THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 19, pp. 12256–12267, May 8, 2015

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

Received for publication, January 19, 2015, and in revised form, March 26, 2015 Published, JBC Papers in Press, March 27, 2015, DOI 10.1074/jbc.M115.639492

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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 $tRNA^{Leu}$, 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* (*Ec*LeuRS). Acidic residues on the β-strand enhance the editing activity of *Ec*LeuRS and sense the size of the $tRNA^{Leu}$ 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 *Ec*LeuRS. 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 *Ec*LeuRS 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)[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* (*Mm*LeuRS) (9), and *Mm*LeuRS is therefore unable to maintain its catalytic fidelity in post-transfer editing functions. However, once the CP1 domain of LeuRS from *Escherichia coli* (*Ec*LeuRS) is inserted into *Mm*LeuRS, 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 $tRNA^{Leu}$ isoacceptors (11, 12). The refined crystal structure of the *Ec*LeuRS and $tRNA^{Leu}$ 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. *Ec*LeuRS establishes nonspecific backbone contacts between $tRNA^{Leu}$ 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 the characteristic α-α-β-α topology in most class Ia and Ib aaRSs (Fig. 1*A*). Both the KMSKS loop and this structural mod-

* This work was supported by National Key Basic Research Foundation of China Grant 2012CB911000, Natural Science Foundation of China Grant 31130064, and Committee of Science and Technology in Shanghai Grant 12JC1409700.

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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; *Ph*LeuRS, *P. horikoshii* LeuRS; hcLeuRS, human cytoplasm LeuRS; *Mm*LeuRS, *M. mobile* LeuRS; *Ec*LeuR, *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. 1*A*). A previous study suggested that the effect of this class Ia/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 $tRNA^{Leu}$ binding during the $tRNA^{Leu}$-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 $tRNA^{Leu}$ is critical for both the aminoacylation and editing activities of LeuRS (23–25). The tertiary interactions between the D- and T-loops that form the elbow region of L-shaped $tRNA^{Leu}$ are critical elements for leucine identification (26), and the nucleotide G42 in the anticodon arms of $tRNA^{Leu}$ 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 $tRNA^{Ile}$ (29).

Here, we systematically investigate the key residues of the conserved SC-fold structural module in prokaryotic and eukaryotic LeuRSs (Fig. 1*B*). Crucial basic and Gly residues in the third α-helix (α3) of the SC-fold were found to modulate the aminoacylation and editing activities of LeuRS. Additionally, several key residues of helix α3 interact with the $tRNA^{Leu}$ anticodon stem, whereas acidic residues of the β-strand control the tRNA-dependent editing activity by sensing the size of the $tRNA^{Leu}$ D-loop. These findings were further confirmed by experiments performed on the chimeric *Mm*LeuRS-CP1/LSD enzyme, a minimalist 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 aminoacylation 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* (*Aa*LeuRS), *Pyrococcus horikoshii (Ph*LeuRS), *E. coli* (*Ec*LeuRS), and *M. mobile* (*Mm*LeuRS) 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 $tRNA^{Leu}_{GAG}$ (*Ec*-RNA^{Leu}_{GAG}), *A. aeolicus* $tRNA^{Leu}_{GAG}$ (*Aa*$tRNA^{Leu}_{GAG}$), and human cytoplasmic $tRNA^{Leu}_{CAG}$ (hctRNA^{Leu}_{CAG}) with accepting capabilities between 1400 and 1600 pmol/A_{260} units were prepared from overproducing strains constructed in our laboratory (30–33). *In vitro* transcription of *P. horikoshii* $tRNA^{Leu}_{GAG}$ (*Ph*$tRNA^{Leu}_{GAG}$), *M. mobile* $tRNA^{Leu}_{UAA}$ (*Mm*$tRNA^{Leu}_{UAA}$), *M. mobile* $tRNA^{Leu}_{CAA}$ (*Mm*$tRNA^{Leu}_{CAA}$), and their mutated derivatives were prepared using T7 RNA polymerase as described previously (20, 27). The *Mm*$tRNA^{Leu}_{UAA}$ transcript and its mutated derivatives (-G12U, -G13U, -G24U, -C25U, -U39G, -C40U, -C41U, -C42U, -C41U/C42U) and the *Mm*$tRNA^{Leu}_{CAA}$ transcript and mutant (+C17a) all had accepting activities between 1200 and 1500 pmol/A_{260} units. [^{3}H]Ile-*Ec*$tRNA^{Leu}_{GAG}$ was obtained using the editing-deficient *Ec*LeuRS-Y330D mutant as described previously (34).

ATP-Pyrophosphate (PPi) Exchange, tRNA Charging, and Deacylation—The ATP-PPi exchange of *Ec*LeuRS (37 °C) and its mutants was performed as described previously (10, 34). Aminoacylation activities of *Mm*LeuRS, *Ec*LeuRS, and their mutants were measured in a reaction containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 4 mM ATP, 10 μM $tRNA^{Leu}$, 40 μM [^{3}H]Leu (11 Ci/mM), and enzyme (5 nM *Ec*LeuRS or 20 nM *Mm*LeuRS and their mutants). Aminoacylation activities of *Aa*LeuRS (20 nM), *Ph*LeuRS (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 *Mm*LeuRS (30 °C) and *Aa*LeuRS (65 °C) and their mutants. Cognate $tRNA^{Leu}$ concentrations ranging from 0.5 to 30 μM were used to determine the K_m value of enzymes for their cognate $tRNA^{Leu}$. For *Mm*LeuRS, *Mm*LeuRS-CP1/LSD and their mutants, *Mm*$tRNA^{Leu}_{UAA}$ were used. The deacylation reaction of *Ec*LeuRS 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 $MgCl_2$, 0.5 mM DTT, and 1 μM [^{3}H]Ile-*Ec*$tRNA^{Leu}$ (300 μCi/μM). Reactions were initiated with enzyme diluted to 20 nM. Because radioactive Nva is commercially unavailable, [^{3}H]Ile was used as a source to prepare mischarged $tRNA^{Leu}$.

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 *Mm*LeuRS-CP1/LSD, *Ec*LeuRS, hcLeuRS, *Aa*LeuRS, and their mutants were measured as described previously (20, 26, 28). *Mm*$tRNA^{Leu}_{CAA}$ and its mutants were used to study the editing ability of the chimeric *Mm*LeuRS-CP1/LSD. The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM $MgCl_2$, 5 mM DTT, 5 units/ml pyrophosphatase (Roche Applied Science), 3 mM ATP, 20 nM [α-^{32}P]ATP (3000 Ci/mM; PerkinElmer Life Sciences), 15 mM Nva, and the presence or absence of 5 μM $tRNA^{Leu}$. Reactions were initiated by the addition of the following concentrations of LeuRSs and their mutants: 1 μM *Mm*LeuRS-CP1/LSD (30 °C), 1 μM hcLeuRS (37 °C), 1 μM *Aa*LeuRS (65 °C), and 0.2 μM *Ec*LeuRS (37 °C). At regular time intervals, 1.5-μl aliquots were quenched in 6 μl of 200 mM sodium acetate (pH 5.0) and spotted in duplicate on polyethyleneimine cellulose plates (PEI, Merck) that had been pre-washed with water. TLC plates were developed in the presence of 0.1 M ammonium acetate and 5% acetic acid to separate [^{32}P]aminoacyl-adenylate, [^{32}P]AMP,

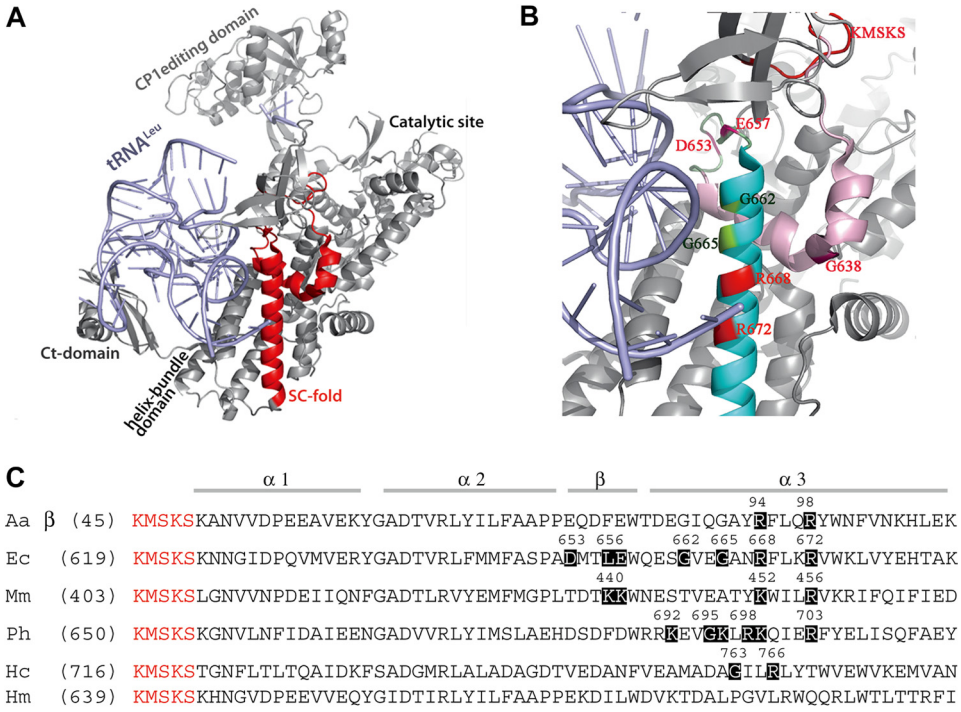

FIGURE 1. **Structure of the SC-fold and alignment of LeuRSs from different species.** *A,* crystal structure of $tRNA^{Leu}_{UAA}$ (*light blue*) in complex with *Ec*LeuRS (*gray*) in the editing conformation (PDB entry 4ARC). The major domains of LeuRS were labeled, and SC-fold is *highlighted* in *red*. *B,* residues of the third helix (α3; *cyan*) and β-strand (*lime*) investigated in this study are *highlighted* and *numbered* in *red*. *C,* sequence alignment based on structural elements of LeuRS enzymes from different species. Mutated residues are shown in *dark boxes,* and KMSKS residues are in *red*. *Aa, A. aeolicus*; *Ec, E. coli*; *Mm, M. mobile*; *Ph, P. horikoshii*; *Hc,* human cytoplasmic, and *Hm,* human mitochondria.

and [^{32}P]ATP. The plates were visualized by phosphorimaging and analyzed using MultiGauge version 3.0 software (Fujifilm). [^{32}P]AMP spot gray densities were compared with those of known [^{32}P]ATP concentrations, and rate constants (k_{obs}) were obtained by plotting [^{32}P]AMP formation *versus* time.

Measurement of tRNA Equilibrium Dissociation Constants by Filter Binding Assays—*Ec*LeuRS-[^{32}P]*Ec*$tRNA^{Leu}_{GAG}$, *Ec*LeuRS-[^{32}P]*Mm* $tRNA^{Leu}_{UAA}$, and hcLeuRS-[^{32}P]hctRNA^{Leu}_{CAG} complex formations were monitored using the classical filter binding method of Berg and co-workers (35, 36). Radiolabeled *M. mobile* or *E. coli* [^{32}P]$tRNA^{Leu}_{GAG}$ (60 nM, 60,000 cpm) and human cytoplasmic [^{32}P]$tRNA^{Leu}_{CAG}$ (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 $MgCl_2$) 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 $MgCl_2$. 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 *Ec*LeuRS revealed that the third α-helix (Ser^{661} to Lys^{683}; 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. 2*A*). However, this construct completely lacked both aminoacylation and editing activities, supporting the hypothesis that this helix is critical for these functions (Fig. 2*B* and Tables 1 and 2).

Considering the interaction between tRNA and aaRS is predominantly mediated by crucial basic residues (37), in *Ec*LeuRS, we investigated the function of residues Arg^{668}, Lys^{671}, and Arg^{672} in the third α-helix of the SC-fold by site-directed mutagenesis (PDB codes 4ARC and 4AQ7; Fig. 1, *B* and *C*). *Ec*LeuRS-K671A did not affect the catalytic efficiency (k_{cat}/K_m) at all (Table 1). However, the k_{cat}/K_m values of *Ec*LeuRS-R668A and *Ec*LeuRS-R668E had decreased to 23 and 5% of the k_{cat}/K_m values of wild-type (WT) *Ec*LeuRS (Table 1). The k_{cat}/K_m values of the double mutant *Ec*LeuRS-R668A/R672A was only 6.4% of that of the native enzyme, and the catalytic efficiency of the *Ec*LeuRS-R668E/R672E double-site mutant was severely diminished, with only 1.4% of tRNA aminoacylation activity remaining (Table 1). Although mutations at Arg^{668} or Arg^{672} decreased the aminoacylation activity to different extents, all of these mutants, including the almost inactive *Ec*LeuRS-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 $tRNA^{Leu}$

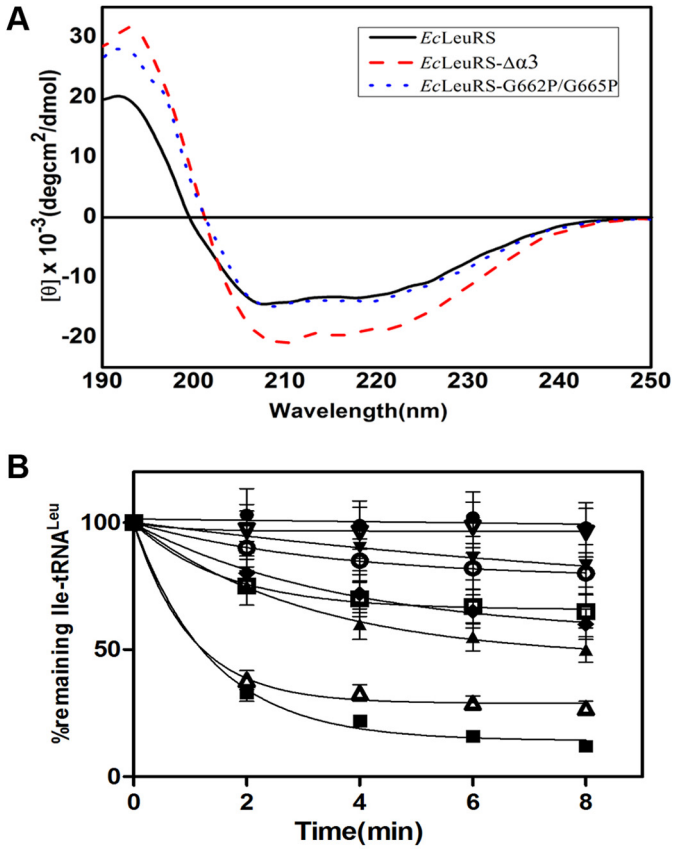

FIGURE 2. **CD spectroscopic analysis of *Ec*LeuRS-Δα3 and hydrolysis of *Ec*LeuRS and its mutants.** *A*, two enzymes were scanned by UV between 190 and 250 nm using CD spectroscopy. The concentration of each enzyme was 0.3 mg/ml in H_2O. The CD signal in millidegrees was converted to mean residue ellipticity [θ]. *B*, deacylation curves of [^{3}H]Ile-*Ec*tRNA^{Leu} (1 μM) induced by *Ec*LeuRS enzymes (5 nM). No enzyme (●); *Ec*LeuRS-R668E/R672E (▼); *Ec*LeuRS (■); *Ec*LeuRS-G662P/G665P (□); *Ec*LeuRS-R668E (○); *Ec*LeuRS-R672E (♦); *Ec*LeuRS-G662P (△); and *Ec*LeuRS-G665P(▲).

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* (*Aa*LeuRS) and *M. mobile* (*Mm*LeuRS). *Aa*LeuRS forms a unique heterodimer with its SC-fold located in the β-subunit. The counterparts of *Ec*LeuRS-Arg^{668} and *Ec*LeuRS-Arg^{672} in *Aa*LeuRS are βArg^{94} and βArg^{98}, respectively, and mutating these residues in *Aa*LeuRS had a similar effect on aminoacylation; *Aa*LeuRS-βR98A did not alter the catalytic efficiency, whereas mutating βArg^{94} to either Ala or Glu decreased k_{cat}/K_m values to 34 and 22% of that of WT *Aa*LeuRS (Table 1). When the Ala mutants were combined in βR94A/R98A, the aminoacylation activity dropped to only 17% of that of WT *Aa*LeuRS, and the double Glu mutant βR94E/R98E exhibited an even lower k_{cat}/K_m value (only 4.4% of that of WT *Aa*LeuRS; Table 1).

In *Mm*LeuRS, Lys^{452} and Arg^{456} correspond to the Arg^{668} and Arg^{672} residues of *Ec*LeuRS, respectively. Substitution of Lys^{452} 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 *Mm*LeuRS-R456A and to 21% for *Mm*LeuRS-R456E of that of the WT *Mm*LeuRS (Table 1). Notably, the K_m values for the above *Mm*LeuRS mutants were not significantly altered. These results suggest that helix α3 in the SC-fold of *Mm*LeuRS interacts with tRNA in a different manner to that of *Ec*LeuRS and *Aa*LeuRS.

The crucial basic residues of helix α3 in the archaeal *P. horikoshii* LeuRS (*Ph*LeuRS) (PDB entry 1WZ2) and eukaryotic human cytoplasmic LeuRS (hcLeuRS) enzymes were investigated. *Ph*LeuRS contains five positively charged residues (Lys^{692}, Lys^{696}, Arg^{698}, Lys^{699}, and Arg^{703}) in helix α3, and alanine-scanning mutagenesis revealed that the K_m value for tRNA of *Ph*LeuRS-K696A was 0.65 μM, an ~3-fold increase compared with the WT value (0.20 μM). The k_{cat} of *Ph*LeuRS-R703A (0.0055 s^{-1}) was much lower than that of native *Ph*LeuRS (0.020 s^{-1}; Table 1). The other *Ph*LeuRS mutants had no effect on tRNA charging activity.

Arg^{766} is the only basic residue in helix α3 of hcLeuRS SC-fold (Fig. 1*C*). 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 *Ec*LeuRS-R668A/R672A and -R668E/R672E were ~11 and 9.1% that of the WT *Ec*LeuRS in the presence of *Ec*tRNA$^{Leu}_{GAG}$ and Nva (Table 2). The k_{obs} values of the single-site mutants *Ec*LeuRS-R668A and *Ec*LeuRS-R672A were 67 and 59% that of wild-type *Ec*LeuRS in the presence of *Ec*tRNA$^{Leu}_{GAG}$ 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 *Ec*LeuRS-R672E and *Ec*LeuRS-R668E were decreased significantly to 20 and 12% that of WT *Ec*LeuRS for *Ec*tRNA$^{Leu}_{GAG}$ 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 *Ec*LeuRS.

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 *Ec*LeuRS-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 *Ec*LeuRS. Further deacylation results indicated the impaired tRNA-dependent transfer editing of the mutants resulted from their decreased hydrolysis ability (Fig. 2*B*).

Finally, we probed the basic residues of *Aa*LeuRS and hcLeuRS. Mutations of either Arg^{94} or Arg^{98} of *Aa*LeuRS 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 *Aa*tRNA$^{Leu}_{GAG}$ and Nva, which was around 10% that of WT *Aa*LeuRS, and the double mutant βR94E/R98E was even lower

TABLE 1

Kinetic constants of LeuRSs and their mutants at basic residues from various species for their cognate tRNAs in aminoacylation 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	k_{cat}	k_{cat}/K_m	Relative value
		μM^{-1}	s^{-1}	$s^{-1}\ \mu M^{-1}$	
*Ec*LeuRS	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
	-Δhelix α3	NM	NM	NM	NM
*Aa*LeuRS	WT	0.30 ± 0.02	1.5 ± 0.3	5.0	1.0
	β-R94A	0.75 ± 0.06	1.3 ± 0.1	1.7	0.34
	β-R94E	0.81 ± 0.07	0.89 ± 0.09	1.1	0.22
	β-R98A	0.31 ± 0.03	1.5 ± 0.1	4.8	0.96
	β-R98E	0.39 ± 0.03	1.0 ± 0.1	2.6	0.52
	β-R94A/R98A	0.81 ± 0.08	0.67 ± 0.06	0.83	0.17
	β-R94E/R98E	0.88 ± 0.08	0.19 ± 0.02	0.22	0.044
*Mm*LeuRS	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
*Ph*LeuRS	WT[b]	0.20 ± 0.14	0.020 ± 0.003	0.11	1.0
	-K692A	0.44 ± 0.04	0.041 ± 0.004	0.090	0.090
	-K696A	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	$(5.5 \pm 0.5) \times 10^{-3}$	0.050	0.42
hcLeuRS	WT[c]	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.
[b] Data are from Ref. 31.
[c] Data are from Ref. 30.

TABLE 2

Observed rate constants of LeuRSs and their mutants at basic residues from various species for AMP synthesis in the presence of Nva and their cognate tRNAs

All rates are the average of three repeats with the standard deviations indicated. NM means not measurable (too low to be measured).

LeuRS	Rate of AMP formation k_{obs}	Relative value
	s^{-1}	
*Ec*LeuRS[a]	3.4 ± 0.5	1.0
-R668A	2.3 ± 0.3	0.67
-R672A	2.0 ± 0.2	0.59
-R668E	0.42 ± 0.02	0.12
-R672E	0.69 ± 0.05	0.20
-R668A/R672A	0.39 ± 0.05	0.11
-R668E/R672E	0.31 ± 0.03	0.091
-Δhelix α3	NM	NM
*Aa*LeuRS	1.4 ± 0.2	1.0
-R94A	0.23 ± 0.02	0.16
-R98A	0.21 ± 0.02	0.15
-R94E	0.19 ± 0.02	0.14
-R98E	0.19 ± 0.02	0.14
-R94E/R98E	0.13 ± 0.01	0.093
hcLeuRS	1.6 ± 0.1	1.0
-R766A	0.16 ± 0.02	0.10

[a] Data are from Ref. 10.

(0.13 s^{-1}; Table 2). Consistently, mutation of the unique basic residue Arg^{766} to Ala in hcLeuRS (hcLeuRS-R766A) led to a 90% decrease in AMP formation in the presence of $hctRNA^{Leu}_{CAG}$ (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.

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

As the refined structure of the *Ec*LeuRS-*Ec*$tRNA^{Leu}_{UAA}$ complex (PDB codes 4ARC and 4AQ7) displayed disorder in the anticodon loop, there was no clear evidence to show whether residues Arg^{668} and Arg^{672} from helix α3 of the SC-fold participated in interactions with the D-stem and anticodon stem of *Ec*$tRNA^{Leu}_{UAA}$ (11). In an attempt to verify and identify the determinants of the editing reaction on the tRNA, we constructed a set of mutants of $tRNA^{Leu}$. Because *Ec*LeuRS recognizes the synthetic transcript of *Mm*$tRNA^{Leu}_{UAA}$ produced *in vitro* as well as the fully modified *Ec*$tRNA^{Leu}_{GAG}$ purified from an *E. coli* overproducing strain (k_{cat}/K_m = 2.6 and 2.2 $\mu M^{-1}\ s^{-1}$, respectively), we chose to mutate the *Mm*$tRNA^{Leu}_{UAA}$ transcript, which is

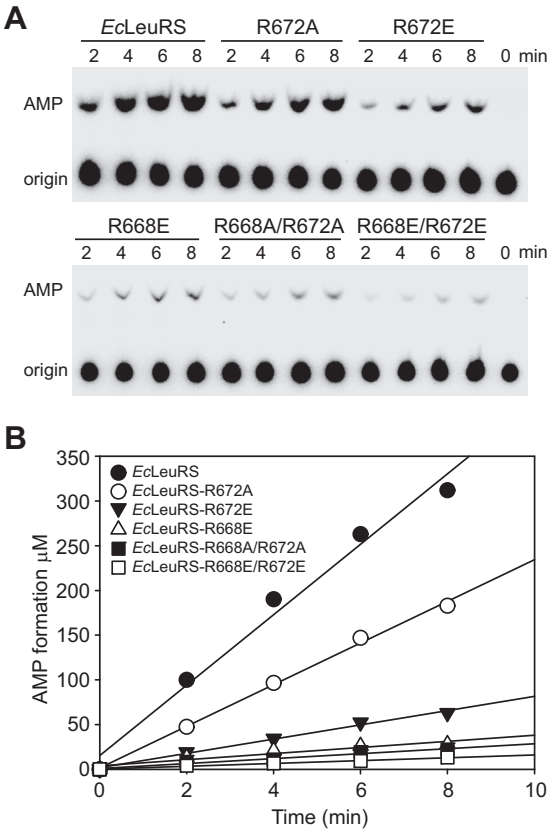

FIGURE 3. **Effects of mutating the helix α3 Arg residues on editing activity in the presence of tRNA.** *A,* AMP formation assays used to measure total editing capability with 0.2 μM *Ec*LeuRS, *Ec*LeuRS-R672A, *Ec*LeuRS-R672E, *Ec*LeuRS-R668E, *Ec*LeuRS-R668A/R672A, and *Ec*LeuRS-R668E/R672E in the presence of 5 μM *Ec*tRNA$^{Leu}_{GAG}$ and 15 mM Nva. *B,* graphical representation of AMP formation by *Ec*LeuRS and mutants. k_{obs} values for AMP formation were calculated from the line gradients (Table 2).

TABLE 3

Observed rate constants of LeuRS and its mutants at basic residues for AMP synthesis in the presence of Nva and absence of tRNA^{Leu}

All rates are the average of three repeats with the standard deviations indicated.

LeuRS	Rate of AMP formation k_{obs}	Relative value
	s^{-1}	
*Ec*LeuRS[a]	0.33 ± 0.04	1.0
-R668A	0.32 ± 0.04	0.67
-R672A	0.54 ± 0.05	1.6
-R668E	0.36 ± 0.03	1.1
-R672E	0.35 ± 0.05	1.1
-R668A/R672A	0.37 ± 0.08	1.1
-R668E/R672E	0.30 ± 0.02	0.91

[a] Data are from Ref. 10.

remarkably similar in sequence to *Ec*tRNA$^{Leu}_{GAG}$ (Fig. 4). We constructed seven mutants with specific nucleotide substitutions in the stems of *Mm*tRNA$^{Leu}_{UAA}$: -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 aminoacylation reaction and the k_{obs} in the editing reaction were measured, and the values for WT *Mm*tRNA$^{Leu}_{UAA}$ 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 *Ec*LeuRS for the -C40U, -C41U, and -C42U variants were 73, 51, and 26% that of *Ec*LeuRS for WT *Mm*tRNA$^{Leu}_{UAA}$

TABLE 4

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

The k_d values were determined using filter binding assays in the presence of 0.01 to 15 μM LeuRS. Rates are the average of three assays with the standard deviations indicated.

Enzyme	k_d	Relative value
	μM	
*Ec*LeuRS	0.20 ± 0.04	1.0
-R668A	0.46 ± 0.04	2.3
-R672A	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

(Table 5). Consistently, the k_{cat}/K_m value of the *Ec*LeuRS for double mutant -C41U/C42U was even lower (12% that of WT *Mm*tRNA$^{Leu}_{UAA}$), with a value of 0.31 $\mu M^{-1}\ s^{-1}$ (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 *Ec*LeuRS for *Mm*tRNA$^{Leu}_{UAA}$-C41U (1.7 μM) and -C42U (1.6 μM) mutants were ~5-fold greater than that of WT *Mm*tRNA$^{Leu}_{UAA}$ (0.27 μM; Table 4). In addition, we showed that the double mutant *Mm*tRNA$^{Leu}_{UAA}$-C41U/C42U was even less capable of editing. In the presence of 15 mM Nva, the k_{obs} value of *Mm*tRNA$^{Leu}_{UAA}$-C41U/C42U was 1.4 s^{-1}, compared with 2.6 s^{-1} for WT *Mm*tRNA$^{Leu}_{UAA}$ (Table 4). Together, these results suggest that LeuRS employs these basic residues of the SC-fold domain to modulate both aminoacylation and editing activities through interaction with the anticodon stem of tRNA^{Leu}.

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

The mutations also diminished the Leu activation activity, as measured using the ATP-PPi exchange reaction (Fig. 4*B*). Compared with the k_{obs} of WT *Ec*LeuRS (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 aminoacylation 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 *Ec*tRNA$^{Leu}_{GAG}$ 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

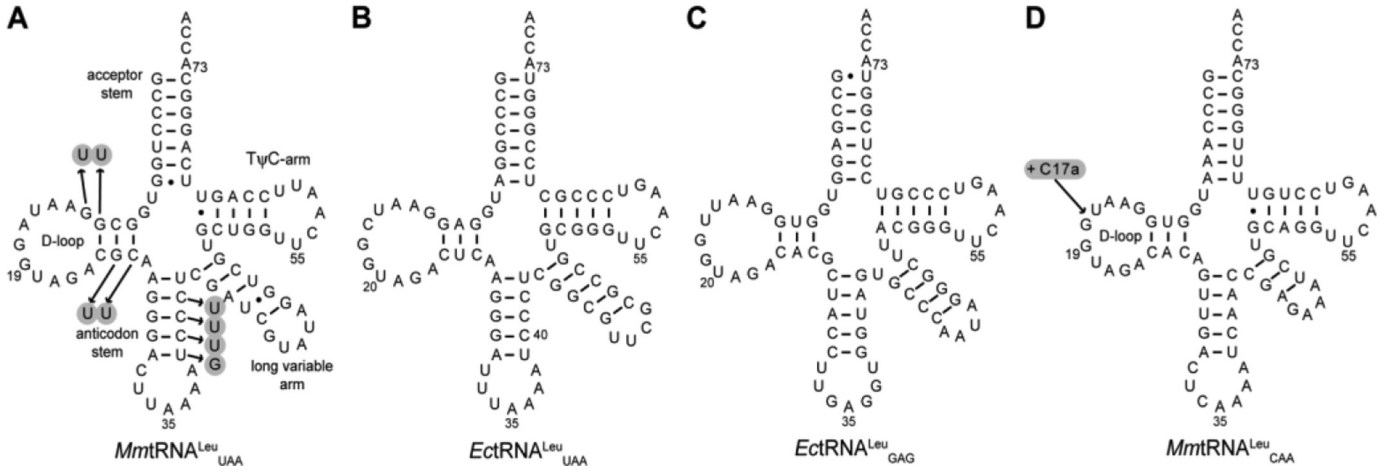

FIGURE 4. Cloverleaf structures of *Mm*tRNA$^{Leu}_{UAA}$ (*A*); *Ec*tRNA$^{Leu}_{UAA}$ (*B*); *Ec*tRNA$^{Leu}_{GAG}$ (*C*); and *Mm*tRNA$^{Leu}_{CAA}$ (*D*). Mutations constructed during this study are indicated with *arrows*.

TABLE 5

Various parameters of *Ec*LeuRS for *Mm*tRNA$^{Leu}_{UAA}$ and its derived variants

The K_m 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 *Mm*tRNA$^{Leu}_{UAA}$. The k_d values are the dissociation constants of the *Ec*LeuRS and *Mm*tRNA$^{Leu}_{UAA}$ complex determined by filter binding assays in the presence of 0.01 to 10 μM *Ec*LeuRS. Parameters are the average of three repeats with the standard deviations indicated.

*Mm*tRNA$^{Leu}_{GAG}$	K_m	k_{cat}	k_{cat}/K_m	Relative value	k_{obs} of AMP formation	k_d
	μM	s^{-1}	$s^{-1}\mu M^{-1}$		s^{-1}	μM
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

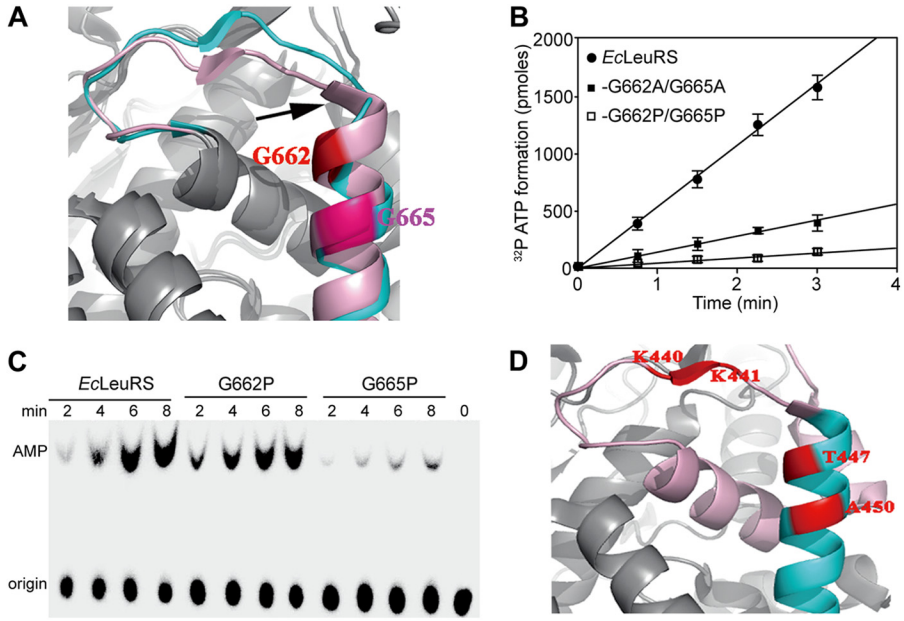

FIGURE 5. **Effects of mutating the helix α3 Gly residues on synthetic and editing activities.** *A,* structure of the *Ec*LeuRS-tRNA^{Leu} complex in aminoacylation (*pink*) and editing (*cyan*) conformations (PDB codes 4AQ7 and 4ARC, respectively) (11). Helix α3 residues Gly^{662} and Gly^{665} are 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 *Ec*LeuRS, *Ec*LeuRS-G662A/G665A, and *Ec*LeuRS-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 *Ec*LeuRS, *Ec*LeuRS-G662P, and *Ec*LeuRS-G665P in the presence of 5 μM *Ec*tRNA$^{Leu}_{GAG}$ and 15 mM Nva. k_{obs} values of AMP formation are reported in Table 7. *D,* structure of *Mm*LeuRS (*gray*; PDB code 3ZIU (39). Residues shown on helix α3 (*cyan*) are equivalent to Gly^{662} and Gly^{665} of *Ec*LeuRS. Other helices of the SC-fold are represented in *pink*, and the β-strand is in *red*.

TABLE 6

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

Enzymes	K_m	k_{cat}	k_{cat}/K_m	Relative value
	μM	s^{-1}	$s^{-1}\ \mu M^{-1}$	
*Ec*LeuRS[a]	2.2 ± 0.1	4.9 ± 0.3	2.2	1.0
-G662P	1.1 ± 0.3	2.2 ± 0.3	2.0	0.91
-G665P	1.3 ± 0.1	2.1 ± 0.1	1.6	0.73
-G662A/G665A	1.2 ± 0.1	0.70 ± 0.07	0.58	0.26
-G662P/G665P	1.4 ± 0.2	0.30 ± 0.03	0.21	0.095
-G662R/G665R	1.3 ± 0.1	0.80 ± 0.08	0.61	0.28
*Ph*LeuRS	0.20 ± 0.01	0.020 ± 0.003	0.11	1.0
-G695P	0.19 ± 0.05	0.010 ± 0.001	0.053	0.48
hcLeuRS	0.74 ± 0.05	2.6 ± 0.2	3.5	1.0
-G763P	0.61 ± 0.06	1.1 ± 0.2	1.8	0.51

[a] Data are from Ref. 10.

impact (Table 7 and Fig. 5*C*). Additionally, the post-transfer editing activity of mutation G665P was also decreased (Fig. 2*B*). These observations suggest that the editing-defective mutant *Ec*LeuRS-G665P, which exhibited a standard synthetic efficiency, could be used to produce mischarged aa-tRNA^{Leu} 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 *Ph*LeuRS and hcLeuRS. Both enzymes contain a single Gly in helix α3, albeit at different positions (PDB code 1WZ2; Fig. 1*C*). Mutations to Pro in *Ph*LeuRS-G695P decreased the catalytic efficiency of aminoacylation to 5.3% that of WT *Ph*LeuRS. Similarly, a mutant replaced Gly^{763} 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 *Mm*LeuRS lacks any Gly residues in helix α3; the two Gly residues found in *Ec*LeuRS are replaced by Thr and Ala (PDB code 3ZIU; Figs. 1*C* and 5*D*). Additionally, the β-strand of the SC-fold of *Mm*LeuRS contains two Lys residues, whereas other LeuRS enzymes usually contain two or three acidic residues in this vicinity (Fig. 1*C*). This suggests that *Mm*LeuRS 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^{653}, Glu^{657}, and Leu^{656} of *Ec*LeuRS with Lys. In the aminoacylation reaction, the catalytic efficiency of mutant *Ec*tRNA$^{Leu}_{GAG}$-D653K and double mutant *Ec*tRNA$^{Leu}_{GAG}$-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 *Ec*tRNA$^{Leu}_{GAG}$-D653K dropped to 45% that of WT *Ec*LeuRS for AMP formation, which was only half that of the native enzyme

TABLE 7

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

All rates are the average of three repeats with the standard deviations indicated.

	Rate of AMP formation $k_{obs}(s^{-1})$			
LeuRS	−*Ec*tRNA$^{Leu}_{GAG}$	Relative value	+ *Ec*tRNA$^{Leu}_{GAG}$	Relative value
*Ec*LeuRS[a]	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	1.5 ± 0.1	0.45
-L656K/E657K	0.30 ± 0.03	0.91	0.31 ± 0.03	0.091

[a] Data are from Ref. 10.

(Table 7). For the double mutant L656K/E657K, the k_{obs} value was decreased to 9.1% that of WT *Ec*LeuRS in the presence of tRNA, which suggested that the mutant had lost its tRNA-dependent editing activity (Fig. 6*A* and Table 7). Therefore, we performed an Ile-tRNA^{Leu} 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 deacylate mischarged *Ec*tRNA$^{Leu}_{GAG}$, suggesting that the SC-fold β-strand is involved in tRNA-dependent editing of LeuRS following the transfer step (Fig. 6*B*).

In the crystal structure of the complex containing *Ec*LeuRS and *Ec*tRNA$^{Leu}_{UAA}$ (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 *Mm*tRNA$^{Leu}_{UAA}$ 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^{Leu} with a CAA anticodon that contains only 10 nucleotides in the D-loop instead of the usual 11 nucleotides in *Ec*tRNA$^{Leu}_{UAA}$ and *Mm*tRNA$^{Leu}_{UAA}$ (Fig. 4). Remarkably, during the editing reaction, *Mm*tRNA$^{Leu}_{CAA}$ could not stimulate AMP formation as well as *Mm*tRNA$^{Leu}_{UAA}$; in the presence of Nva and *Ec*LeuRS, the k_{obs} was only 0.91 s^{-1} compared with 2.6 s^{-1} for both *Mm*tRNA$^{Leu}_{CAA}$ and *Ec*tRNA$^{Leu}_{UAA}$. 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 *Mm*tRNA$^{Leu}_{CAA}$ (17a), just before the crucial nucleotides G18 and G19. In the resulting mutant *Mm*tRNA$^{Leu}_{CAA}$ (+C17a), the loop was identical to that of *Mm*tRNA$^{Leu}_{UAA}$ and *Ec*tRNA$^{Leu}_{UAA}$ (Fig. 4). Consistently, in the presence of Nva, the k_{obs} value of *Ec*LeuRS for mutant (+C17a) was increased to 2.1 s^{-1}, reaching a level comparable with *Mm*tRNA$^{Leu}_{UAA}$ and *Ec*tRNA$^{Leu}_{UAA}$ (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^{Leu} D-loop. The editing activity of *Ec*LeuRS was magnified when the native size of the D-loop of tRNA^{Leu} was introduced into *Mm*tRNA$^{Leu}_{CAA}$. These results show that the β-strand adjacent to the SC-fold influences the editing activities of *Ec*LeuRS, probably via the conserved acidic residues.

TABLE 8

Kinetic constants of *Ec*LeuRS, chimeric LeuRS and their mutants for tRNA^{Leu} in aminoacylation 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). WT indicates the chimeric enzyme *Mm*LeuRS-CP1/LSD obtained by inserting the LSD and CP1 domain of *Ec*LeuRS into *Mm*LeuRS.

Enzymes	LeuRS	K_m	k_{cat}	k_{cat}/K_m	Relative value
		μM	s^{-1}	$s^{-1}\ \mu M^{-1}$	
*Ec*LeuRS[a]	WT	2.2 ± 0.1	4.9 ± 0.3	2.2	1.0
	-L656K/E657K	0.66 ± 0.07	1.1 ± 0.01	1.7	0.77
	-D653K	2.2 ± 0.2	3.1 ± 0.3	1.4	0.64
*Mm*LeuRS-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

[a] Data are from Ref. 10.

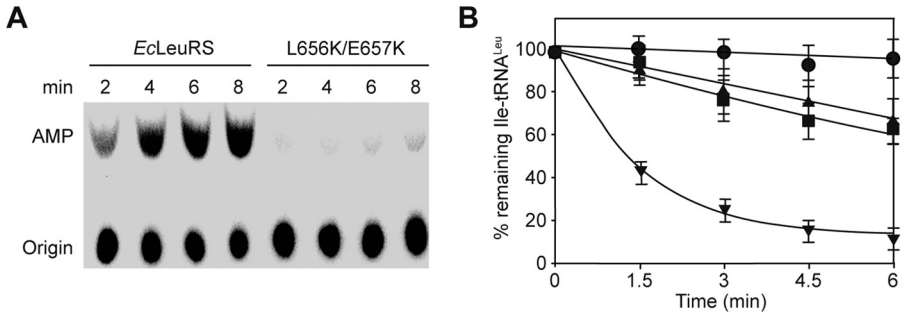

FIGURE 6. **Residues of the SC-fold β-strand are involved in tRNA-dependent editing.** *A,* TLC showing AMP formation catalyzed by 0.2 μM *Ec*LeuRS and *Ec*LeuRS-L656K/E657K in the presence of 5 μM *Ec*tRNA$^{Leu}_{GAG}$ and 15 mM Nva. k_{obs} values are reported in Table 7. *B,* deacylation curves of [^{3}H]Ile-*Ec*tRNA^{Leu} (1 μM) induced by *Ec*LeuRS enzymes (5 nM). No enzyme (●), *Ec*LeuRS (▼), *Ec*LeuRS-D653K (■), and *Ec*LeuRS-L656K/E657K (▲).

TABLE 9

Observed rate constants of *Ec*LeuRS and chimeric LeuRS and its mutant for AMP formation in the presence of Nva and *Mm*tRNA$^{Leu}_{CAA}$

Rates are the average of three repeats with the standard deviations indicated.

LeuRS	*Mm*tRNA$^{Leu}_{CAA}$	k_{obs} of AMP formation	Relative value
		s^{-1}	
*Ec*LeuRS		0.33 ± 0.04[a]	1.0
	+WT	0.91 ± 0.10	2.8
	+ (+C17a)	2.1 ± 0.2	6.4
*Mm*LeuRS-CP1/LSD		(3.7 ± 0.8) × 10^{-2}	1.0
	+WT	(4.1 ± 0.8) × 10^{-2}	1.1
	+ (+ C17a)	0.10 ± 0.04	2.7
*Mm*LeuRS-CP1/LSD-K440L/K441E		(3.7 ± 0.8) × 10^{-2}	1.0
	+WT	0.11 ± 0.02	3.0
	+ (+ C17a)	0.31 ± 0.04	8.4

[a] Data are from Ref. 10.

Engineering of the Chimeric MmLeuRS-CP1/LSD and MmtRNA$^{Leu}_{CAA}$ to Improve Fidelity—We previously engineered a variant of *Mm*LeuRS containing an insertion of CP1 and LSD from *Ec*LeuRS, and this chimera exhibited improved tRNA-dependent editing activity for isoacceptor *Mm*tRNA$^{Leu}_{UAA}$ (21). In this study, the k_{obs} value of AMP formation during the editing reaction of the chimera was 0.037 and 0.041 s^{-1} in the absence or presence of the isoacceptor *Mm*tRNA$^{Leu}_{CAA}$, respectively (Table 9 and Fig. 7*A*). This result suggests the tRNA-dependent editing pathway was inefficient in the presence of the *Mm*tRNA$^{Leu}_{CAA}$ isoacceptor. Therefore, *Mm*LeuRS-CP1/LSD was able to discriminate between the AUG and CAA isoacceptors during the editing reaction. Considering the contribution of the SC-fold to the catalytic function and fidelity of LeuRS, we first introduced into *Mm*LeuRS two Glu residues in place of the two unusual basic residues (Lys^{440} and Lys^{441}) found in the β-strand. However, the double mutant *Mm*LeuRS-CP1/LSD-K440E/K441E did not possess any aminoacylation activity (Table 7), suggesting that both Lys residues contribute to the catalytic activity in *Mm*LeuRS. We further substituted Lys^{440} with Leu and Lys^{441} with Glu to mimic the residues in *Ec*LeuRS. Remarkably, the k_{obs} value of *Mm*LeuRS-CP1/LSD-K440L/K441E for *Mm*tRNA$^{Leu}_{CAA}$ was twice that of the starting *Mm*LeuRS-CP1/LSD chimera in the editing reaction (0.11 s^{-1} *versus* 0.041 s^{-1} in Table 9 and Fig. 7, *A* and *B*). In contrast, the k_{cat}/K_m values of the double mutant -K440L/K441E exhibited only 33% compared with the starting chimera *Mm*LeuRS-CP1/LSD (Table 7). We next measured the editing capacity of the double mutant K440L/K441E in the presence of *Mm*tRNA$^{Leu}_{CAA}$ with the (+C17a) insertion in the D-loop, and the k_{obs} value (0.31 s^{-1}) was almost 3-fold higher than that of *Mm*tRNA$^{Leu}_{CAA}$ (0.11 s^{-1}), which confirmed the importance of the size of the D-loop (Table 9 and Fig. 7, *C* and *D*).

Taken together, these results show that the SC-fold β-strand influences both aminoacylation and editing activities of *Ec*LeuRS via conserved acidic residues. Revealingly, the editing activity of *Mm*LeuRS that ordinarily lacks these residues was enhanced when acidic residues were introduced into this enzyme.

DISCUSSION

Conserved SC-fold Is Crucial for Both Aminoacylation and Editing of LeuRSs—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-

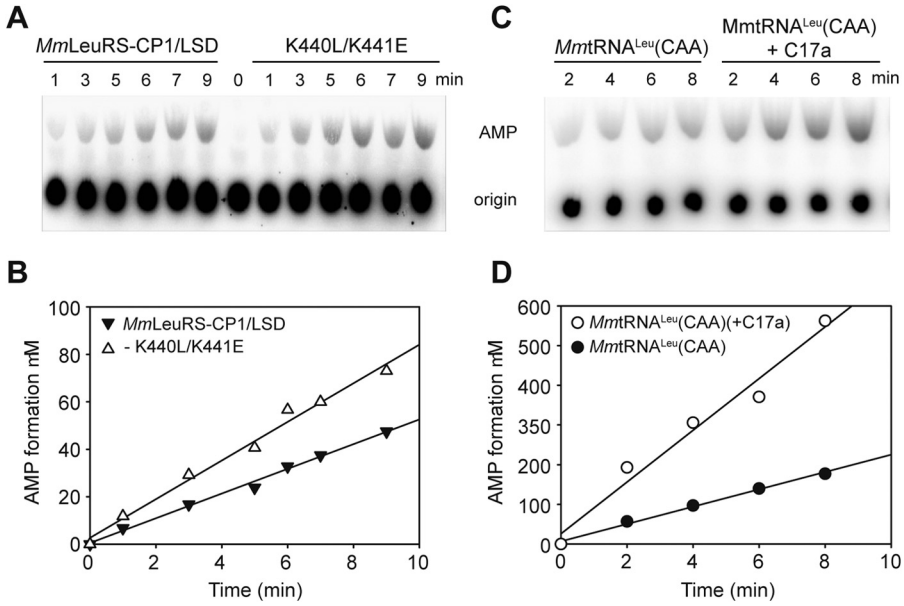

FIGURE 7. **Editing of the engineered *Mm*LeuRS-CP1/LSD for *Mm*tRNA$^{Leu}_{CAA}$ and mutated derivatives.** *A,* AMP formation assay used to measure total editing activity of 1 μM *Mm*LeuRS-CP1/LSD and mutated derivative K440L/K441E in the presence of 5 μM *Mm*tRNA$^{Leu}_{CAA}$ and 15 mM Nva. *B,* graphical representation of AMP formation as a function of time for *Mm*LeuRS-CP1/LSD and derivative K440L/K441E. *C,* AMP formation assays used to measure total editing activity with 1 μM *Mm*LeuRS-CP1/LSD-K440L/K441E in the presence of 5 μM *Mm*tRNA$^{Leu}_{CAA}$ and mutant (+C17a) with 15 mM Nva. *D,* graphical representation of AMP formation as a function of time for *Mm*tRNA$^{Leu}_{CAA}$ and mutant (+C17a). k_{obs} values for AMP formation were calculated from the slopes (Table 9).

cally 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 *Ec*LeuRS 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 *Ph*LeuRS, only Arg^{703} and Lys^{696} were essential for catalytic activity. In contrast, hcLeuRS contains only one basic residue (Arg^{766}) 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 *Ec*LeuRS, suggesting the synthetic active site pocket may undergo conformational changes during catalysis. In this study we identified a mutant (*Ec*LeuRS-G665P) with diminished editing properties reminiscent of a previously characterized CP1-editing site mutant (*Ec*LeuRS-Y330D) (34). Compared with the basic residues, mutants with impaired aminoacylation activity, both *Ec*LeuRS-G665P and *Ec*LeuRS-Y330D, retained tRNA aminoacylation activity, and these variants could therefore be used in the future to mischarged aa-tRNA^{Leu} for deacylation studies.

The crystal structure of *Ec*LeuRS in complex with tRNA^{Leu} (PDB codes 4ARC and 4AQ7) revealed Gly residues that may contribute to conformational changes of the SC-fold β-strand containing several acidic residues (Glu^{657} and Asp^{653}) involved in the fidelity of *Ec*LeuRS (Fig. 4*A*). This structure also highlighted the proximity of residue Met^{654} and tRNA^{Leu}, which is an even shorter distance than between Glu^{657}, Asp^{653}, and the tRNA (PDB code 4AQ7) (11). However, mutation of Met^{654} to Ala or Lys did not affect either the synthetic or editing properties of LeuRS, suggesting Met^{654} may not be functionally important or may act in a different way (data not shown).

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: *Mm*LeuRS 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 *Mm*LeuRS nor hmLeuRS contain the Gly or acidic residues found in helix α3 of the SC-fold (Fig. 1*C*). 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^{Leu} provide valuable new information that may be of use in the engineering of both synthetase enzymes and tRNAs. Incorporation of two key *Ec*LeuRS β-strand residues, including a conserved acidic residue, into the chimeric *Mm*LeuRS-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 *Mm*tRNA$^{Leu}_{CAA}$ 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 *Mm*tRNA$^{Leu}_{CAA}$ that is naturally smaller than the D-loop of other tRNA^{Leu} molecules, the editing activity of the synthetic *Mm*LeuRS 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 proofreading 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 to contribute to the catalytic site, the SC-fold carrying this module also forms a functional link between the catalytic activity and tRNA binding. This feature senses the size of the D-loop of tRNA^{Leu} and controls the editing reaction. In addition, engineering of the chimeric *Mm*LeuRS-CP1/LSD and tRNA provides evidence that the SC-fold module has co-evolved with the CP1 editing domain in LeuRS enzymes.

Acknowledgments—We thank Drs. Michael T. Bethune and Shuai Jiang at Caltech and Drs. Xiaolong Zhou and Rujuan Liu in our laboratory for their good suggestions.

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