The C-Terminal Domain of the Archaeal Leucyl-tRNA Synthetase Prevents Misediting of Isoleucyl-tRNA^{Ile}†

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ABSTRACT: In the archaeal leucyl-tRNA synthetase (LeuRS), the C-terminal domain recognizes the long variable arm of tRNA^{Leu} for aminoacylation, and the so-called editing domain deacylates incorrectly formed Ile-tRNA^{Leu}. We previously reported, for *Pyrococcus horikoshii* LeuRS, that a deletion mutant lacking the C-terminal domain (LeuRS_Δ(811−967)) retains normal editing activity, but has severely reduced aminoacylation activity. In this study, we found that LeuRS_Δ(811−967), but not the wild-type LeuRS, exhibited surprisingly robust deacylation activity against Ile-tRNA^{Ile}, correctly formed by isoleucyl-tRNA synthetase ("misediting"). Structural superposition of tRNA^{Ile} onto the LeuRS·tRNA^{Leu} complex indicated that Ile911, Lys912, and Glu913 of the LeuRS C-terminal domain clash with U20 of tRNA^{Ile}, which is bulged out as compared to the corresponding nucleotide of tRNA^{Leu}. The deletion of amino acid residues 911−913 of LeuRS enhanced the Ile-tRNA^{Ile} deacylation activity, without affecting the Ile-tRNA^{Leu} deacylation activity. These results demonstrate that the clashing between U20 of tRNA^{Ile} and residues 911−913 of the LeuRS C-terminal domain is the structural mechanism that prevents misediting. In contrast, the deletion of the C-terminal domains of the isoleucyl- and valyl-tRNA synthetases impaired both the aminoacylation (Ile-tRNA^{Ile} and Val-tRNA^{Val} formation, respectively) and editing (Val-tRNA^{Ile} and Thr-tRNA^{Val} deacylation, respectively) activities, and did not cause misediting (Val-tRNA^{Val} and Thr-tRNA^{Thr} deacylation, respectively) activity. Thus, the requirement of the C-terminal domain for misediting prevention is unique to LeuRS, which does not recognize the anticodon of the cognate tRNA, unlike the common aminoacyl-tRNA synthetases.

The accuracy of protein synthesis depends on the specific recognition of amino acids and tRNAs by aminoacyl-tRNA synthetases (aaRSs[1]) (*1*, *2*). The aminoacylation of tRNA occurs in two steps. In the first step, the amino acid is condensed with ATP to create an aminoacyl adenylate. In the second step, the activated aminoacyl moiety is transferred to the 3′-end of its cognate tRNA (*3*). Leucyl-tRNA synthetase, isoleucyl-tRNA synthetase, and valyl-tRNA synthetase (LeuRS, IleRS, and ValRS, respectively) are thought to have evolved from a common ancestral enzyme that did not discriminate between these three similar amino acids, leucine, isoleucine, and valine, during their early evolution from the other class-I aaRSs (*4*). All of them charge tRNAs with the NAN anticodon.

LeuRS should discriminate the cognate leucine from the noncognate isoleucine. IleRS should distinguish between isoleucine and valine, while ValRS should discriminate valine from threonine. However, these enzymes reportedly misaminoacylate the noncognate amino acids at various rates (*3*, *5*−*7*). To maintain high translational fidelity, these aaRSs catalyze proofreading (editing) reactions, in which the misformed tRNA is deacylated. Note that they should avoid "misediting" (deacylation of aa-tRNAs correctly formed by other aaRSs); LeuRS, IleRS, and ValRS should not edit Ile-tRNA^{Ile}, Val-tRNA^{Val}, and Thr-tRNA^{Thr}, respectively. A large domain (named the editing domain) is responsible for the editing activity (*5*, *7*−*11*). The crystal structures revealed that the editing domain is a discrete region, located more than 30 Å away from the aminoacylation site (*7*, *12*−*16*). The editing domains of ValRS, IleRS, and LeuRS share sequence and structural homology (*7*, *11*−*16*). The completely conserved Asp residue is crucial for the editing reaction (*6*, *7*, *17*, *18*).

Four tRNA-complex crystal structures, the bacterial *Staphylococcus aureus* IleRS·tRNA^{Ile} complex (*15*), the bacterial *Thermus thermophilus* ValRS·tRNA^{Val} complex (*12*), the archaeal *Pyrococcus horikoshii* LeuRS·tRNA^{Leu} complex (*19*), and the *T. thermophilus* LeuRS-tRNA^{Leu} complex (*20*), have been determined thus far (Figure 1). The structures

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; ThrRS, threonyl-tRNA synthetase; TCA, trichloroacetic acid.

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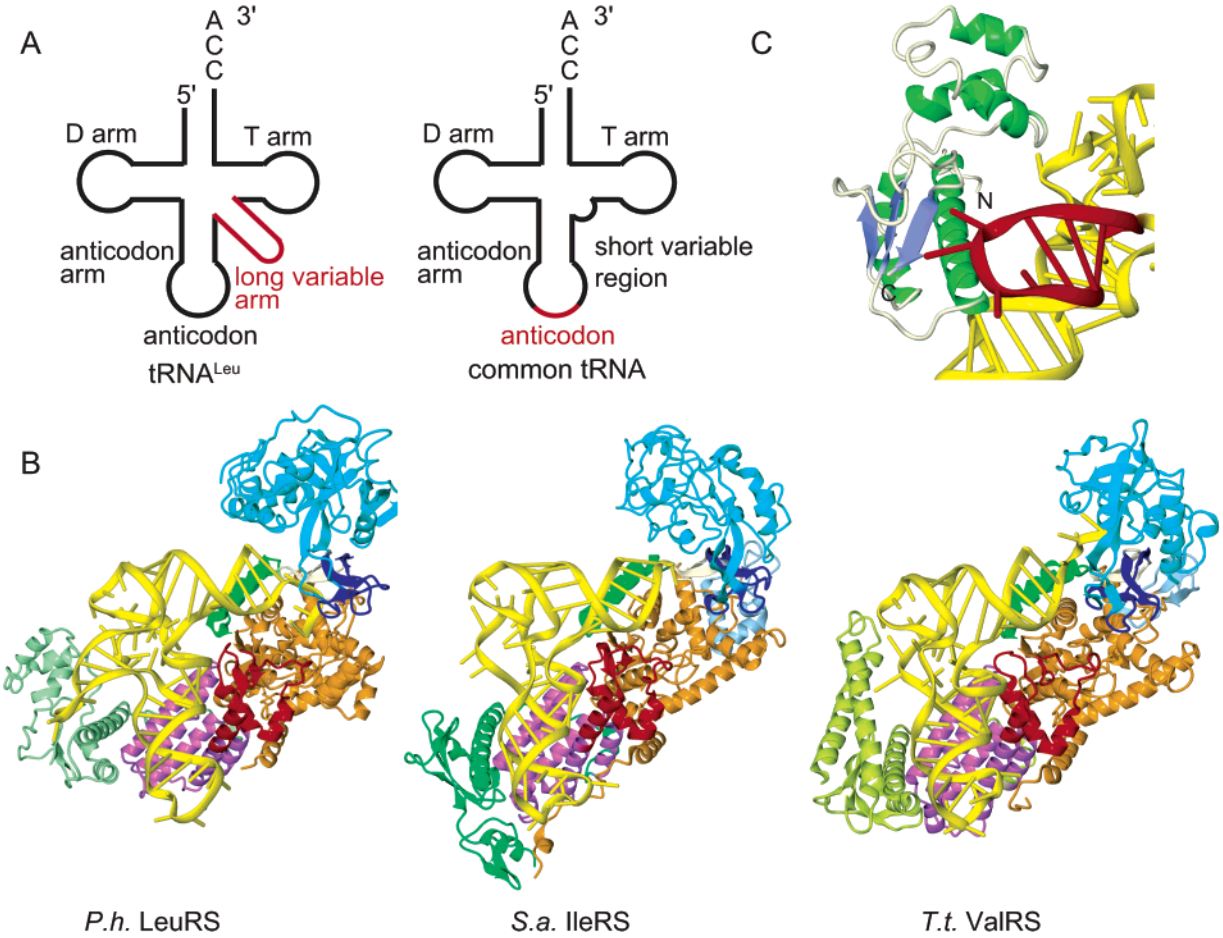

FIGURE 1: aaRS-tRNA complex structures. (A) Cloverleaf models of $tRNA^{Leu}$ and a common tRNA. The regions that are mainly recognized by the cognate aminoacyl-tRNA synthetases are colored red. (B) Ribbon models of the *P. horikoshii* LeuRS-$tRNA^{Leu}$, *S. aureus* IleRS-$tRNA^{Ile}$, and *T. thermophilus* ValRS-$tRNA^{Val}$ complex structures. The color-coded structures are as follows: tRNA (yellow), the Rossmann-fold aminoacylation domain (orange), the CP core (white), the CP1 hairpin (blue), the CP1 editing domain (cyan), the CP2 domain (green), the CP3 domain (pale blue), the SC-fold domain (red), the α-helix bundle domain (magenta), and the C-terminal domain (light green, dark green, and yellow green in LeuRS, IleRS, and ValRS, respectively). (C) The recognition mode of the $tRNA^{Leu}$ long variable arm tip by the LeuRS C-terminal domain. $tRNA^{Leu}$ is colored yellow, and its long variable arm is colored red. The α-helices and the *β*-strands are colored green and blue, respectively. The N- and C-terminal extremities (Glu822 and Glu976) of the C-terminal domain are marked.

suggested that, in the editing reaction, the 3′-terminal CCA region of tRNA flips from the aminoacylation site to the editing site, while the rest of the tRNA remains bound. Our binary complex structures of *P. horikoshii* LeuRS·$tRNA^{Leu}$ in the aminoacylation mode suggested that the tRNA acceptor stem end undergoes a conformational change for translocation of the aminoacylated 3′-end from the aminoacylation domain to the editing domain (*19*). The change in the recognition mode of the discriminator A73 seems to be important for the conformational change of the tRNA acceptor stem.

The tRNA-complex structures also revealed the mechanisms by which the enzymes recognize their cognate tRNAs. Similar to most aaRSs, IleRS and ValRS recognize the anticodon bases of their cognate tRNAs via the α-helix bundle domains within the enzyme main bodies of the enzymes (Figure 1) (*12*, *15*). In addition, their C-terminal domains contact other parts of the tRNA. In contrast, the *P. horikoshii* (archaeal/eukaryal type) LeuRS and the *T. thermophilus* (bacterial type) LeuRS do not contact the anticodon bases of the $tRNA^{Leu}$ (*19*, *20*). The C-terminal domain of *P. horikoshii* LeuRS recognizes the bases on the tip of the long variable arm (*19*), which is unique to $tRNA^{Leu}$. The C-terminal domain of *T. thermophilus* LeuRS recognizes the tertiary base pair between the D- and T-arms (*20*). The C-terminal domains of IleRS, ValRS, and archaeal/eukaryal LeuRS share partial structural homology, suggesting an evolutional linkage (*19*). The common core architecture of the C-terminal domains is composed of a *β*-sheet surrounded by α-helices, including a quite long one (Figure 1B). In addition, each domain has its own specific insertion, which is important for its respective manner of tRNA recognition. In contrast, the C-terminal domain of bacterial LeuRS does not share structural homology with those of the other enzymes (*20*).

Previously, we found that the C-terminally truncated *P. horikoshii* LeuRS can catalyze the first step of the aminoacylation reaction (Leu-AMP formation), but cannot catalyze the second step (transfer of Leu from Leu-AMP to $tRNA^{Leu}$) (*7*). In contrast, its editing (Ile-$tRNA^{Leu}$ deacylation) activity is similar to that of the wild-type enzyme. These findings indicated that the C-terminal domain of the archaeal LeuRS functions to recognize the cognate tRNA in the aminoacylation reaction, but not in the editing reaction.

In this study, we found that the C-terminally truncated *P. horikoshii* LeuRS deacylates Ile-$tRNA^{Ile}$, the correct product of IleRS, which was not deacylated by the wild-type LeuRS. These findings revealed that the C-terminal domain of *P.*

horikoshii LeuRS rejects the correctly formed Ile-tRNA^{Ile} in the editing reaction, to prevent misediting. From the structural superposition of tRNA^{Ile} onto the *P. horikoshii* LeuRS-tRNA^{Leu} complex, and the mutational analysis based on it, we clarified the mechanism of Ile-tRNA^{Ile} rejection by the C-terminal domain. U20 of tRNA^{Ile} is bulged out and would clash against the archaeal-LeuRS specific insertion in the C-terminal domain. In contrast, the C-terminal domain-truncated *T. thermophilus* IleRS and ValRS can form the aminoacyl-adenylate, but can perform neither the aminoacylation, correct editing, nor misediting reaction. Thus, the requirement of the C-terminal domain for misediting prevention is unique to the archaeal LeuRS, while the need for the C-terminal domain in the aminoacylation reaction is common among the three enzymes.

EXPERIMENTAL PROCEDURES

Protein Preparation. The genes encoding the full-length (amino acid residues 1−967), the C-terminally truncated (amino acid residues 1−810, 1−964, 1−965, and 1−966), and the three-residue deleted (amino acid residues 1−910: 914−967) *P. horikoshii* LeuRSs were cloned into the pET16b vector (Novagen) (*21*, *22*). Each of the LeuRS proteins has a His_6 tag at its N-terminus. The gene encoding the full-length ThrRS (amino acid residues 1−659) was cloned into the pET28c vector (Novagen). The ThrRS protein has a His_6 tag at its N-terminus. The genes encoding the full-length (amino acid residues 1−1045) and the C-terminally truncated *T. thermophilus* IleRS (amino acid residues 1−790) were cloned into the pET28c vector (Novagen). Each of these IleRSs has a His_6 tag at its N-terminus. The genes encoding the full-length (amino acid residues 1−862) and the C-terminally truncated *T. thermophilus* ValRS (amino acid residues 1−707) were cloned into the pET26b vector (Novagen). Each of these ValRSs has a His_6 tag at its C-terminus. The D332A, D328A, and D279A mutations were introduced by PCR methods in LeuRS, IleRS, and ValRS, respectively. The enzymes were overexpressed in the *Escherichia coli* strain BL21(DE3) codon PLUS (Stratagene), and were purified in a similar manner, as described previously (*7*, *11*, *17*). The enzymes were dialyzed against 150 mM Tris-HCl buffer (pH 7.5) containing 150 mM KCl and 10 mM $MgCl_2$. The final purity of the proteins was monitored by SDS−PAGE and UV spectroscopy.

tRNA Preparation. *P. horikoshii* tRNA^{Leu}, *P. horikoshii* tRNA^{Ile}, *T. thermophilus* tRNA^{Ile}, *T. thermophilus* tRNA^{Val}, and *T. thermophilus* tRNA^{Thr} were transcribed *in vitro* with T7 RNA polymerase and were purified by phenol/chloroform treatment. They were further purified by anion exchange chromatography with a ResourceQ column, using 20 mM Tris-HCl buffer (pH 8.0) containing 8 mM $MgCl_2$ and 280 mM NaCl as a starting buffer, with a linear gradient of 280−1000 mM NaCl. The tRNA-containing fractions were pooled, ethanol-precipitated, and dried. They were dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, and were quantified by the absorbance at 260 nm wavelength, assuming that 1 Abs_{260} value corresponds to a concentration of 44 mg/L for a tRNA solution.

Amino Acid Activation Assay. We refer to the 60 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ as "TM buffer". The cognate amino acid-dependent PPi-ATP isotopic exchange reactions were carried out at 65 °C, in TM buffer containing 2 mM amino acid (valine or isoleucine), 4 mM ATP, 4 mM [^{32}P]sodium pyrophosphate (1.65 Ci/mmol), and 100 nM wild-type or C-terminally truncated enzyme (ValRS or IleRS). Aliquots were removed at specific time points, and the amount of [^{32}P]ATP synthesized was measured, as described previously (*23*).

Aminoacylation Assay. Leu-tRNA^{Leu} formation reactions were carried out at 37 °C, in TM buffer containing 2 mM ATP, 3 μM tRNA^{Leu}, 20 μM [^{14}C]leucine (300 cpm/pmol), and 80 nM LeuRS enzymes. Ile-tRNA^{Leu} misformation reactions were carried out at 65 °C, in TM buffer containing 1 mM DTT, 3 mM ATP, 3 μM tRNA^{Leu}, 40 μM [^{14}C]-isoleucine (300 cpm/pmol), and 2 μM LeuRS enzymes. Leu-tRNA^{Ile} and Ile-tRNA^{Ile} formation reactions were carried out at 50 °C, in TM buffer containing 2 mM ATP, 3 μM *P. horikoshii* tRNA^{Ile}, 20 μM [^{14}C]leucine or [^{14}C]isoleucine (300 cpm/pmol), and 500 nM LeuRS enzymes or 10 nM *T. thermophilus* IleRS. Ile-tRNA^{Ile} formation reactions were carried out at 50 °C, in TM buffer containing 2 mM ATP, 3 μM *T. thermophilus* tRNA^{Ile}, 20 μM [^{14}C]isoleucine (300 cpm/pmol), and 20 nM IleRS enzymes. Val-tRNA^{Ile} misformation reactions were carried out at 37 °C, in TM buffer containing 3 mM ATP, 10 μM *T. thermophilus* tRNA^{Ile}, 20 μM [^{14}C]valine (300 cpm/pmol), and 200 nM IleRS enzymes. Val-tRNA^{Val} formation reactions were carried out at 37 °C, in TM buffer containing 3 mM ATP, 5 μM tRNA^{Val}, 20 μM [^{14}C]valine (300 cpm/pmol), and 30 nM ValRS enzymes. Thr-tRNA^{Val} misformation reactions were carried out at 37 °C, in TM buffer containing 3 mM ATP, 5 μM tRNA^{Val}, 20 μM [^{14}C]threonine (300 cpm/pmol), and 500 nM ValRS enzymes. Aliquots were removed at specific time points and were quenched on filter papers (Whatman, 3 mm) equilibrated with 10% trichloroacetic acid (TCA). The filters were washed 3 times with 5% ice-cold TCA and once with 100% ethanol. The radioactivities of the precipitates were quantitated by scintillation counting.

Deacylaction Assay. [^{14}C]Ile-tRNA^{Leu}, [^{14}C]Val-tRNA^{Ile}, and [^{14}C]Thr-tRNA^{Val} were prepared by incubating reaction mixtures, containing TM buffer, 4 mM ATP, 10 μM tRNA (tRNA^{Leu}, *T. thermophilus* tRNA^{Ile}, or tRNA^{Val}), 20 μM [^{14}C]-labeled amino acid (300 cpm/pmol) (isoleucine, valine, or threonine), and 2 μM full-length, editing-deficient mutant enzyme (D332A mutant LeuRS, D328A mutant IleRS, or D279A mutant ValRS), at 65 °C for 5 min. [^{14}C]Ile-tRNA^{Ile}, [^{14}C]Val-tRNA^{Val}, [^{14}C]Thr-tRNA^{Thr}, and [^{14}C]Leu-tRNA^{Leu} were prepared by incubating reaction mixtures, containing TM buffer, 4 mM ATP, 10 μM tRNA (*P. horikoshii* tRNA^{Ile}, tRNA^{Val}, tRNA^{Thr}, or tRNA^{Leu}), 20 μM [^{14}C]-labeled amino acid (300 cpm/pmol) (isoleucine, valine, threonine, or leucine), and 2 μM full-length, wild-type enzyme (*T. thermophilus* IleRS, ValRS, ThrRS, or LeuRS), at 65 °C for 5 min. The [^{14}C]aa-tRNAs thus produced were purified as described (*5*, *24*). The Ile-tRNA^{Leu}, Val-tRNA^{Ile}, and Thr-tRNA^{Val} deacylation reactions (post-transfer editing) by LeuRS, IleRS, and ValRS, respectively, were carried out at 37 °C, in TM buffer containing 2 μM [^{14}C]aa-tRNA and 60 nM wild-type or mutant enzyme (30 nM enzyme in the case of ValRS). The Ile-tRNA^{Ile}, Leu-tRNA^{Leu}, Val-tRNA^{Val}, and Thr-tRNA^{Thr} misdeacylation reactions, by LeuRS, LeuRS, IleRS, and ValRS, respectively, were carried out at 37 °C,

in TM buffer containing 2 μM [^{14}C]aa-tRNA and wild-type or mutant enzyme (20 or 100 nM LeuRS, 100 nM LeuRS, 60 nM IleRS, and 60nM ValRS, respectively). Aliquots were removed at specific time points and were quenched on filter papers (Whatman, 3 mm) equilibrated with 10% TCA. The filters were washed and the radioactivities of the precipitates were quantitated as described above.

RESULTS

LeuRS_Δ(811−967) Cannot Aminoacylate the Cognate tRNA^{Leu}, but Can Deacylate Ile-tRNA^{Leu}. We previously reported that the C-terminal domain-truncated, archaeal *P. horikoshii* LeuRS (LeuRS_Δ(811−967)) could still catalyze the first step of the aminoacylation reaction, the leucyl-adenylate formation, like the full-length LeuRS (LeuRS_FL) (*7*). However, LeuRS_Δ(811−967) could not charge the activated leucine to tRNA^{Leu} (*7*). The Leu-tRNA^{Leu} formation activity of the D332A mutant of LeuRS_FL was not affected, while it produced a significant amount of Ile-tRNA^{Leu} and could not deacylate Ile-tRNA^{Leu} (Figure 2A, 2B, and 2C) (*7*). These results demonstrated that the Asp residue (Asp332), located in the editing active site, is crucial for the editing reaction by the archaeal LeuRS. The Asp residue is completely conserved among LeuRS (both archaeal/eukaryal type and bacterial type), IleRS, and ValRS, and interacts with the α-amino group of the editing substrate analogue in these aaRSs (*6*, *16*, *17*). The mutation of the Asp residue to alanine abolished the editing activity, as also found in the bacterial and eukaryal LeuRSs, IleRS, and ValRS (*6*, *17*, *18*). Here, we measured the activity of the D332A mutant of LeuRS_Δ(811−967) (Figure 2A, 2B, and 2C). It could not form either Leu-tRNA^{Leu} or Ile-tRNA^{Leu}, and it could not deacylate Ile-tRNA^{Leu}, like the D332A mutant of LeuRS_FL. Thus, we confirmed that the deacylation of Ile-tRNA^{Leu} by LeuRS_Δ(811−967) is actually catalyzed by its editing active site, which was inactivated by the D332A mutation.

LeuRS_Δ(811−967) Misedits the Correctly Formed, Noncognate Ile-tRNA^{Ile}. We measured the deacylation activities of the wild-type and mutant LeuRSs against Ile-tRNA^{Ile}, which is the correct product of IleRS. Surprisingly, the LeuRS_Δ(811−967) deacylated Ile-tRNA^{Ile} at a significant rate at a 20 nM enzyme concentration, although the LeuRS_FL did not deacylate it (Figure 2D). The impairment of deacylation by the D332A mutant of LeuRS_Δ(811−967) indicated that the deacylation by LeuRS_Δ(811−967) is catalyzed by the editing active site. Therefore, we can conclude that the C-terminal domain of the archaeal LeuRS functions to prevent the misediting of the correctly formed, noncognate Ile-tRNA^{Ile}.

LeuRS_Δ(811−967) Does Not Recognize tRNA^{Ile} in the Aminoacylation Reaction. Next, we examined whether LeuRS_Δ(811−967) misrecognizes the noncognate tRNA^{Ile} and forms Leu-tRNA^{Ile} in the aminoacylation reaction. None of the LeuRS wild-type and mutants tested, including the editing-deficient ones, exhibited Leu-tRNA^{Ile} formation, even at a high enzyme concentration (500 nM) (Figure 2E). They also did not charge isoleucine to tRNA^{Ile} (data not shown). As a positive control, we confirmed that *T. thermophilus* IleRS attached isoleucine to *P. horikoshii* tRNA^{Ile} and produced Ile-tRNA^{Ile} at a 10 nM enzyme concentration (Figure 2E). Thus, LeuRS_Δ(811−967) does not misrecognize tRNA^{Ile} in the aminoacylation reaction, although it misdeacylates Ile-tRNA^{Ile} in the editing reaction.

LeuRS_Δ(811−967) Does Not Deacylate Leu-tRNA^{Leu}. We measured the misdeacylation activities of correctly formed Leu-tRNA^{Leu} by the wild-type and mutant LeuRSs. None of them misdeacylated Leu-tRNA^{Leu} (Figure 2F). Since LeuRS_Δ(811−967) was able to recognize tRNA^{Leu} in the editing reaction (it can deacylate Ile-tRNA^{Leu}) (Figure 2C), these results show that it retains the amino acid discrimination ability in the editing reaction. Therefore, we can conclude that the C-terminal deletion of LeuRS affects the tRNA recognition, but not the amino acid recognition, in the editing reaction.

Comparison of the tRNA^{Leu} and tRNA^{Ile} Structures. To understand how the LeuRS C-terminal domain discriminates between tRNA^{Leu} and tRNA^{Ile}, we analyzed the 3D structures of *P. horikoshii* tRNA^{Leu} and *P. horikoshii* tRNA^{Ile}. A comparison of the cloverleaf structures revealed that there are three significant differences between these two molecules (Figure 3A). First, tRNA^{Leu} possesses a long variable arm, while tRNA^{Ile} has a short variable region. Second, the D-stem is longer in tRNA^{Ile}, since C13-G22 in tRNA^{Ile} can form a base pair, while G13-A22 in tRNA^{Leu} cannot. Third, tRNA^{Ile} lacks an additional nucleotide in the 20b position, while tRNA^{Leu} has A20b in this position. *E. coli* tRNA^{Ile}, which shares sequence homology with *P. horikoshii* tRNA^{Ile}, and whose 3D structure has been determined in complex with *S. aureus* IleRS in the editing mode (*15*), also has all of these features seen in *P. horikoshii* tRNA^{Ile} (Figure 3A). Therefore, the 3D structures of *E. coli* tRNA^{Ile} and *P. horikoshii* tRNA^{Ile} should be very similar. We superposed the structure of *E. coli* tRNA^{Ile} onto that of the LeuRS-bound *P. horikoshii* tRNA^{Leu}, which revealed a significant difference in their D-loops (Figure 3B and 3C). The U20 and U20a bases in *E. coli* tRNA^{Ile} are directed away from the tRNA core structure. In contrast, in *P. horikoshii* tRNA^{Leu}, the U20 and C20a bases, as well as the additional A20b, face inward toward the tRNA core.

The U20 nucleotide in *E. coli* tRNA^{Ile}, which faces outward, would clash against the C-terminal domain of LeuRS (Figure 3C). This clashing may be the rejection mechanism of tRNA^{Ile} by the LeuRS C-terminal domain in the editing reaction. Residues 911−913 (Ile911, Lys912, and Glu913) of LeuRS (colored magenta in Figure 3C) may be responsible for the rejection mechanism, since U20 in *E. coli* tRNA^{Ile} would clash against them. These residues are located on a loop in the region (colored pink in Figure 3C) that is inserted specifically in the archaeal LeuRS, within the common core architecture of the C-terminal domain.

To validate our hypothesis that residues 911−913 function in rejecting tRNA^{Ile}, we prepared the mutant lacking these three residues (LeuRS_Δ(911−913)). The structural model of the deletion mutant is shown in Figure 3D. Here, the clashing between tRNA^{Ile} and the LeuRS C-terminal domain is not observed.

LeuRS_Δ(911−913) Misedits Ile-tRNA^{Ile}. We examined the Ile-tRNA^{Leu} (correct editing) and Ile-tRNA^{Ile} (misediting) deacylation activities of LeuRS_Δ(911−913) (Figure 4). The deacylation activity of LeuRS_Δ(911−913) against Ile-tRNA^{Leu} was comparable to those of LeuRS_FL and LeuRS_Δ(811−967) (Figure 4A), revealing that the region

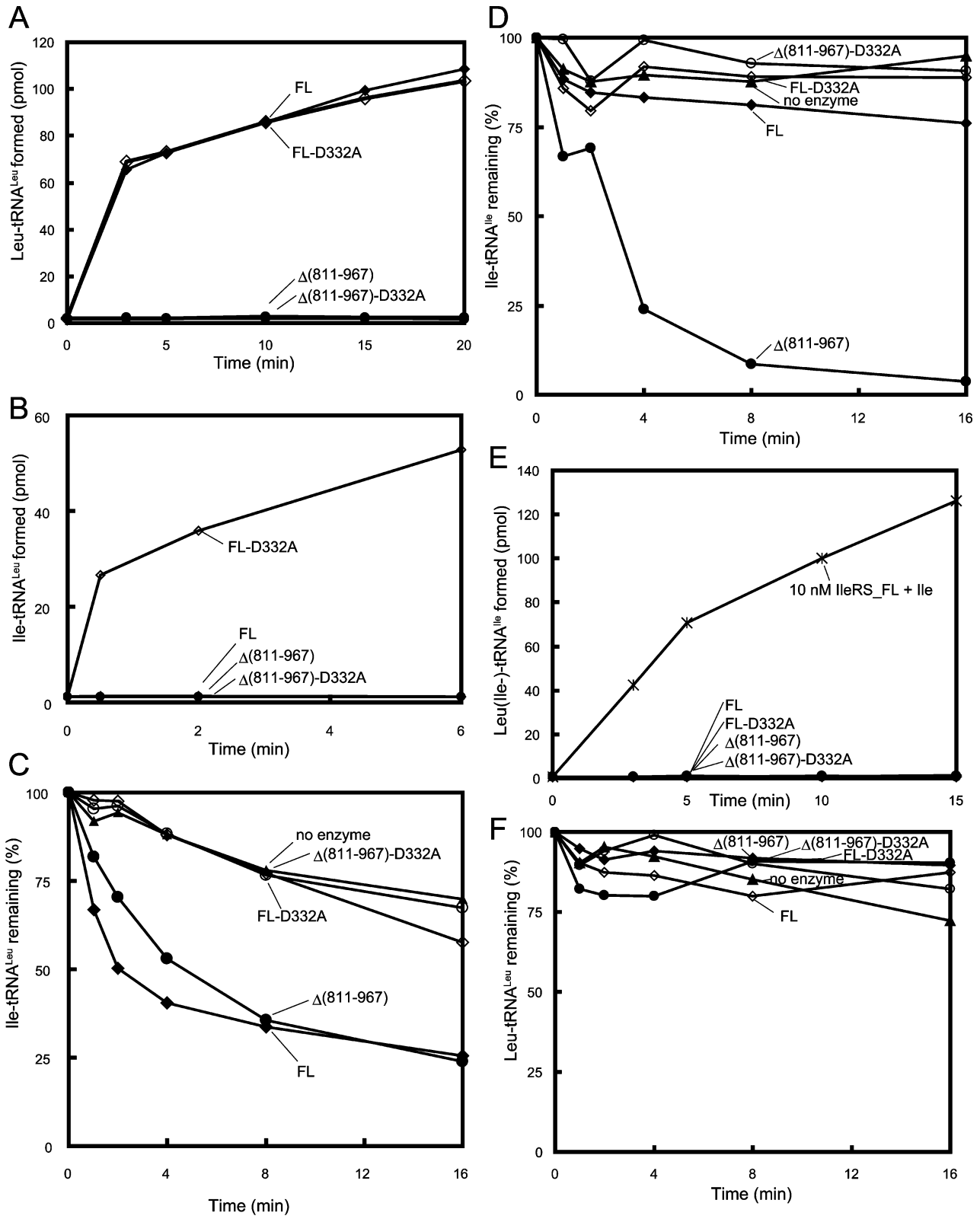

FIGURE 2: LeuRS_Δ(811−967) cannot aminoacylate the cognate tRNA^{Leu}, but can deacylate Ile-tRNA^{Leu}. (A) Leu-tRNA^{Leu} formation by LeuRSs. (B) Ile-tRNA^{Leu} misformation by LeuRSs. (C) Ile-tRNA^{Leu} deacylation by LeuRSs. (D) Ile-tRNA^{Ile} deacylation by LeuRSs. (E) Leu-tRNA^{Ile} misaminoacylation by LeuRSs. (F) Leu-tRNA^{Leu} misdeacylation by LeuRSs. In this paper, consistent symbols are used throughout all of the figures. Full-length, wild-type, ◆; full-length, editing-defective mutant, ◇; C-terminal domain-deleted mutant, ●; C-terminal domain-deleted, editing-defective mutant, ○; no enzyme, ▲.

is not important for tRNA^{Leu} recognition in the editing reaction. For the Ile-tRNA^{Ile} misdeacylation experiments, we used a higher enzyme concentration (100 nM) than that used in the experiment shown in Figure 2D (20 nM). Under the high enzyme concentration conditions, LeuRS_FL exhibited detectable misdeacylation activity, as compared with the absence of the enzyme, while LeuRS_Δ(811−967) deacylated Ile-tRNA^{Ile} much more rapidly (Figure 4B). Thus, we could validate the effects of the mutations. LeuRS_Δ(911−913) deacylated Ile-tRNA^{Ile} more rapidly than LeuRS_FL

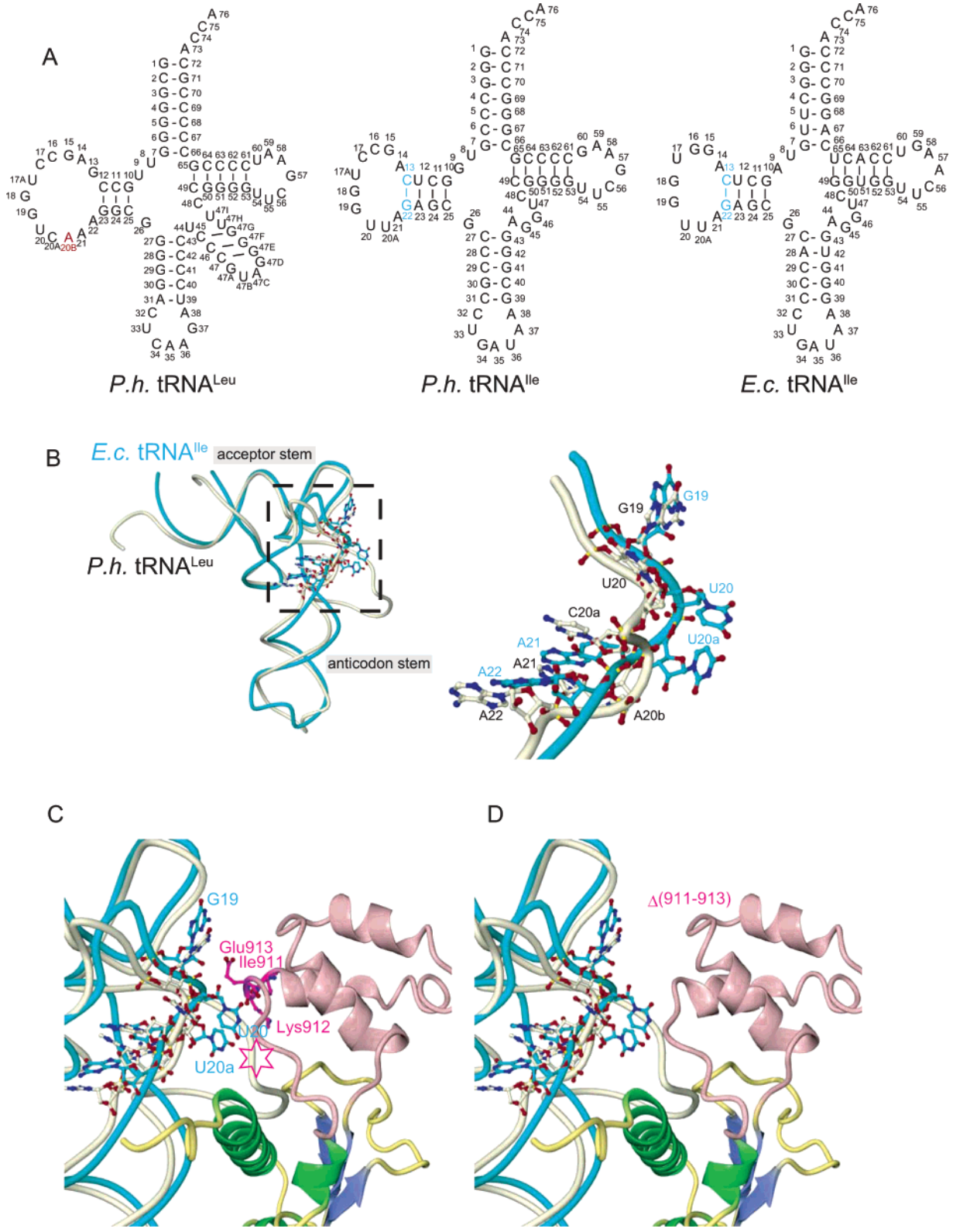

FIGURE 3: Structural mechanism for the prevention of Ile-tRNA^{Ile} misediting by the LeuRS C-terminal domain. (A) Cloverleaf models of *P. horikoshii* tRNA^{Leu}, *P. horikoshii* tRNA^{Ile}, and *E. coli* tRNA^{Ile}. (B) (Left) Structural superposition of *P. horikoshii* tRNA^{Leu} (colored white) and *E. coli* tRNA^{Ile} (colored cyan), taken from the LeuRS-tRNA^{Leu} complex and the IleRS-tRNA^{Ile} complex, respectively. (Right) Close-up view of the region around the U20 nucleotides. (C) Clashing between the LeuRS C-terminal domain and *E. coli* tRNA^{Ile}, which is superposed onto the *P. horikoshii* LeuRS-tRNA^{Leu} complex, based on the tRNA structures. The nucleotide residue U20 and the amino acid residues 911−913 in the LeuRS C-terminal domain are clashing. The LeuRS C-terminal domain is shown by a ribbon model, with α-helices, β-strands, and loops colored green, blue, and yellow, respectively. The archaeal LeuRS-specific insertion is colored pink. The amino acid residues 911−913 are shown by magenta ball and stick models. (D) Structural model of the C-terminal domain of LeuRS_Δ(911−913) bound with tRNA^{Leu} and tRNA^{Ile}. The clashing is not observed, due to the deletion of residues 911−913.

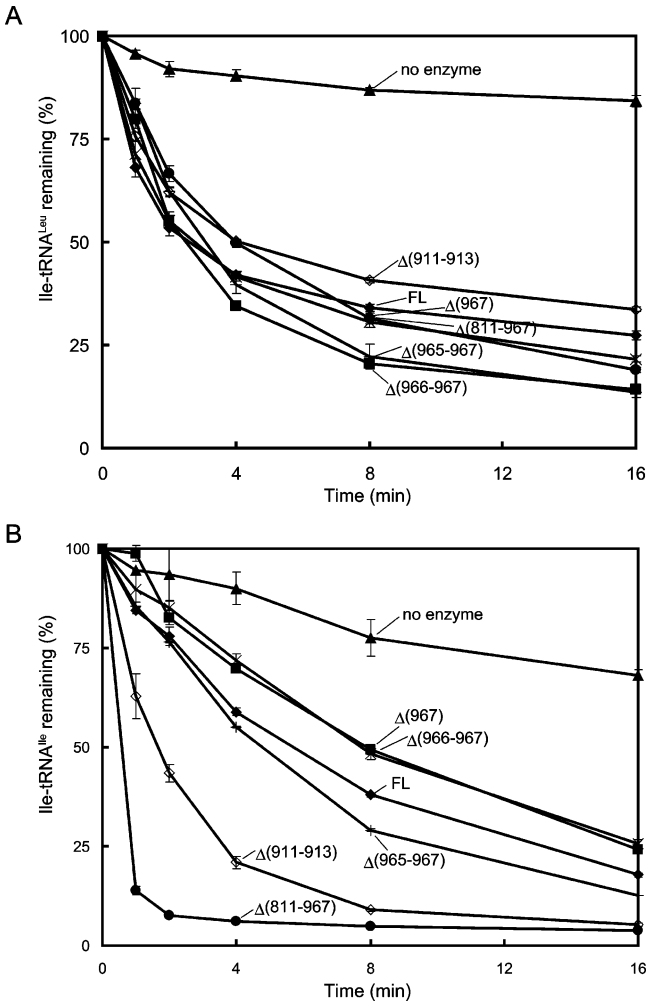

FIGURE 4: LeuRS_Δ(911−913) shows normal Ile-tRNA^{Leu} deacylation and enhanced Ile-tRNA^{Ile} deacylation. (A) Ile-tRNA^{Leu} deacylation (correct editing) by LeuRSs. (B) Ile-tRNA^{Ile} deacylation (misediting) by LeuRSs.

(Figure 4B). Therefore, we can conclude that residues 911−913 function in rejecting tRNA^{Ile}, to prevent misediting. Since LeuRS_Δ(811−967) deacylates Ile-tRNA^{Ile} more rapidly than LeuRS_Δ(911−913), another region in the C-terminal domain may also contribute to the rejection. However, considering the remarkable enhancement of the misediting activity by the quite minor change of LeuRS_Δ(911−913) (only a three-residue deletion), the main contributors are these three residues.

The Leu-tRNA^{Leu} Formation Activity of LeuRS_Δ(911−913) Is Decreased 2-fold. We examined whether LeuRS_Δ(911−913) and LeuRS_FL can recognize tRNA^{Ile} in the aminoacylation reaction and form Leu-tRNA^{Ile}. However, such an activity was not observed, even at high enzyme and substrate concentrations (data not shown). Therefore, the tRNA must be selected in a much stricter way in the aminoacylation reaction than in the editing reaction. Next, we performed a kinetic analysis of tRNA^{Leu} recognition in the Leu-tRNA^{Leu} formation reactions by LeuRS_FL and LeuRS_Δ(911−913) (Table 1). The K_m values were 1.67 and 1.79 μM, and the k_{cat} values were 0.331 and 0.177 s^{-1}, for LeuRS_FL and LeuRS_Δ(911−913), respectively. The K_m values are almost the same, and the k_{cat} value is decreased by about 2-fold in the deletion mutant. The relative k_{cat}/K_m value of LeuRS_Δ(911−913) is 0.500. Thus, the deletion

Table 1: Kinetic Analysis of Leu-tRNA^{Leu} Formation

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m	k_{cat}/K_m (relative)
LeuRS_FL	1.67	0.331	0.198	1
LeuRS_Δ(911−913)	1.79	0.177	0.099	0.500

of residues 911−913 resulted in a 2-fold decrease in the Leu-tRNA^{Leu} formation activity. Residues 911−913 are located within the archaeal LeuRS-specific insertion (Figure 3C). The insertion contributes to the conformational change of a region within the common core architecture of the C-terminal domain, which opens the binding pocket for the tip bases of the tRNA^{Leu} long variable arm (*19*). Therefore, the slight decrease of the Leu-tRNA^{Leu} formation activity by the deletion of residues 911−913 may occur because the conformational change is not induced properly in the deletion mutant. Thus, the three-residue region functions in both tRNA^{Leu} selection in the aminoacylation reaction and tRNA^{Ile} rejection in the editing reaction.

LeuRS_Δ(967), LeuRS_Δ(966−967), and LeuRS_Δ(965−967) Deacylate Ile-tRNA^{Leu}, but Not Ile-tRNA^{Ile}. The crystal structures of *P. horikoshii* LeuRS:tRNA^{Leu} revealed that some of the residues at the C-terminal extremity hydrogen bond with the nucleotide bases at the tip of the tRNA^{Leu} long variable arm in the aminoacylation reaction (Figure 1C) (*19*). We previously reported that C-terminally deleted mutants, which lack two or three residues at the C-terminal extremity (LeuRS_Δ(966−967) and LeuRS_Δ(965−967), respectively), have severe impairments in Leu-tRNA^{Leu} synthesis, possibly because they cannot recognize tRNA^{Leu} in the aminoacylation reaction (*19*). In contrast, the C-terminal single residue deleted mutant (LeuRS_Δ(967)) exhibited Leu-tRNA^{Leu} formation activity similar to that of the wild-type enzyme. Here, we examined whether these mutants have deacylation activities against Ile-tRNA^{Leu} and Ile-tRNA^{Ile}. LeuRS_Δ(967), LeuRS_Δ(966−967), and LeuRS_Δ(965−967) showed similar deacylation activities against Ile-tRNA^{Leu}, as compared with those of LeuRS_FL, LeuRS_Δ(811−967), and LeuRS_Δ(911−913) (Figure 4A). These results revealed that the three mutants retain the cognate tRNA^{Leu} selection capability in the editing reaction. LeuRS_Δ(967), LeuRS_Δ(966−967), and LeuRS_Δ(965−967) showed Ile-tRNA^{Ile} misdeacylation rates similar to that of LeuRS_FL at the high enzyme concentration, indicating that the C-terminal residue truncations did not affect the noncognate tRNA^{Ile} rejection capability of LeuRS in either a positive or negative manner.

Thus, we succeeded in separating the tRNA^{Leu} recognition impairment in the aminoacylation reaction and the newly acquired tRNA^{Ile} misrecognition in the editing reaction: LeuRS_Δ(966−967) and LeuRS_Δ(965−967) are severely impaired in the tRNA^{Leu} recognition in the aminoacylation reaction, while they retain the noncognate tRNA^{Ile} rejection capability in the editing reaction. LeuRS_Δ(811−967) has both impairments. In contrast, LeuRS_Δ(911−913) has about a 3-fold reduction in the tRNA^{Ile} rejection capability (LeuRS_Δ(911−913) deacylates Ile-tRNA^{Ile} about three times as rapidly as LeuRS_FL at a high enzyme concentration), while the tRNA^{Leu} recognition in the aminocylation reaction is decreased 2-fold. Thus, we can say that the C-terminal domain of the archaeal LeuRS plays two roles (tRNA^{Leu} selection in the aminoacylation reaction and

Table 2: Summary of the Assay Results of *P. horikoshii* LeuRS in This and Previous Studies[a]

	Leu-AMP formation	Leu-tRNA^{Leu} formation	Ile-tRNA^{Leu} formation	Leu-tRNA^{Ile} formation	Ile-tRNA^{Ile} formation	Ile-tRNA^{Leu} deacylation	Ile-tRNA^{Ile} deacylation	Leu-tRNA^{Leu} deacylation
LeuRS_FL	+++[b]	+++	−	−	−	+++	−	−
LeuRS_FL-D332A		+++	+++	−	−	−	−	−
LeuRS_Δ(811−967)	+++[b]	−	−	−	−	+++	+++	−
LeuRS_Δ(811−967)-D332A		−	−	−	−	−	−	−
LeuRS_Δ(911−913)		+		−		+++	+	
LeuRS_Δ(967)		+++[b]				+++	−	
LeuRS_Δ(966−967)		−[b]				+++	−	
LeuRS_Δ(965−967)		−[b]				+++	−	

[a] +++, strong activity; +, weak activity; −, not detected. Blank columns indicate that the corresponding assays were not performed. [b] From the previous studies (*7*, *19*).

tRNA^{Ile} rejection in the editing reaction) using the different regions. The tRNA^{Leu} recognition in the aminoacylation reaction is mainly performed by some residues at the C-terminal extremity, while the noncognate tRNA^{Ile} rejection in the editing reaction is mainly attributable to residues 911−913.

The assay results of *P. horikoshii* LeuRS from this and previous studies are summarized in Table 2.

IleRS_Δ(791−1045) Can Activate Isoleucine, but Can neither Aminoacylate tRNA^{Ile}, Edit Val-tRNA^{Ile}, nor Misedit Val-tRNA^{Val}. To elucidate the function of the C-terminal domain of IleRS, which shares structural homology with that of the archaeal LeuRS, we examined the activity of the wild-type and C-terminal domain-truncated *T. thermophilus* IleRSs (IleRS_FL and IleRS_Δ(791−1045)) (Figure 5). IleRS_Δ(791−1045) showed Ile-AMP formation activity similar to that of IleRS_FL (Figure 5A). These results revealed that the C-terminal domain of IleRS is dispensable for the amino acid activation reactions, as in the case of archaeal LeuRS. Next, we measured the Ile-tRNA^{Ile} and Val-tRNA^{Ile} formation activities. We also prepared and analyzed the editing-deficient D328A mutants of IleRS_FL and D328A IleRS_Δ(791−1045). The full-length enzymes produced significant amounts of Ile-tRNA^{Ile} (Figure 5B). In contrast, both of the C-terminally deleted mutants, regardless of their editing deficiencies, exhibited severe aminoacylation defects. Furthermore, Val-tRNA^{Ile} misformation was observed in D328A IleRS_FL, but not in D328A IleRS_Δ(791−1045), IleRS_Δ(791−1045), or IleRS_FL (Figure 5C). These results revealed that the charging activity of the activated amino acid to the cognate tRNA is impaired by the deletion of the C-terminal domain, possibly because it cannot recognize the cognate tRNA^{Ile}. Next, we measured the Val-tRNA^{Ile} deacylation activities. Neither IleRS_Δ(791−1045), D328A IleRS_Δ(791−1045), nor D328A IleRS_FL deacylated Val-tRNA^{Ile}, while IleRS_FL did (Figure 5D). These findings suggested that the C-terminal domain of IleRS is necessary for the recognition of the cognate tRNA in the editing reaction, unlike the case of the archaeal LeuRS. In contrast to the case of LeuRS_Δ(811−967), IleRS_Δ(791−1045) did not deacylate the correctly formed Val-tRNA^{Val}, which was slowly deacylated by 60 nM IleRS_FL (Figure 5E). These results revealed that the C-terminal domain of IleRS is not required for the rejection of Val-tRNA^{Val} in the editing reaction. The assay results of *T. thermophilus* IleRS from this study are summarized in Table 3.

ValRS_Δ(708−862) Can Activate Valine, but Can neither Aminoacylate tRNA^{Val}, Edit Thr-tRNA^{Val}, nor Misedit Thr-tRNA^{Thr}. Finally, we examined the activity of the wild-type and C-terminal domain-truncated *T. thermophilus* ValRS (ValRS_FL and ValRS_Δ(708−862)) (Figure 6). ValRS_Δ(708−862) exhibited similar Val-AMP formation activity to that of ValRS_FL (Figure 6A), revealing that the C-terminal domain is not important in the amino acid activation reaction. Next, we measured the Val-tRNA^{Val} and Thr-tRNA^{Val} formation activities. Both of the C-terminally deleted mutants, regardless of their editing deficiencies, exhibited severe defects in Val-tRNA^{Val} formation (Figure 6B). Furthermore, Thr-tRNA^{Val} misformation was observed with D279A ValRS_FL, but not with D279A ValRS_Δ(708−862) (Figure 6C). These findings revealed that the charging activity is impaired by the truncation of the C-terminal domain. Neither ValRS_Δ(708−862), D279A ValRS_FL, nor D279A ValRS_Δ(708−862) deacylated Thr-tRNA^{Val}, while ValRS_FL did (Figure 6D), suggesting that the C-terminal domain of ValRS is necessary for the cognate tRNA editing. ValRS_Δ(708−862), as well as ValRS_FL, did not deacylate the correctly formed Thr-tRNA^{Thr} (Figure 6E). Thus, unlike the archaeal LeuRS, the main body of the ValRS enzyme is sufficient for the rejection of Thr-tRNA^{Thr}, and the C-terminal domain is not necessary for the prevention of misediting. The assay results of *T. thermophilus* ValRS from this study are summarized in Table 4.

DISCUSSION

LeuRS is unique among the aaRSs, in that it does not contact the anticodon bases for cognate tRNA recognition. Most aaRSs utilize the anticodon bases to achieve specific recognition of their cognate tRNAs. The recognition strategy using anticodon bases is useful, since most amino acids are assigned to one codon box in the genetic code. For example, valine is encoded by the codon GUN. ValRS can specifically select tRNA^{Val} by choosing the tRNA with the anticodon NAC. However, in the case of leucine, as many as six codons are assigned, and LeuRS should recognize up to six tRNA^{Leu} isoacceptors with different anticodons. Therefore, it may be difficult for LeuRS to achieve tRNA selectivity by recognizing anticodon bases. Nature has adopted a different strategy for specific tRNA^{Leu} recognition: the variable region has been extended in tRNA^{Leu}, and the archaeal/eukaryal LeuRS recognizes the unique long arm (*25*).

Among the homologous LeuRS, IleRS, and ValRS enzymes, the core structures of the C-terminal domains are also homologous. The C-terminal domains consist of the common

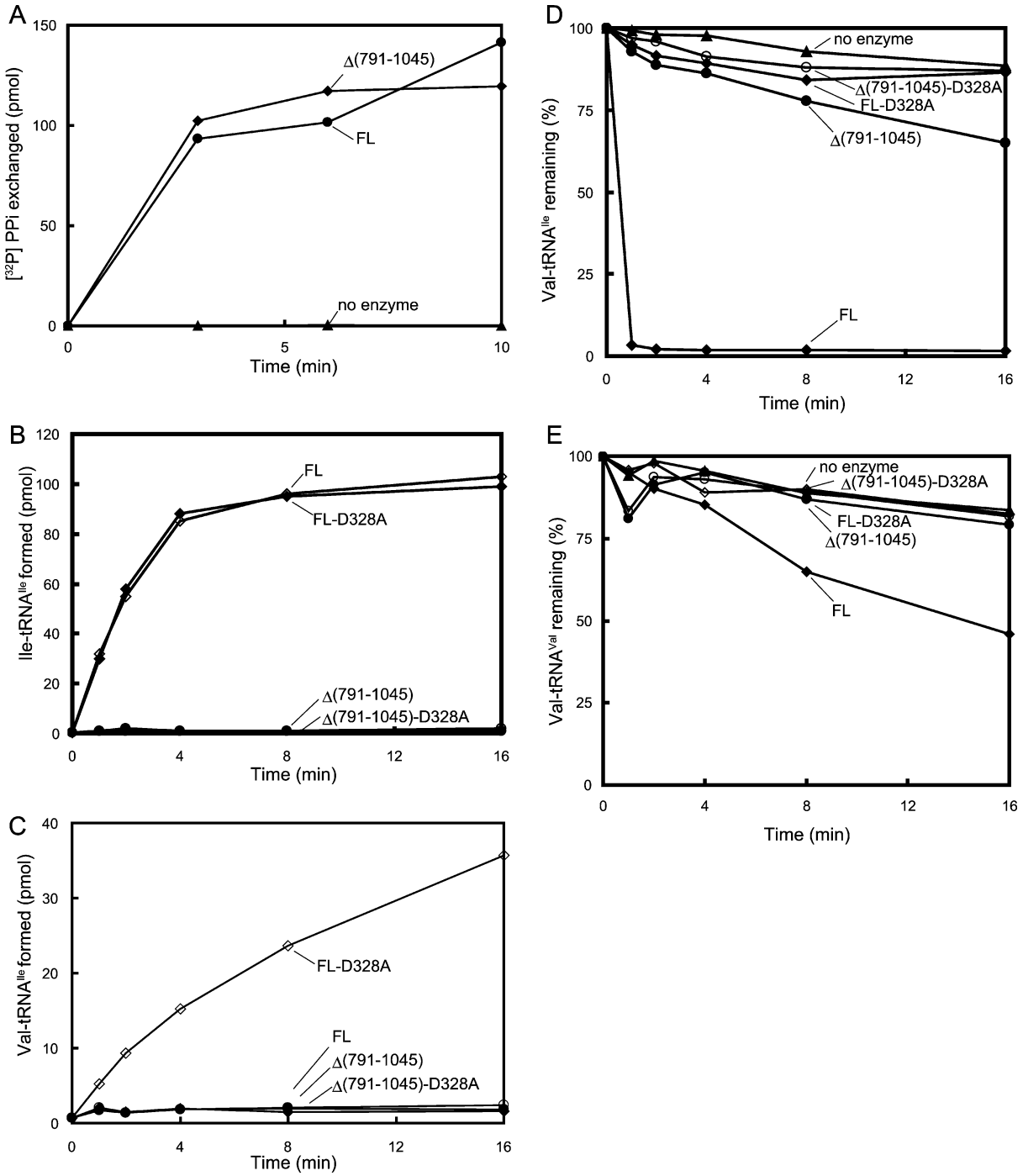

FIGURE 5: IleRS_Δ(791−1045) can activate the amino acid, but can neither aminoacylate $tRNA^{Ile}$ nor deacylate Val-$tRNA^{Ile}$ and Val-$tRNA^{Val}$. (A) Ile-AMP formation by IleRSs, monitored by isoleucine-dependent PPi-exchange. (B) Ile-$tRNA^{Ile}$ formation by IleRSs. (C) Val-$tRNA^{Ile}$ misformation by IleRSs. (D) Val-$tRNA^{Ile}$ deacylation by IleRSs. (E) Val-$tRNA^{Val}$ misdeacylation by IleRSs.

Table 3: Summary of the Assay Results of *T. thermophilus* IleRS in This Study[a]

	Ile-AMP formation	Ile-$tRNA^{Ile}$ formation	Val-$tRNA^{Ile}$ formation	Val-$tRNA^{Ile}$ deacylation	Val-$tRNA^{Val}$ deacylation
IleRS_FL	+++	+++	−	+++	+
IleRS_FL-D328A		+++	+++	−	−
IleRS_Δ(791−1045)	+++	−	−	−	−
IleRS_Δ(791−1045)-D328A		−	−	−	−

[a] +++, strong activity; +, weak activity; −, not detected. Blank columns indicate that the corresponding assays were not performed.

core architecture, with a β-sheet and two peripheral α-helices (Figure 1B). The additional region specific to each of the three enzymes is added to the core architecture. The anticodon bases of $tRNA^{Ile}$ are recognized by the IleRS-specific additional region in the C-terminal domain as well as by the α-helix bundle domain and the N-terminal extension in the main body of the enzyme (Figure 1B). The anticodon bases of $tRNA^{Val}$ are recognized by the core region

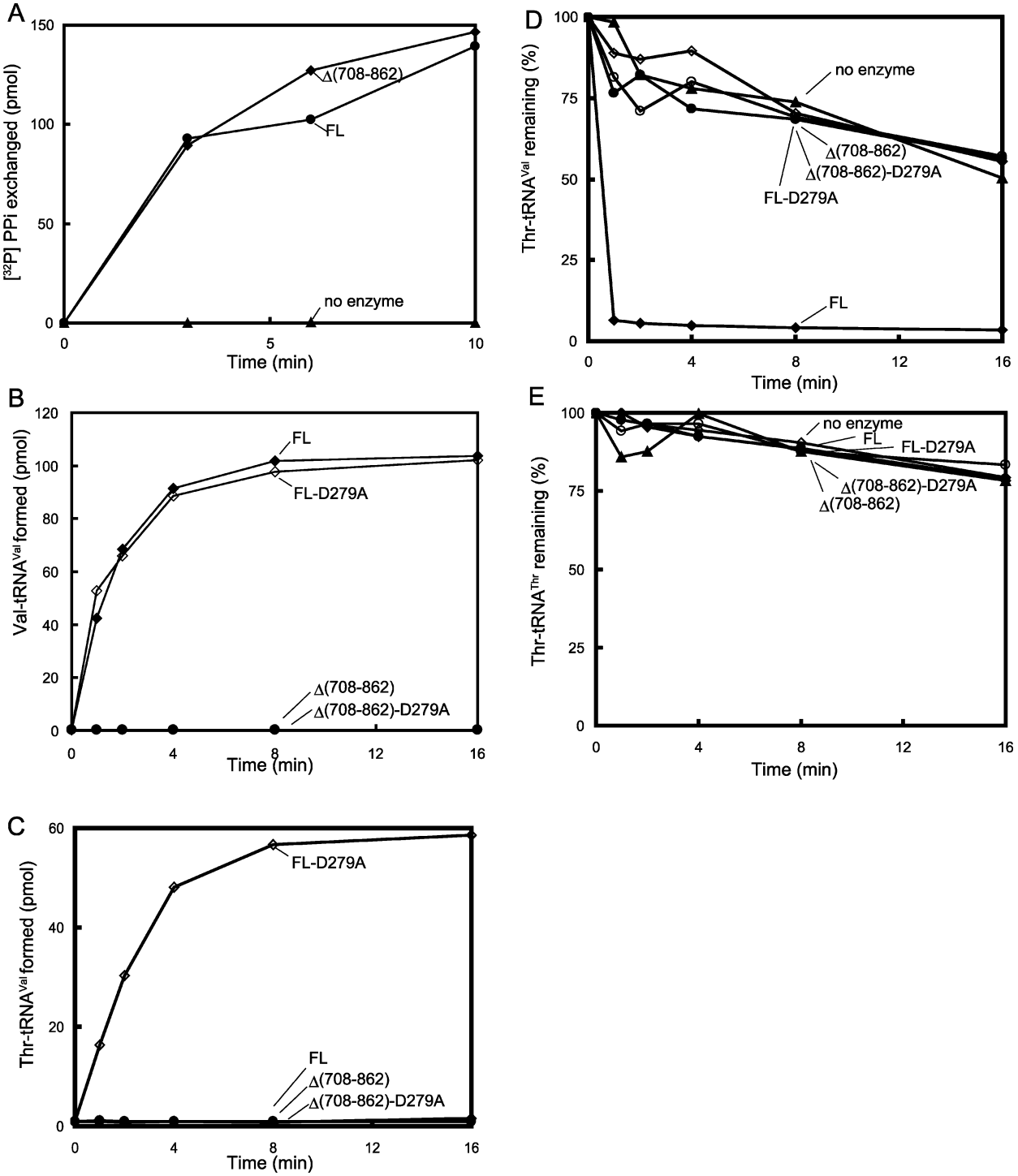

FIGURE 6: ValRS_Δ(708−862) can activate the amino acid, but can neither aminoacylate $tRNA^{Val}$ nor deacylate Thr-$tRNA^{Val}$ and Thr-$tRNA^{Thr}$. (A) Val-AMP formation by ValRSs, monitored by isoleucine-dependent PPi-exchange. (B) Val-$tRNA^{Val}$ formation by ValRSs. (C) Thr-$tRNA^{Val}$ misformation by ValRSs. (D) Thr-$tRNA^{Val}$ deacylation by ValRSs. (E) Thr-$tRNA^{Thr}$ misdeacylation by ValRSs.

in the C-terminal domain as well as by the α-helix bundle domain in the main body of the enzyme (Figure 1B). The anticodon bases of $tRNA^{Leu}$ are not contacted by LeuRS (Figure 1B). The tip of the long variable arm of $tRNA^{Leu}$ is recognized by a part of the core region of the C-terminal domain, which adopts a different structure because of the interactions with the LeuRS-specific additional region in the C-terminal domain (Figure 1C).

In this study, we have demonstrated that the C-terminal domains of LeuRS, IleRS, and ValRS are necessary for the second step of the aminoacylation reactions. In addition, previous biochemical studies demonstrated that the cognate tRNA nucleotides that interact with the C-terminal domain are important for the reactions, whereas the positions of the recognized nucleotides are not the same among LeuRS, IleRS, and ValRS. Actually, the three aaRSs show severely reduced activity with cognate tRNA mutants that have mutations into the recognized nucleotides, and a detectable activity with noncognate tRNA mutants transplanted with the cognate nucleotides (the "identity" nucleotides) (*25*−

Table 4: Summary of the Assay Results of *T. thermophilus* ValRS in This Study[a]

	Val-AMP formation	Val-tRNA^{Val} formation	Thr-tRNA^{Val} formation	Thr-tRNA^{Val} deacylation	Thr-tRNA^{Thr} deacylation
ValRS_FL	+++	+++	−	+++	−
ValRS_FL-D279A		+++	+++	−	−
ValRS_Δ(708−862)	+++	−	−	−	−
ValRS_Δ(708−862)-D279A		−	−	−	−

[a] +++, strong activity; −, not detected. Blank columns indicate that the corresponding assays were not performed.

27). Therefore, the recognition of the identity nucleotides by the C-terminal domain is crucial for the cognate tRNA selection in the aminoacylation reactions of these three enzymes.

We have also shown that the C-terminal domains are necessary for the editing reactions in IleRS and ValRS. These findings indicate that the recognition of the same or a similar set of identity nucleotides by the C-terminal domain is also used for the cognate tRNA selection in the editing reactions of IleRS and ValRS. In contrast, the C-terminal domain was dispensable for the editing reaction of the archaeal LeuRS. Thus, the recognition of the identity nucleotides by the C-terminal domain is not used for the cognate tRNA^{Leu} selection by the archaeal LeuRS. There is a significant point that distinguishes the archaeal/eukaryal LeuRS from IleRS/ValRS; the orientations of the editing domain differ between them. In the archaeal/eukaryal LeuRS, the domain is rotated by ∼180° (rotational state II), with the two-β-stranded linker untwisted by a half-turn, as compared to those in IleRS and ValRS (rotational state I) (*7*). The pathways taken by the tRNA 3′-ends to approach the editing active sites are different. The tRNA^{Leu} 3′-end goes around the outside of both the enzyme's main body and the two-β-stranded linker, while the tRNA$^{Ile/Val}$ 3′-end goes in between them (*7*, *12*, *15*, *19*). Therefore, in LeuRS, even if the tRNA has less contact with the enzyme, its 3′-end could be accommodated in the editing active site, unlike in IleRS/ValRS, where more extensive contact is required. These differences in the editing domain orientations may be the reason why the C-terminal domain is necessary for editing reactions in IleRS/ValRS, but not in LeuRS.

The finding that LeuRS_Δ(811−967), which has lost the C-terminal domain and thereby the major contact with the long variable arm of tRNA^{Leu}, can still edit Ile-tRNA^{Leu} raised the possibility that Ile-tRNA^{Ile} may also be edited by this C-terminally truncated enzyme. Actually, LeuRS_Δ(811−967) misedited Ile-tRNA^{Ile}. A structural superposition suggested that amino acid residues 911−913 of the full-length *P. horikoshii* LeuRS would clash with the U20 nucleotide of the modeled tRNA^{Ile} bound to LeuRS, and thus prevent the misediting. In fact, LeuRS_Δ(911−913) deacylated Ile-tRNA^{Ile}. Therefore, the clashing between the bulged U20 in tRNA^{Ile} and Ile911-Lys912-Glu913 on the LeuRS-specific additional region in the C-terminal domain contributes toward preventing the noncognate tRNA^{Ile} rejection in the editing reaction. LeuRS would not edit a tRNA with a bulged U20.

Thus, the different structural elements in the C-terminal domain of the archaeal LeuRS play a role for achieving the tRNA specificity in either aminoacylation or editing. Some residues at the C-terminal extremity function in the cognate tRNA^{Leu} selection in the aminoacylation reaction by hydrogen bonding with the bases at the tip of the long variable arm. On the other hand, Ile911-Lys912-Glu913, on the LeuRS-specific additional region in the C-terminal domain, contribute to preventing the noncognate tRNA^{Ile} rejection in the editing reaction, by clashing with U20 of tRNA^{Ile}. In contrast, IleRS and ValRS seem to require the cognate identity nucleotides for the editing reaction in addition to the aminoacylation reaction, which is likely to prevent them from misediting Val-tRNA^{Val} and Thr-tRNA^{Thr}, respectively.

After it is synthesized by an aaRS, the aa-tRNA is bound with EF-Tu and is delivered to the ribosome. EF-Tu and the class Ia/b aaRSs without an editing domain, such as cysteinyl-tRNA synthetase, can simultaneously bind to one cognate tRNA molecule (*28*). After it is aminoacylated by the aaRS, the 3′-end of such a tRNA may be immediately transferred to EF-Tu, thus avoiding the risk of being misedited by another aaRS. In contrast, a tRNA^{Ile} molecule cannot be simultaneously bound by IleRS and EF-Tu, because of potential clashing between EF-Tu and the editing domain of IleRS (*28*). As a consequence, the synthesized Ile-tRNA^{Ile} should dissociate from IleRS for its capture by EF-Tu, and thus may potentially contact LeuRS. Therefore, the archaeal LeuRS C-terminal domain is important to prevent misediting of Ile-tRNA^{Ile}.

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