 ± 0.2  | 4.8 ± 0.4            | 2.0                              | 0.91          |
|         | -R672E | 2.8 ± 0.2  | 0.53 ± 0.05          | 0.19                             | 0.086         |
|         | -R668A/R672A | 2.2 ± 0.2   | 0.31 ± 0.03          | 0.14                             | 0.064         |
|         | -R668E/R672E | 5.8 ± 0.5   | 0.18 ± 0.01          | 0.030                            | 0.013         |
|         | -K671A | 2.4 ± 0.2  | 4.8 ± 0.5            | 2.0                              | 0.91          |
|         | -hctLeuRS-R766A | 1.7 ± 0.2   | 0.56 ± 0.06          | 0.33                             | 0.090         |

| Enzyme  | Mutant | $k_d$ (μM) | $k$ (s$^{-1}$) | Relative value |
|---------|--------|------------|----------------|---------------|
| EcLeuRS | WT$^a$  | 0.30 ± 0.02 | 1.5 ± 0.3      | 5.0           |
|         | -R94A  | 0.75 ± 0.06 | 1.3 ± 0.1      | 1.7           |
|         | -R94E  | 0.81 ± 0.07 | 0.89 ± 0.09    | 1.1           |
|         | -R98A  | 0.31 ± 0.03 | 1.5 ± 0.1      | 4.8           |
|         | -R98E  | 0.39 ± 0.03 | 1.0 ± 0.1      | 2.6           |
|         | -R94A/R98A | 0.81 ± 0.08 | 0.67 ± 0.06    | 0.83          |
|         | -R94E/R98E | 0.88 ± 0.08 | 0.19 ± 0.02    | 0.22          |

| Enzyme  | Mutant | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | Relative value |
|---------|--------|------------|----------------------|----------------------------------|---------------|
| MmLeuRS | WT     | 1.5 ± 0.2  | 3.3 ± 0.2            | 2.2                              | 1.0           |
|         | -K452A | 1.7 ± 0.2  | 3.2 ± 0.2            | 1.9                              | 0.86          |
|         | -K452E | 1.9 ± 0.2  | 2.8 ± 0.1            | 1.5                              | 0.70          |
|         | -R456A | 2.3 ± 0.2  | 1.3 ± 0.1            | 0.60                             | 0.30          |
|         | -R456E | 2.5 ± 0.2  | 0.85 ± 0.49          | 0.34                             | 0.15          |

| Enzyme  | Mutant | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | Relative value |
|---------|--------|------------|----------------------|----------------------------------|---------------|
| PhLeuRS | WT$^a$  | 0.20 ± 0.14| 0.020 ± 0.003        | 0.11                             | 1.0           |
|         | -K92A  | 0.44 ± 0.04| 0.041 ± 0.004        | 0.090                            |               |
|         | -K96A  | 0.65 ± 0.10| 0.017 ± 0.002        | 0.030                            | 0.24          |
|         | -R698A | 0.52 ± 0.05| 0.067 ± 0.006        | 0.13                             | 1.2           |
|         | -K699A | 0.60 ± 0.05| 0.050 ± 0.007        | 0.080                            | 0.75          |
|         | -R703A | 0.12 ± 0.04| (5.5 ± 0.5) $\times 10^{-3}$ | 0.050                            | 0.42          |

| Enzyme  | Mutant | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | Relative value |
|---------|--------|------------|----------------------|----------------------------------|---------------|
| hclLeuRS| WT$^a$  | 0.74 ± 0.05| 2.6 ± 0.2            | 3.5                              | 1.0           |
|         | -R766A | 1.7 ± 0.2  | 0.56 ± 0.06          | 0.33                             | 0.090         |

$^a$ Data are from Ref. 10.

Basic Residues Participate in the LeuRS Affinity for the Anticodon Stem of tRNA$^{Leu}_{Leu}$—Filter binding assays revealed that the lower catalytic efficiencies ($k_{cat}/K_m$) of the above mutants were due to a lower affinity for tRNA$^{Leu}_{Leu}$. Indeed, the dissociation constants ($k_d$) of native EcLeuRS and hclLeuRS for their cognate tRNAs were 0.20 and 0.18 μM, respectively, and this was increased more than 10-fold for EcLeuRS-R668A/R672A and 27-fold for EcLeuRS-R668E/R672E (Table 4). The $k_d$ of mutant hclLeuRS-R766A was 15-fold higher than hclLeuRS for hctRNA$^{Leu}_{Leu}$ (Table 4). As the $k_d$ value reflects the affinity of LeuRS for tRNA$^{Leu}_{Leu}$ in the ground state before the aminocacylation catalysis, these results indicated that these residues were involved in the initial binding of enzyme with tRNA$^{Leu}_{Leu}$. Moreover, the unchanged $K_m$ values of mutants for tRNA$^{Leu}_{Leu}$ suggested that the affinity of enzyme for tRNA$^{Leu}_{Leu}$ in the transition state of the aminocacylation reaction was unaffected.

As the refined structure of the EcLeuRS-EctRNA$^{Leu}_{UAA}$ complex (PDB codes 4ARC and 4AQ7) displayed disorder in the anticodon loop, there was no clear evidence to show whether residues Arg$^{766}$ and Arg$^{672}$ from helix α3 of the SC-fold participated in interactions with the D-stem and anticodon stem of EctRNA$^{Leu}_{UAA}$ (11). In an attempt to verify and identify the determinants of the editing reaction on the tRNA, we constructed a set of mutants of tRNA$^{Leu}_{Leu}$. Because EcLeuRS recognizes the synthetic transcript of MmRNA$^{Leu}_{UAA}$ produced in vitro as well as the fully modified EctRNA$^{Leu}_{UAA}$ purified from an E. coli over-producing strain ($k_{cat}/K_m = 2.6$ and $2.2$ μM$^{-1}$ s$^{-1}$, respectively), we chose to mutate the MmRNA$^{Leu}_{UAA}$ transcript, which is...

(0.13 s$^{-1}$; Table 2). Consistently, mutation of the unique basic residue Arg$^{766}$ to Ala in hclLeuRS (hclLeuRS-R766A) led to a 90% decrease in AMP formation in the presence of hctRNA$^{Leu}_{Leu}$ (Table 2). Overall, our results show that both prokaryotic and eukaryotic LeuRSs employ crucial basic residues of helix α3 of the SC-fold to modulate the catalytic efficiency and fidelity of the enzyme.
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### TABLE 4

Dissociation constants of LeuRSs and their mutants from *E. coli* and human cytoplasm for their cognate tRNAs.

| Enzyme                  | $k_d$ (µM) | Relative value |
|-------------------------|------------|---------------|
| EcLeuRS                 | 0.20 ± 0.04| 1.0           |
| -R668A                  | 0.46 ± 0.04| 2.3           |
| -R672E                  | 0.85 ± 0.07| 4.3           |
| -R668A/R672A            | 2.3 ± 0.2  | 12            |
| -R668E/R672E            | 5.3 ± 0.5  | 27            |
| -R668E                  | 0.50 ± 0.05| 2.5           |
| -R672E                  | 0.24 ± 0.02| 1.2           |
| -G662P/G665P            | 0.039 ± 0.005| 0.20      |
| -L656K/E657K            | 0.24 ± 0.02| 1.2           |
| hcLeuRS                 | 0.18 ± 0.02| 1.0           |
| -R766A                  | 2.74 ± 0.25| 15            |

Data are from Ref. 10.

Remarkably similar in sequence to *EctRNA*$_{GAG}^{Leu}$ (Fig. 4), we constructed seven mutants with specific nucleotide substitutions in the stems of *MmntRNA*$_{UAA}^{Leu}$ -G24U and -C25U in the D-stem and -U39G, -C40U, -C41U, -C42U, and -C41U/C42U in the anticodon stem (Fig. 4). The catalytic efficiency ($k_{cat}/K_m$) in the aminoclaylation reaction and the $k_{obs}$ in the editing reaction were measured, and the values for WT *MmntRNA*$_{UAA}^{Leu}$ and the -G24U, -C25U, and -U39G mutants were indistinguishable, indicating that these nucleotides do not critically interact with LeuRS (data not shown). However, the catalytic efficiencies ($k_{cat}/K_m$) of EcLeuRS for the -C40U, -C41U, and -C42U variants were 73, 51, and 26% that of EcLeuRS for WT *MmntRNA*$_{UAA}^{Leu}$ (Table 5). Consistently, the $k_{cat}/K_m$ value of the EcLeuRS for double mutant -C41U/C42U was even lower (12% that of WT *MmntRNA*$_{UAA}^{Leu}$), with a value of 0.31 µM$^{-1}$ s$^{-1}$ (Table 5).

Further analysis revealed that the decreased catalytic efficiency may result from an altered affinity for the tRNA substrate. In the filter binding assay, the $k_d$ values of EcLeuRS for *MmntRNA*$_{UAA}^{Leu}$ -C41U (1.7 µM) and -C42U (1.6 µM) mutants were ~5-fold greater than that of WT *MmntRNA*$_{UAA}^{Leu}$ (0.27 µM; Table 4). In addition, we showed that the double mutant *MmntRNA*$_{UAA}^{Leu}$ -C41U/C42U was even less capable of editing. In the presence of 15 mM Nva, the $k_{obs}$ value of *MmntRNA*$_{UAA}^{Leu}$ -C41U/C42U was 1.4 s$^{-1}$, compared with 2.6 s$^{-1}$ for WT *MmntRNA*$_{UAA}^{Leu}$ (Table 4). Together, these results suggest that LeuRS employs these basic residues of the SC-fold domain to modulate both aminoclaylation and editing activities through interaction with the anticodon stem of tRNA*$^{Leu}$.

Two Gly Residues in Helix α3 Participate in the Aminoclaylation and Editing Functions of LeuRS—Analysis of the SC-fold of EcLeuRS showed that both the β-strand and helix α3 undergo substantial conformational changes during aminoclaylation and editing (PDB code 4AQ7 and 4ARC; Fig. 5A). Mutating the first two Gly residues of α3 to Ala (EcLeuRS-G662A/G665A) was accompanied by a substantial drop in aminoclaylation activity (Table 6), whereas mutating to Pro did not impact the aminoclaylation activity of EcLeuRS-G662P or EcLeuRS-G665P (Table 6). However, the catalytic efficiency of the double mutant EcLeuRS-G662P/G665P was decreased to 9.5% that of WT EcLeuRS with *EctRNA*$_{GAG}^{Leu}$, but exhibited similar secondary structure with WT EcLeuRS (Table 6 and Fig. 2A).

The mutations also diminished the Leu activation activity, as measured using the ATP-PPi exchange reaction (Fig. 4B). Compared with the $k_{obs}$ of WT EcLeuRS (57 s$^{-1}$), the activity of the double mutant G662P/G665P (3.6 s$^{-1}$) was impacted to a much greater extent than the double mutant G662A/G665A (16.5 s$^{-1}$). These results suggest that the decreased aminoclaylation activity may be the consequence of a reduction in the formation of adenylate. Additionally, these double mutants were also shown to be defective in their editing capacity. The $k_{obs}$ value of the double mutant G662P/G665P in the presence of Nva and *EctRNA*$_{GAG}^{Leu}$ was only 9.4% that of the WT enzyme. Mutation G665P alone reduced the $k_{obs}$ to 12% that of the WT enzyme, whereas the G662P mutation had only a minor
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FIGURE 5. Effects of mutating the helix α3 Gly residues on synthetic and editing activities. A, structure of the EcLeuRS-ECtRNA^Leu^u^A^ complex in aminoacylation (pink) and editing (cyan) conformations (PDB codes 4AQ7 and 4ARC, respectively) (11). Helix α3 residues Gly^G662^ and Gly^G665^ are numbered and highlighted in red and pink. Arrows indicate conformational changes of β-strand of the SC-fold facilitated by the Gly residues. B, Leu activation properties measured in the ATP/PPi exchange reaction with 10 nM EcLeuRS, EcLeuRS-G662A/G665A, and EcLeuRS-G662P/G665P. Leu was used at a final concentration of 5 mM. C, AMP formation assays used to measure total editing activity of 1 μM EcLeuRS, EcLeuRS-G662P, and EcLeuRS-G665P in the presence of 5 μM EcEtRNA^Leu^u^A^ and 15 mM Nva. k_{obs} values of AMP formation are reported in Table 7. D, structure of MmLeuRS (gray; PDB code 3ZIU (39). Residues shown on helix α3 (cyan) are equivalent to Gly^G662^ and Gly^G665^ of EcLeuRS. Other helices of the SC-fold are represented in pink, and the β-strand is in red.

TABLE 5
Various parameters of EcLeuRS for MmEtRNA^Leu^ and its derived variants

The K_{cat} and k_{cat} values were determined using the tRNA charging assay described under “Experimental Procedures.” The k_{obs} values are the observed rate constants for AMP formation with Nva and MmEtRNA^Leu^GAG. The k_{d} values are the dissociation constants of the EcLeuRS and MmEtRNA^Leu^GAG complex determined by filter binding assays in the presence of 0.01 to 10 μM EcLeuRS. Parameters are the average of three repeats with the standard deviations indicated.

| MmEtRNA^Leu^GAG | K_{cat} | k_{cat} | k_{cat}/K_{cat} | Relative value | k_{obs} of AMP formation | k_{d} |
|------------------|--------|--------|----------------|---------------|-------------------------|------|
| WT               | 1.6 ± 0.1 | 4.2 ± 0.5 | 2.6 | 1.0 | 2.6 ± 0.3 | 0.27 ± 0.03 |
| -G12U            | 1.7 ± 0.2 | 3.9 ± 0.3 | 2.3 | 0.88 | 2.7 ± 0.2 | 0.27 ± 0.02 |
| -G13U            | 1.7 ± 0.2 | 4.0 ± 0.5 | 2.4 | 0.92 | 2.6 ± 0.2 | 0.29 ± 0.03 |
| -C40U            | 1.5 ± 0.1 | 3.5 ± 0.3 | 1.9 | 0.73 | 2.4 ± 0.3 | 0.38 ± 0.02 |
| -C41U            | 1.4 ± 0.1 | 1.9 ± 0.1 | 1.3 | 0.51 | 2.5 ± 0.3 | 1.7 ± 0.2 |
| -C42U            | 2.7 ± 0.2 | 1.8 ± 0.1 | 0.67 | 0.26 | 1.9 ± 0.2 | 1.6 ± 0.2 |
| -C41U/C42U       | 4.2 ± 0.4 | 1.3 ± 0.1 | 0.31 | 0.12 | 1.4 ± 0.1 | 1.6 ± 0.2 |
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**TABLE 6**

Kinetic constants of LeuRSs from various species and their mutants at Gly residues for their cognate tRNAs in aminoacylation reaction

| Enzymes | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | Relative value |
|---------|------|--------|-------------|----------------|
| EcLeuRS  | 2.2 ± 0.1 | 4.9 ± 0.3 | 2.2 | 1.0 |
| EcLeuRS G665P  | 1.1 ± 0.3 | 2.2 ± 0.3 | 2.0 | 0.91 |
| EcLeuRS G662P  | 1.3 ± 0.1 | 2.1 ± 0.1 | 1.6 | 0.73 |
| MmLeuRS  | 1.2 ± 0.1 | 0.70 ± 0.07 | 0.58 | 0.26 |
| MmLeuRS G665P  | 1.4 ± 0.2 | 0.30 ± 0.03 | 0.21 | 0.095 |
| MmLeuRS G662P  | 1.3 ± 0.1 | 0.80 ± 0.08 | 0.61 | 0.28 |
| PhLeuRS  | 0.2 ± 0.01 | 0.020 ± 0.003 | 0.11 | 1.0 |
| PhLeuRS G695P  | 0.09 ± 0.05 | 0.010 ± 0.001 | 0.053 | 0.48 |
| hcLeuRS  | 0.74 ± 0.05 | 2.62 ± 0.2 | 3.5 | 1.0 |
| hcLeuRS G763P  | 0.61 ± 0.06 | 1.12 ± 0.2 | 1.8 | 0.51 |

* Data are from Ref. 10.

**TABLE 7**

Observed rate constants of EcoliLeuRS and its mutants at G of helix α3, crucial residues in β-sheet for AMP synthesis in the presence of Nva

| LeuRS | $-E_{tRNA}^{tRNA\text{Leu}}$ | Relative value | $+E_{tRNA}^{tRNA\text{Leu}}$ | Relative value |
|-------|--------------------------|---------------|-----------------|---------------|
| EcLeuRS  | 0.33 ± 0.04 | 1.0 | 3.4 ± 0.5 | 1.0 |
| -G662P  | 0.29 ± 0.03 | 0.88 | 2.4 ± 0.3 | 0.70 |
| -G665P  | 0.059 ± 0.007 | 0.18 | 0.42 ± 0.05 | 0.12 |
| -G662P/G665P  | 0.027 ± 0.003 | 0.082 | 0.32 ± 0.03 | 0.094 |
| -D653K  | 0.33 ± 0.03 | 1.0 | 1.2 ± 0.1 | 0.45 |
| -L656K/E657K  | 0.30 ± 0.03 | 0.91 | 0.31 ± 0.03 | 0.091 |

* Data are from Ref. 10.

impact (Table 7 and Fig. 5C). Additionally, the post-transfer editing activity of mutation G665P was also decreased (Fig. 2B). These observations suggest that the editing-defective mutant EcLeuRS-G665P, which exhibited a standard synthetic efficiency, could be used to produce misacetylated aa-tRNA<sub>Leu</sub> molecules.

To further explore the hypothesis that Gly residues might facilitate conformational changes of the SC-fold module during the transition between the aminoacylation and editing stages, we examined the sequences of PhLeuRS and hcLeuRS. Both enzymes contain a single Gly in helix α3, albeit at different positions (PDB code 1W2Z; Fig. 1C). Mutations to Pro in PhLeuRS-G695P decreased the catalytic efficiency of aminoacylation to 5.3% of that of WT PhLeuRS. Similarly, a mutant replaced Gly<sup>663</sup> of hcLeuRS with Pro, and hcLeuRS-G763P had only half-catalytic efficiency of hcLeuRS (Table 6). Although not conserved, these data suggest that the Gly residues play a substantial role in retaining aminoacylation activity.

Sequence alignment revealed that MmLeuRS lacks any Gly residues in helix α3; the two Gly residues found in EcLeuRS are replaced by Thr and Ala (PDB code 3ZlU; Figs. 1C and 5D). Additionally, the β-strand of the SC-fold of MmLeuRS contains two Lys residues, whereas other LeuRS enzymes usually contain two or three acidic residues in this vicinity (Fig. 1C). This suggests that MmLeuRS employs a different strategy for interaction with its cognate tRNA, which may be linked to the absence of the CP1 domain and its associated editing function. Indeed, without the need to transfer the tRNA from the synthetic to the editing site, the role of the SC-fold may have diverged in these enzymes, and this may explain the altered charge distribution and the absence of flexibility-inducing Gly residues.

**β-Strand of the SC-fold Participates in the Editing Function of LeuRS**—To study the role of the key residues of the SC-fold β-strand, we replaced residues Asp<sup>653</sup>, Gln<sup>657</sup>, and Leu<sup>656</sup> of EcLeuRS with Lys. In the aminoacylation reaction, the catalytic efficiency of mutant EcRNA<sub>Leu</sub><sup>666/665A/667</sup>-D653K and double mutant EcRNA<sub>Leu</sub><sup>666/667</sup>-L656K/E657K was not significantly different from that of the native enzyme (Table 8). However, in the editing step, in the presence of Nva and tRNA, the $k_{obs}$ value of EcRNA<sub>Leu</sub><sup>666/665A/667</sup>-D653K dropped to 45% that of WT EcLeuRS for AMP formation, which was only half that of the native enzyme (Table 7). For the double mutant L656K/E657K, the $k_{obs}$ value was decreased to 9.1% that of WT EcLeuRS in the presence of tRNA, which suggested that the mutant had lost its tRNA-dependent editing activity (Fig. 6A and Table 7). Therefore, we performed an Ile-tRNA<sup>Leu</sup> deacylation assay in the presence of mutant D653K or L656K/E657K. The deacylation curves showed that both mutants were severely affected in their capacity to deacetyl mischarged EcRNA<sub>Leu</sub><sup>666/665A/667</sup>, suggesting that the SC-fold β-strand is involved in tRNA-dependent editing of LeuRS following the transfer step (Fig. 6B).

In the crystal structure of the complex containing EcLeuRS and EcRNA<sub>Leu</sub><sup>UAA</sup> (11), nucleotides G12 and G13 are located in the vicinity of the β-strand of the SC-fold. Although these residues are not within bonding distance of the acidic residues 653 and 657, we mutated them to U in MmRNA<sub>Leu</sub><sup>UAA</sup> to probe their role during the complete aminoacylation/editing cycle. However, both aminoacylation and editing activity properties were indistinguishable from the WT tRNA (Table 4).

We further explored the possibility that, due to its appropriate placement in the structure of the complex, the SC-fold may sense the size of the D-loop during the editing step. We chose to use a different MM tRNA<sup>Leu</sup> with a CAA anticodon that contains only 10 nucleotides in the D-loop instead of the usual 11 nucleotides in EcRNA<sub>Leu</sub><sup>Leu</sup> and MmRNA<sub>Leu</sub><sup>Leu</sup> (Fig. 4). Remarkably, during the editing reaction, MmRNA<sub>Leu</sub><sup>Leu</sup> could not stimulate AMP formation as well as MmRNA<sub>Leu</sub><sup>UAA</sup>; in the presence of Nva and EcLeuRS, the $k_{obs}$ value was only 0.91 s<sup>-1</sup> compared with 2.6 s<sup>-1</sup> for both MmRNA<sub>Leu</sub><sup>UAA</sup> and EcRNA<sub>Leu</sub><sup>UAA</sup>. This result strongly suggested that the size of the D-loop is a key determinant of editing activity. To confirm this hypothesis, we inserted in the D-loop after cytosine 17 of MmRNA<sub>Leu</sub><sup>UAA</sup> (17a), just before the crucial nucleotides G18 and G19. In the resulting mutant MmRNA<sub>Leu</sub><sup>UAA</sup> (C17a), the loop was identical to that of MmRNA<sub>Leu</sub><sup>UAA</sup> and EcRNA<sub>Leu</sub><sup>UAA</sup> (Fig. 4). Consistently, in the presence of Nva, the $k_{obs}$ value of EcLeuRS for mutant (C17a) was increased to 2.1 s<sup>-1</sup>, reaching a level comparable with MmRNA<sub>Leu</sub><sup>UAA</sup> and EcRNA<sub>Leu</sub><sup>UAA</sup> (Tables 7 and 9). This result further indicates that the β-strand of the SC-fold may participate in editing by sensing the size of the tRNA D-loop. The editing activity of EcLeuRS was magnified when the native size of the D-loop of tRNA<sup>Leu</sup> was introduced into MmRNA<sub>Leu</sub><sup>UAA</sup>. These results show that the β-strand adjacent to the SC-fold influences the editing activities of EcLeuRS, probably via the conserved acidic residues.
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### Table 8

| Enzymes                  | LeuRS     | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹ μM⁻¹) | Relative value |
|--------------------------|-----------|------------|-----------------|--------------------------|---------------|
| EcLeuRS                  | WT        | 2.2 ± 0.1  | 4.9 ± 0.3       | 1.4                      | 0.64          |
|                          | -L656K/E657K | 0.66 ± 0.07 | 3.1 ± 0.3       | 1.4                      | 0.64          |
| MmLeuRS-CP1/LSD          | WT        | 2.8 ± 0.3  | 2.1 ± 0.2       | 0.78                     | 1.0           |
|                          | -K440E/K441E | NM       | NM              | NM                      | NM            |
|                          | -K440L/K441E | 5.2 ± 0.5 | 0.69 ± 0.02     | 0.13                     | 0.33          |

*Data are from Ref. 10.

### Table 9

| LeuRS                  | MmtrNA$^{tRNA}_{C_A}$ | $k_{obs}$ of AMP formation (s⁻¹) | Relative value |
|------------------------|-----------------------|----------------------------------|---------------|
| EcLeuRS                | +WT                   | 0.53 ± 0.04²                   | 1.0           |
|                        | (+C17a)               | 0.91 ± 0.10                    | 2.8           |
| MmLeuRS-CP1/LSD        | +WT                   | (3.7 ± 0.8) x 10⁻²             | 1.0           |
|                        | (+C17a)               | (4.1 ± 0.8) x 10⁻²             | 1.1           |
| MmLeuRS-CP1/LSD-K440L/K441E | +WT       | 0.10 ± 0.04                    | 2.7           |
|                        | (+C17a)               | 0.11 ± 0.02                    | 3.0           |

*Data are from Ref. 10.

### Figure 6

**Residues of the SC-fold β-strand are involved in tRNA-dependent editing.** A, TLC showing AMP formation catalyzed by 0.2 μM EcLeuRS and EcLeuRS-L656K/E657K in the presence of 5 μM EtRNA$^{tRNA}_{C_A}$ and 15 mM Nva. $k_{obs}$ values are reported in Table 7. B, deacylation curves of [3H]Ile-EtRNA$^{tRNA}_{C_A}$ (1 μM) induced by EcLeuRS enzymes (5 nM). No enzyme ( ), EcLeuRS ( ), EcLeuRS-D653K ( ), and EcLeuRS-L656K/E657K ( ).

### DISCUSSION

Conserved SC-fold Is Crucial for Both Aminoacylation and Editing of LeuRS—Systematic comparison of class I synthetases identified a region connecting the Rossmann fold and the anticodon binding domain (7, 15–18) named the SC-fold. This module is poorly conserved in sequence and is widely considered to function in a structure-dependent manner rather than in a sequence-dependent manner. However, this study showed that several crucial SC-fold residues contribute to both aminoacylation and editing functions of LeuRS and interact specifi-
Physically with the tRNA. Although archaeal and eukaryotic cytoplasmic LeuRSs are architecturally distinct from bacterial LeuRSs (38), all share crucial elements, including one or two crucial basic residues on helix α3 of the SC-fold. Interestingly, many bacterial and archaeal LeuRSs contain additional basic residues, such as EcLeuRS that harbors an extra Lys residue at position 671 between the two crucial basic residues. However, mutation of this additional basic residue had no effect on the aminoacylation activity of the enzyme (Table 1). Moreover, of the five basic residues on helix α3 of PhLeuRS, only Arg703 and Lys696 were essential for catalytic activity. In contrast, hcLeuRS contains only one basic residue (Arg766) that is involved in both synthetic and editing catalytic steps. These results suggest that despite the evident separation in the three kingdoms of life, evolutionary pressure has caused the SC-fold to retain these functional elements.

Specific Gly residues form the second critical element that modulates the aminoacylation and editing properties of LeuRS, and mutation of these residues affected the Leu activation activity in EcLeuRS, suggesting the synthetic active site pocket may undergo conformational changes during catalysis. In this study we identified a mutant (EcLeuRS-G665P) with diminished editing properties reminiscent of a previously characterized CP1-editing site mutant (EcLeuRS-Y330D) (34). Compared with the basic residues, mutants with impaired aminoacylation activity, both EcLeuRS-G665P and EcLeuRS-Y330D, retained tRNA aminoacylation activity, and these variants could therefore be used in the future to mischarged aa-tRNA<sub>Leu</sub> for deacylation activity, and these variants could therefore be.

![Graphical representation of](image)

**FIGURE 7. Editing of the engineered MmLeuRS-CP1/LSD for MmRNA<sub>Leu</sub> and mutated derivatives.** A, AMP formation assay used to measure total editing activity of 1 μM MmLeuRS-CP1/LSD and mutated derivative K440L/K441E in the presence of 5 μM MmRNA<sub>Leu</sub> and 15 mM Nva. B, graphical representation of AMP formation as a function of time for MmLeuRS-CP1/LSD and derivative K440L/K441E. C, AMP formation assays used to measure total editing activity with 1 μM MmLeuRS-CP1/LSD-K440L/K441E in the presence of 5 μM MmRNA<sub>Leu</sub> and mutant (+ C17a) with 15 mM Nva. D, graphical representation of AMP formation as a function of time for MmRNA<sub>Leu</sub> and mutant (+ C17a). k<sub>off</sub> values for AMP formation were calculated from the slopes (Table 9).

- **Enzymes Lacking the SC-fold Crucial Residues Exhibit Impaired Editing Capacity**—To date, only two LeuRSs lacking the editing activity have been described (9, 10, 38, 39) as follows: MmLeuRS lacks the entire CP1 editing domain, and human mitochondrial LeuRS (hmLeuRS) has lost only the catalytic residues of the CP1 domain. As a consequence, both enzymes are deprived of post-transfer editing activity. Sequence alignment revealed that neither MmLeuRS nor hmLeuRS contain the Gly or acidic residues found in helix α3 of the SC-fold (Fig. 1C). Additionally, hmLeuRS utilizes a Thr instead of the first Gly of helix α3, and it has a Leu in place of the crucial Glu in the β-strand. The lack of editing activity in these enzymes further suggested that they may be functionally important and may be involved in mediating communication between the SC-fold and the CP1 editing domain.

- **Improving the Aminoacylation Fidelity by Modifying the Editing Determinants of Both Synthetase and tRNA**—The discovery of residues of the SC-fold that influence the editing capacity of LeuRS and the interaction with the anticodon stem and D-loop of tRNA<sub>Leu</sub> provide valuable new information that may be of use in the engineering of both synthetase enzymes and tRNAs. Incorporation of two key EcLeuRS β-strand residues, including a conserved acidic residue, into the chimeric MmLeuRS-CP1-LSD enzyme significantly enhanced the editing activity, which opens up exciting possibilities for improving the fidelity of this and other artificial synthetases.

Similarly, we engineered the tRNA to improve the fidelity of LeuRS by enhancing the tRNA-dependent editing capacity. We
observed that the isoacceptor MtrnaLeu

C_{AAA} was only weakly able to stimulate the editing activity of the chimeric LeuRS, compared with other isoacceptors. By inserting a single nucleotide in the D-loop of MtrnaLeu

C_{AAA} that is naturally smaller than the D-loop of other tRNA

Leu molecules, the editing activity of the synthetic MmLeuRS enzyme was markedly enhanced. In concert with enzyme engineering, such tRNA modification approaches are paving the way for improving the fidelity of artificial synthetase enzymes. Although error-prone systems are presumably advantageous for proteome diversity in some living organisms (9, 19), constructing artificial enzymes with high fidelity from naturally occurring enzymes lacking proof-reading activity remains challenging. Remarkably, the work described here suggests that at least two nonexclusive pathways may exist to achieve this goal.

In summary, this study reveals the functional importance of the conserved SC-fold module in both the aminoacylation and editing activities of LeuRS enzymes. The SC-fold is therefore not only a connecting linker between the tRNA-binding module and the catalytic site. Furthermore, the SC-fold interacts with the inner side of the L-shaped tRNA, thereby positioning the anticodon stem. Given that the KMSKS motif is presumed with the inner side of the L-shaped tRNA, thereby positioning the conserved SC-fold module in both the aminoacylation and reading activity remains challenging. Remarkably, the work described here suggests that at least two nonexclusive pathways may exist to achieve this goal.

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