Degenerate Connective Polypeptide 1 (CP1) Domain from Human Mitochondrial Leucyl-tRNA Synthetase*

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The connective polypeptide 1 (CP1) editing domain of leucyl-tRNA synthetase (LeuRS) from various species either harbors a conserved active site to exclude tRNA mis-charging with noncognate amino acids or is evolutionarily truncated or lost because there is no requirement for high translational fidelity. However, human mitochondrial LeuRS (hmtLeuRS) contains a full-length but degenerate CP1 domain that has mutations in some residues important for post-transfer editing. The significance of such an inactive CP1 domain and a translational accuracy mechanism with different noncognate amino acids are not completely understood. Here, we identified the essential role of the evolutionarily divergent CP1 domain in facilitating hmtLeuRS’s catalytic efficiency and endowing enzyme with resistance to AN2690, a broad-spectrum drug acting on LeuRSs. In addition, the canonical core of hmtLeuRS is not stringent for noncognate norvaline (Nva) and valine (Val). hmtLeuRS has a very weak tRNA-independent pre-transfer editing activity for Nva, which is insufficient to remove mis-activated Nva. Moreover, hmtLeuRS chimeras fused with a functional CP1 domain from LeuRSs of other species, regardless of origin, showed restored post-transfer editing activity and acquired fidelity during aminoacylation. This work offers a novel perspective on the role of the CP1 domain in optimizing aminoacylation efficiency.

In the three domains of life, genetic information is delivered from DNA to mRNA and is subsequently translated into protein. The fidelity of the whole process is vital for maintaining cellular physiology. Aminoacyl-tRNA synthetases (aaRSs)3 are a family of canonical and diverse enzymes that catalyze the esterification of the amino acids to the cognate tRNAs in a two-step reaction, supplying the building blocks, aminoacyl-tRNAs (aa-tRNAs) for protein biosynthesis. First, the corresponding amino acid is activated with the hydrolysis of ATP, forming an enzyme-aminocacyl-adenylate complex, and subsequently, the activated aminoacyl moiety is transferred to the terminal adenine (A76) of the 3’ end of tRNA (1–3). Based on sequence alignments and structural and functional analyses, the 20 aaRSs are divided exclusively into class I and class II, each consisting of 10 enzymes (4–6). Class I aaRSs utilize a Rossman dinucleotide-binding fold, characterized by two conserved motifs, HIGH and KMSKS, as the framework to perform their tRNA charging activities (4, 5). However, class II aaRSs use an anti-parallel β-sheet active-site architecture with signature motifs, motifs 1–3, to dimerize and to bind ATP and amino acids (4, 6). During evolution, half of the aaRSs have gradually incorporated an editing domain to remove the wrong amino acid charged on the cognate tRNA, possessing a “double-sieve” mechanism to correct errors in aminoacylation and guarantee the accuracy of protein synthesis. Amino acids larger than the cognate amino acid are excluded via the aminoacylation active site, i.e., “coarse sieve”, and the editing site performs as the “fine sieve” to hydrolyze the incorrect but isosteric amino acid, based on size and structure (3, 7).

Leucyl-tRNA synthetase (LeuRS), along with isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS), belongs to sub-group Ia, which shares a homologous connective polypeptide 1 (CP1) domain tethered to the halves of the Rossman fold by two flexible β-strands. LeuRS could mis-activate a series of analogs, including valine (Val), isoleucine (Ile), methionine (Met), and intermediate metabolites, like norvaline (Nva) and L-α-aminobutyric acid (ABA) (8, 9). The high fidelity in discriminating the cognate substrate from the large pool of structurally similar noncognate amino acids is largely maintained by proofreading (editing). Based on rectifying errors in the aminoacyl-adenosine monophosphate (aa-AMP) level or the aa-tRNA level, total editing can be divided into pre-transfer editing and post-transfer editing. In addition, pre-transfer editing is further classified as tRNA-dependent and tRNA-independent pre-transfer editing, depending on whether the tRNA can

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3 The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; CP1, connective peptide 1; LeuRS, leucyl-tRNA synthetase; hmtLeuRS, human mitochondrial leucyl-tRNA synthetase; Nva, norvaline; Val, Valine; PDB, Protein Data Bank; aa, aminoacyl; AN2690, 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole.
domains provide a direct safeguard to guarantee accuracy in functionally active in post-transfer editing. These active CP1s served Asp residue, the GTG and Thr-rich regions, and are duees are highly homologous and conserved, possessing the conservation activity of Escherichia coli LeuRS (EcLeuRS) (12, 13). Residues that are essential for the hydrolytic role of the CP1 domain have been identified by structural and biochemical analyses, and these include a Thr-rich peptide and a GTG motif (constituting the editing catalytic pocket that can perform the hydrolytic role) and an absolutely conserved Asp residue (such as Asp345 in EsLeuRS), which forms a salt bridge with the α-amino group of the noncognate amino acid) (14–16).

LeuRSs from various species differ in the presence pattern and editing activity of the CP1 domain (Fig. 1). For most LeuRSs, such as LeuRSs from E. coli, Aquifex aeolicus (AaLeuRS) to Saccharomyces cerevisiae cytoplasm (ScLeuRS), and Homo sapiens cytoplasm (HsLeuRS), the active site residues are highly homologous and conserved, possessing the conserved Asp residue, the GTG and Thr-rich regions, and are functionally active in post-transfer editing. These active CP1 domains provide a direct safeguard to guarantee accuracy in tRNALeu aminoacylation. Nevertheless, the CP1 domains of some LeuRSs from Mycoplasma species have been severely truncated, and these LeuRSs are thought to possess no post-transfer editing activity. As a particular example, Mycoplasma mobile encodes a minimized LeuRS (MmLeuRS), which completely lacks a CP1 domain, having a nonapeptide linker (MmLinker, 227-KEEIDGTK235) instead. It is thought that LeuRSs in Mycoplasma parasites gradually lost the important residues in the editing domain to achieve translational inaccuracy and consequent phenotypic plasticity to accommodate host defense (13, 17). Thus, truncation or loss of the CP1 domain is evolutionarily beneficial for their growth or survival. However, LeuRSs from the mitochondria of some higher eukaryotes (such as Mus musculus and H. sapiens) are unique because they have inherent substitutions at key residues in the hydrolytic active sites (18).

hmtLeuRS is encoded by the nuclear LARS2 gene and is then imported into the mitochondria. By contrast, human mitochondrial tRNALeu(546) (including tRNALeu(CUN) and tRNALeu(UUR)) are encoded by the mitochondrial genome. The precursor of hmtLeuRS, with a full length of 903 residues, is transported with the guidance of an N-terminal signal peptide and cleaved between Ser39 and Ile40 (19). Although hmtLeuRS preserves the key conserved Asp residue in the CP1 domain, the GTG region and Thr-rich region have variations to different extents (Fig. 1), leading to a degenerated CP1 that has lost its post-transfer editing activity (18). Compared with the truncation or complete loss of the CP1 domain in Mycoplasma parasites, which favors their survival, the reason why hmtLeuRS retains this degraded domain is unclear. Furthermore, a previous study by Lue and Kelley (18) compared the initial velocity of ATP-PPi exchange among Ile, Val, Met, Thr, Ser, Ala, and Gly and found that among them Ile is the most efficiently activated by hmtLeuRS, albeit much more weakly compared with EcLeuRS. They indicated that hmtLeuRS could discriminate Ile efficiently via a precise synthetic core (18, 20). However, it was suggested recently that the main targets of LeuRS editing include Nva (21). Thus, whether hmtLeuRS is able to rigorously discriminate Nva and other Leu analogs (such as ABA) during aminoacylation needs to be further explored.

Inhibition of aaRSs would halt protein translation and stall the growth of organisms; thus, they have long been pursued as anti-parasitic drug targets. Benzoxaborole 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN2690) is in phase 3 clinical trials for the treatment of onychomycosis, an infection of nails caused by fungi (22). AN2690 inhibits LeuRSs from diverse species, including prokaryotic EcLeuRS, eukaryotic Candida albicans LeuRS (CaLeuRS), ScLeuRS, and HsLeuRS, by forming an adduct with the 2’- and 3’-OH group of the terminal adenosine (A76) of non-aminoacylated tRNALeu in the editing-active site (23). It blocks the translocation of tRNA and the turnover of the enzyme. AN2690 mostly interacts with the main chains and side chains of some residues in the CP1 domain, including the Thr-rich region, the GTG region, and the region around the conserved Asp residue (22, 23). To date, two LeuRSs have been proven to be insensitive to this compound, eukaryotic Giardia lamblia LeuRS (GLLeuRS) and prokaryotic AaLeuRS (22, 24). Based on the degeneration of the
CP1 domain of hmtLeuRS, we wanted to clarify whether hmtLeuRS is a target of AN2690 or not.

In this study, we systematically studied the role of the editing-defunct CP1 domain in aminoacylation by comparing the activities of hmtLeuRS and CP1 domain-deleted/substituted chimeric variants. The CP1 domain in hmtLeuRS improved its catalytic rate and made it resistant to AN2690. As revealed by others, Ile could be effectively discriminated at the synthetic core of hmtLeuRS as is the case for EcLeuRS; however, our study showed that Nva and Val could be mis-activated by hmtLeuRS and may pose threats to the programmed insertion of Leu in newly synthesized peptides. Moreover, we found hmtLeuRS only retains tRNA-independent pre-transfer editing, which is insufficient to remove mis-activated Nva in vitro. The substitution of CP1 domains from other LeuRSs could assist chimeric enzymes to regain their post-transfer editing activities and correct the tendency to mis-incorporate noncognate Leu analogs. Taken together, these results emphasized the crucial role of the hmtLeuRS CP1 domain in enhancing the catalytic rate and revealed the mis-activation and editing features of hmtLeuRS for noncognate amino acids.

Experimental Procedures

Materials—L-Leu, L-Nva, L-Val, L-ABA, L-Met, Tris-HCl, MgCl₂, NaCl, KCl, ATP, tetrasodium pyrophosphate (Na₄PPi), inorganic pyrophosphate, dithiothreitol (DTT), sodium acetate (pH 5.2), and activated charcoal were purchased from Sigma. L-[¹⁴C]Leu, [³²P]Na₄PPi, and [α-³²P]ATP were obtained from PerkinElmer Life Sciences. Isopropyl 1-thio-D-galactopyranoside and AN2690 were purchased from Amescro (Solon, OH) and Milestone Pharmatech (Hangzhou, China), respectively. Pyrophosphatase was purchased from Roche Diagnostics (Basel, Switzerland). Nitrocellulose membranes (0.22 μm), polyethyleneimine cellulose plates, and Amicon ultra-15 filters were obtained from Merck (Darmstadt, Germany). Nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) mixtures were purchased from Sangon (Shanghai, China). The DNA fragment rapid purification kit and plasmid extraction kit were constructed previously in our laboratory (25). The CP1 domain (Ser227–Arg442) was amplified separately from E. coli BL21 (DE3) cells (25). Proteins were purified by nickel-nitrilotriacetic acid affinity and gel filtration chromatography with Superdex™ 75. Purity was confirmed by SDS-PAGE, and their final concentrations were determined by active site titration, as described previously (29).

Circular Dichroism (CD) Spectroscopy—The secondary structure of the chimeric LeuRSs was determined by CD spectroscopy, as reported previously (9). Enzyme samples at 0.2 mg/ml were analyzed on Jasco J-715 spectropolarimeter with a nitrogen purge at room temperature. Spectra were accumulated over three scans, using a 1-mm path length cuvette.

Acquisition of tRNAs—The tRNA constructs of hmtLeuRS, which has a hammerhead ribozyme sequence between a T7 RNA polymerase promoter sequence and the tRNA sequence, were constructed previously (30, 31). In vitro transcriptions were performed as reported previously (32, 33). E. coli tRNA EcLeu (CAG) (EctRNAEcLeu) was isolated from an overexpression strain constructed in our laboratory (34). hmtLeuRS can leucylate EctRNAEcLeu efficiently (19, 25); therefore, EctRNA EcLeu was used to assay the aminoacylation activity of hmtLeuRS.

ATP-PP₄ Exchange Reaction—The ATP-PP₄ exchange reaction was performed at 37 °C in buffer containing 2 mM [²⁸P]Na₄PP₄ (22 cpm/pmol), 100 mM HEPES-KOH (pH 7.8), 10 mM MgCl₂, 10 mM KF, 2 mM ATP and different amino acids. The reaction was initiated by the addition of 300 nm LeuRS. For Km determinations, Leu (0.02–0.5 mM), Nva (0.5–100 mM), ABA (5–160 mM), Val (5–100 mM), Ile (10–150 mM), and Met (5–150 mM) were used. At regular time intervals, samples were quenched by adding 200 μl of a solution containing 2% activated charcoal, 3.5% HClO₄, and 50 mM Na₂PP₄. The concen-
CP1 Domain Contributes to Aminoacylation

The net effect of the editing reaction is the consumption of ATP. Therefore, editing could be measured by monitoring the breakdown of ATP (to AMP and PP) in the presence of noncognate amino acids. The AMP formation assay was performed in a reaction mixture containing 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 12 mM MgCl₂, 0.5 mM DTT, 3 mM ATP, 5 units/ml pyrophosphatase, 20 mM \(^{32}\)P-ATP (3000 Ci/mmol), and 20 mM Nva (or 100 mM Val, 1 mM Leu), with or without 5 \(\mu\)M transcribed hmtRNA\(^{14}\text{eu}\) at 37°C. Reactions were initiated by the addition of 3 \(\mu\)M hmtLeuRS. 1.5-\(\mu\)M aliquots at various time intervals were quenched in 6 \(\mu\)l of 200 mM NaAc, and then 1.5 \(\mu\)l of the quenched aliquots were spotted on pre-activated polyethyleneimine cellulose plates. A solution containing 0.1 mM NH₄Ac and 5% acetic acid was used as the flowing phase to separate aa-\(^{32}\)PAMP, \(^{32}\)PAMP, and \(^{32}\)P-ATP on the TLC plates.

Nonenzymatic Spontaneous Hydrolysis of Adenylates—The rate of nonenzymatic hydrolysis of adenylates was measured by a pulse-chase experiment in which a large excess of unlabeled ATP was added to the reaction buffer following the initiation of Nva-AMP and Val-AMP. The excess of unlabeled ATP induces the release of the noncognate aminoacyl-adenylate from the active site into solution, where its spontaneous hydrolysis is monitored (11, 35). A solution of 3 \(\mu\)M hmtLeuRS was first incubated with 20 mM Nva, 100 \(\mu\)M ATP, and 0.25 \(\mu\)M \([\alpha-\text{\textsuperscript{32}}\text{P}]\)ATP for 15 min at 37°C to prepare Nva-\(^{32}\)PAMP, in a buffer containing 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 12 mM MgCl₂, 0.5 mM DTT, 5 units/ml pyrophosphatase. A relatively large amount of unlabeled ATP was then added such that the final concentration of unlabeled ATP was a 500-fold molar excess of the labeled ATP. The hydrolysis activity was quenched at various time points (2-10 min) by mixing 1.5 \(\mu\)l of the reaction mixture with 6 \(\mu\)l of 200 mM NaAc and 0.1% SDS. Separation of Nva-\(^{32}\)PAMP and \(^{32}\)PAMP by TLC was then performed, and the reactions were quantified as described above.

Results

Significance of the Degenerate CP1 Domain in Amino Acid Activation and Aminoacylation—To determine functional significance of the degenerate CP1 of hmtLeuRS, we obtained the two variants of hmtLeuRS, hmtLeuRS-\(\Delta\)CP1, and hmtLeuRS-MmLin linker by cloning and expressing their genes. The two hmtLeuRS variants had the same CD spectrogram as hmtLeuRS, indicating that they have a stable secondary structure (Fig. 2B).

We assayed and compared the kinetic constants of the two variants with those of hmtLeuRS in the amino acid activation reaction (Table 1). The \(K_m\) values of both variants for Leu were decreased by less than 2-fold after CP1 truncation, showing the affinity between the variant and Leu is slightly increased; however, the \(k_{cat}\) values of hmtLeuRS-\(\Delta\)CP1 and hmtLeuRS-MmLin linker sharply decreased to 3.3 \(\times\) 10⁻² and 12.1 \(\times\) 10⁻² s⁻¹, respectively, when compared with that of hmtLeuRS (1.15 s⁻¹). The catalytic efficiency of hmtLeuRS-\(\Delta\)CP1 (1.7 s⁻¹ mm⁻¹) or hmtLeuRS-MmLin linker (7.6 s⁻¹ mm⁻¹) only retained ~4 or 20% compared with that of the wild-type enzyme (37.8 s⁻¹ mm⁻¹).

In subsequent aminoacylation assays, both variants had almost an unchanged \(K_m\) for tRNA; however, hmtLeuRS-\(\Delta\)CP1 and hmtLeuRS-MmLin linker displayed obvious decreases in \(k_{cat}\) values (0.40 \(\times\) 10⁻² and 1.46 \(\times\) 10⁻² s⁻¹, respectively), accounting for only 9 and 33% that of the native enzyme (4.51 \(\times\) 10⁻² s⁻¹).
10^{-2} \text{s}^{-1}). Although in aminoacylation the catalytic efficiency of hmtLeuRS-Mmlinker (8.8 s^{-1} \text{mM}^{-1}) was much higher than that of hmtLeuRS-ΔCP1 (1.9 s^{-1} \text{mM}^{-1}), they only retained 9 and 42% of that of native enzyme (21.0 s^{-1} \text{mM}^{-1}), respectively (Table 2). The data also showed that the nonapeptide Mmlinker was a good substitute for the degenerate CP1 domain of hmtLeuRS in synthesis of aminoacyl-tRNA.

To further explore the importance of the degenerate CP1 domain, we replaced it with its counterparts from both bacterial and eukaryotic LeuRSs (LeuRSs of E. coli, A. aeolicus, and S. cerevisiae cytoplasmic form and H. sapiens cytoplasmic form), and we obtained four chimeric variants, including hmtLeuRS-EcCP1, hmtLeuRS-AaCP1, hmtLeuRS-ScCP1, and hmtLeuRS-HsCP1. Their proper secondary structures were confirmed by CD spectrogams (Fig. 2B). We compared Leu activation and tRNA charging activities of wild-type hmtLeuRS and these chimeras. The variants showed reduced catalytic activities to different extents (Fig. 3, A and B). Further analysis of the activities of these variants revealed that the activity was related to the origin of the CP1 domain. Chimeric variants with a CP1 domain from bacterial LeuRSs showed slightly more efficient catalysis than those with CP1 domains from eukaryotic LeuRSs (especially in Leu activation). Sequence, insertion point, and orientation variation between CP1 domains from prokaryotic and eukaryal/archaeal LeuRSs when substituting for hmtLeuRS’s CP1 might affect the activity of the chimeras (Fig. 3C) (36).

The above data showed that the presence of the CP1 domain in hmtLeuRS, despite the degeneracy in sequence and deficiency in post-transfer editing (18), is necessary for the amino acid activation and aminoacylation activities of hmtLeuRS. Its deletion or replacement by other CP1 domains led to severe impairment of enzyme function. Therefore, hmtLeuRS must be under evolutionary pressure to keep its CP1 domain.

AN2690 Does Not Affect hmtLeuRS—AN2690 is a broad-spectrum LeuRS inhibitor, targeting the active site of the CP1 editing domain of various LeuRSs. Indeed, our previous analysis revealed that it inhibits the aminoacylation and post-transfer editing activity of HsLeuRS (26). However, does the degeneracy of hmtLeuRS’s CP1 domain affect its response to AN2690? To address this question, we compared the aminoacylation activity of hmtLeuRS in the absence or presence of AN2690. The data showed that AN2690 did not affect the aminoacylation activity of hmtLeuRS (Fig. 4A). To further reveal the significance of degeneracy of the CP1 domain in the AN2690 resistance of hmtLeuRS, we assayed the aminoacylation activity of hmtLeuRS-EcCP1, which has a functional and AN2690-sensitive CP1, in the absence or presence of AN2690 (Fig. 4B). Indeed, addition of the EcCP1 domain restored sensitivity to AN2690. Furthermore, post-transfer editing of hmtLeuRS-EcCP1 to Nva-tRNA^{eu} was obviously impaired by AN2690 (the restoration of post-transfer editing of hmtLeuRS-EcCP1 is described later in the text). However, the effect of AN2690 on the post-transfer editing of hmtLeuRS could not be determined because hmtLeuRS naturally lacks post-transfer editing of Nva-tRNA^{eu} (Fig. 4C), which is consistent with the results of Lue and Kelley (18). Thus, the above data showed that AN2690 could not inhibit the activity of hmtLeuRS because of its degenerate CP1 domain. However, the presence of a functional CP1 domain restored sensitivity to AN2690.

HmtLeuRS Could Obviously Mis-activate Nva and Val—Lue and Kelley (18, 20) assayed and compared the initial rates of activation of several proteinic amino acids by hmtLeuRS and found that, among them, Ile could be mis-activated at the highest rate and the canonical active core of hmtLeuRS could more efficiently discriminate Ile at the activation step compared with EcLeuRS. However, the kinetic parameters for other Leu ana-
aminoacylation. The catalytic rate constants (kcat) of hmtLeuRS and its chimeras showed that hmtLeuRS could effectively discriminate ABA and Met at the first step of aminoacylation of Nva and ABA (Table 3). The results showed that hmtLeuRS has been determined the kinetic parameters for several analogs of Leu, including Nva and ABA, (Table 3). They have a different insertion point; they also change the rotational state to accommodate tRNALeu (35).

The mis-activation discrimination factors were 4730 and 300 nM hmtLeuRS and its chimeras. The mis-activation features of hmtLeuRS, we assayed its ATP-PPi exchange activity and measured the kinetic parameters for several analogs of Leu, including Nva and ABA (Table 3). The results showed that hmtLeuRS could effectively discriminate ABA and Met at the first step of aminoacylation. The catalytic rate constants (kcat) of hmtLeuRS for ABA and Met were about 11% (0.13 s⁻¹) and 5% (0.061 s⁻¹) that for Leu (1.15 s⁻¹), respectively; however, the Km values for ABA and Met increased by about 530-fold (16.2 mM) and 670-fold (20.5 mM) that for Leu (30.4 μM), respectively (Table 3). The mis-activation discrimination factors were 4730 and 12,600 for ABA and Met, which are much higher than the threshold of the general error level in protein biosynthesis (3300) (37). These results indicated ABA and Met should not pose a threat to the catalytic fidelity of hmtLeuRS.

Meanwhile, although Nva and Val, the other two analogs of Leu, had weaker affinity with the enzyme (higher Km values, 6.2 mM for Nva and 19.6 mM for Val), they were activated with comparable catalytic rate constants (1.31 s⁻¹ for Nva and 0.62 s⁻¹ for Val), compared with that for Leu (1.15 s⁻¹) (Table 3). They could be activated by hmtLeuRS efficiently. The discrimination factors for Nva and Val were 180 and 1180, respectively, which were much lower than the mistranslation frequency (3300); therefore, hmtLeuRS might have evolved a pre-transfer proofreading pathway to remove mis-activated amino acids and maintain the catalytic fidelity or allow the generation of mis-charged tRNA without proofreading.

hmtLeuRS Has No tRNA-dependent Pre-transfer Editing—Editing is used to ensure a precise recognition between aaRS and its cognate amino acid, and it minimizes errors in mis-incorporation of amino acids in peptides or proteins. Theoretically, the net effect of the editing reaction is the consumption of ATP. Both hydrolysis of mis-aminocarboxyl-adenylate in pre-transfer editing and removal of mis-charged tRNAs after aminoacylation in post-transfer editing contribute to the formation of AMP (24).

By monitoring AMP formation by TLC in the presence of noncognate amino acids, we could characterize the editing activity of hmtLeuRS.

hmtLeuRS can mis-activate Nva and Val and has lost its post-transfer editing activity; therefore, we determined whether it possesses tRNA-dependent pre-transfer editing and the contributions of tRNA-independent and -dependent pre-transfer editing to total editing in the presence of these two Leu analogs.

First, we determined the AMP formation rate of hmtLeuRS in the presence of Leu. The observed AMP formation rate (kobs) was 1.41 × 10⁻³ s⁻¹ without tRNAs and 1.88 × 10⁻³ s⁻¹ or 1.79 × 10⁻³ s⁻¹ in the presence of hmttRNALeu (CUN) or hmttRNALeu (UUR) transcripts (Table 4). Considering the cognate substrate cannot trigger hmtLeuRS’s editing reaction, AMP should come from the hydrolysis of ATP in Leu activation. Then we monitored the editing of hmtLeuRS toward non-cognate Nva and Val in the absence and presence of hmttRNALeu transcripts. For Nva, the kobs value without tRNA was 2.79 × 10⁻² s⁻¹, ~20-fold greater than that for Leu (1.41 × 10⁻³ s⁻¹); and in the presence of hmttRNALeu (CUN) or hmttRNALeu (UUR), it was 3.29 × 10⁻² or 2.78 × 10⁻² s⁻¹,
(the $k_{\text{obs}}$ of EcLeuRS for Nva was $3.3 \times 10^{-1}$ and $3.42 \text{ s}^{-1}$ in the absence and presence of 5 mM Ec$tRNA^{\text{Leu}}$, respectively) (24), hmtLeuRS possesses weak editing function.

For Val, in the absence of tRNA, the $k_{\text{obs}}$ was $6.34 \times 10^{-4} \text{ s}^{-1}$, only 45% that for Leu ($1.41 \times 10^{-3} \text{ s}^{-1}$) (Table 4), and we suggested the accumulated AMP might be derived from mis-activation rather than editing. hmt$tRNA^{\text{Leu}}$ (CUN) ($5.42 \times 10^{-4} \text{ s}^{-1}$) or hmt$tRNA^{\text{Leu}}$ (UUR) ($5.21 \times 10^{-4} \text{ s}^{-1}$) triggered no more AMP formation, and the $k_{\text{obs}}$ value was still lower than that for Leu in the absence of tRNA ($1.41 \times 10^{-3} \text{ s}^{-1}$) (Table 4). Therefore, hmtLeuRS has no pre-transfer editing (including tRNA-independent pre-transfer editing and tRNA-dependent pre-transfer editing) for Val.

AMP could be produced by hmtLeuRS catalysis or by spontaneous hydrolysis of Nva-AMP. To measure the rate of possible spontaneous hydrolysis, we carried out a pulse-chase experiment (11, 35). The $k_{\text{obs}}$ value of the conversion of Nva-AMP to AMP was $1.18 \times 10^{-4} \text{ s}^{-1}$ (Fig. 6), which was 236-fold lower than the $k_{\text{obs}}$ determined for AMP formation ($2.79 \times 10^{-2} \text{ s}^{-1}$) for no tRNA) by hmtLeuRS in the presence of Nva (Table 4), indicating that the spontaneous hydrolysis of Nva-AMP was negligible. The above data showed that hmtLeuRS is deficient in tRNA-dependent editing, including tRNA-dependent pre-transfer and post-transfer editing, and it only retains tRNA-independent pre-transfer editing for Nva.

tRNA-independent Pre-transfer Editing Is Insufficient to Remove Mis-activated Nva—To understand whether hmtLeuRS could achieve fidelity toward Nva and Val, we examined the mis-acylation of hmtLeuRS using a TLC-based assay. By labeling the 3′ end of Ec$tRNA^{\text{Leu}}$ with [α-32P]ATP, we could monitor the formation of Nva-[32P]AMP and Val-[32P]AMP from aminoacyl-tRNAs by nuclease S1 digestion. The data showed that hmtLeuRS catalyzes the formation of similar amounts of Nva-tRNA1$^{\text{Leu}}$ and Leu-tRNA1$^{\text{Leu}}$, suggesting that tRNA-independent pre-transfer editing from the synthetic domain in hmtLeuRS could not remove Nva efficiently (Fig. 7). For Val, there was only a trace amount of Val mis-charged to tRNA1$^{\text{Leu}}$ by hmtLeuRS, although its concentration (100 mM) was 5-fold higher than those of Nva and Leu (20 mM) (Fig. 7). Considering Val was mis-activated by hmtLeuRS (Table 3) and hmtLeuRS possessed no tRNA-independent pre-transfer editing toward it (Table 4), it is suggested that the mis-activated Val was not efficiently transferred to tRNA. In summary, the tRNA-independent pre-transfer editing of hmtLeuRS was insufficient to ensure specificity in the aminoacylation reaction toward Nva.

Restoration of hmtLeuRS’s Post-transfer Editing Activity—The homology in the CP1 domain of hmtLeuRS and many other LeuRSs showed that the deficient CP1 domain of hmtLeuRS might be an evolutionary remnant of an active editing domain. We performed reverse mutations of the degenerate Thr-rich region and GTG regions in hmtLeuRS to generate an editing-active hmtLeuRS. However, despite extensive efforts, we failed to obtain such a post-transfer editing-active enzyme. Subsequently, we utilized the above CP1 domain chimeras and determined their de-acylation activity, together with wild-type hmtLeuRS. The data showed that all of them could significantly

respective (Table 4 and Fig. 5). tRNA does not affect the pre-transfer editing of hmtLeuRS; therefore, hmtLeuRS only has tRNA-independent pre-transfer editing and has no tRNA-dependent pre-transfer editing for Nva. Compared with EcLeuRS

### TABLE 3

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>$K_m$</th>
<th>$k_{\text{cat}}$</th>
<th>$k_{\text{cat}}/K_m$</th>
<th>Discrimination factor</th>
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<td>Leu</td>
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<td>1.15 ± 0.25</td>
<td>37.8</td>
<td>1</td>
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<td>Nva</td>
<td>6200 ± 130</td>
<td>1.31 ± 0.17</td>
<td>2.1 × 10^{-3}</td>
<td>180</td>
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<tr>
<td>Val</td>
<td>19600 ± 900</td>
<td>0.62 ± 0.09</td>
<td>3.2 × 10^{-2}</td>
<td>1180</td>
</tr>
<tr>
<td>ABA</td>
<td>16200 ± 1100</td>
<td>0.13 ± 0.02</td>
<td>8.0 × 10^{-3}</td>
<td>4730</td>
</tr>
<tr>
<td>Met</td>
<td>20500 ± 1600</td>
<td>0.061 ± 0.008</td>
<td>3.0 × 10^{-3}</td>
<td>12600</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Rate of AMP formation $k_{\text{obs}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No tRNA</td>
</tr>
<tr>
<td>Leu</td>
<td>(1.41 ± 0.08) × 10^{-3}</td>
</tr>
<tr>
<td>Nva</td>
<td>(2.79 ± 0.14) × 10^{-2}</td>
</tr>
<tr>
<td>Val</td>
<td>(6.34 ± 2.86) × 10^{-4}</td>
</tr>
</tbody>
</table>
restore the post-transfer editing activities toward Nva-tRNA^{Leu}_{\text{hmt}} (Fig. 8).

To determine whether an editing-functional CP1 domain could improve fidelity during aminoacylation, we examined the mis-acylation behavior of hmtLeuRS-EcCP1, because EcCP1 is evolutionarily close to CP1 of hmtLeuRS. In the presence of tRNA^{Leu}_{\text{hmt}} and Nva, the formation of Nva-tRNA^{Leu}_{\text{hmt}} of hmtLeuRS-EcCP1 was considerably weak compared with that of hmtLeuRS, because of the prevention of accumulation of Nva-AMP by post-transfer editing conferred by an active editing domain of EcLeuRS (Fig. 9, A and B). When the conserved Asp residue was mutated to Ala in hmtLeuRS-EcCP1 (hmtLeuRS-EcCP1-D345A), the production of Nva-tRNA^{Leu}_{\text{hmt}} dramatically increased (Fig. 9, C and D). hmtLeuRS-EcCP1 catalyzed the synthesis of Val-tRNA^{Leu}_{\text{hmt}} at a relatively lower rate compared with that of hmtLeuRS; hmtLeuRS-EcCP1-D345A produced Val-tRNA^{Leu}_{\text{hmt}} at a slightly higher rate than hmtLeuRS-EcCP1. In conclusion, the fusion of a functional CP1 domain endowed accuracy on an error-prone hmtLeuRS during aminoacylation.

Discussion

CP1 Domain Benefits hmtLeuRS in Catalytic Rate—The CP1 editing module found in class Ia aaRSs is present in nearly all species and is highly conserved throughout evolution. The editing domains of LeuRS and many other aaRSs in Mycoplasma parasites naturally have mutations or deletions (17). It causes mis-translation, phenotype diversity, and plasticity, facilitating the pathogens’ escape from host resistance (13, 17). Compared with the retrogressive CP1 domain of LeuRSs in the mitochondria of several species, such as *H. sapiens*, *Xenopus laevis*, and *Caenorhabditis elegans* (18), some fungal mitochondrial LeuRSs (*C. albicans* and *Schizosaccharomyces pombe*) still remain intact in the essential residues of the CP1 domain. An exception is the LeuRS from the mitochondria of *S. cerevisiae* (ScmtLeuRS), which has variation in the GTG region; but it retains post-transfer editing activity (38).

Although defunct in editing, the reason why hmtLeuRS retains a CP1 domain is unknown. In this study, we confirmed the function of hmtLeuRS’s CP1 domain in the aminoacylation reaction rather than editing. By comparison of hmtLeuRS and CP1 deletion/substitution enzymes, we found that the CP1 domain contributes significantly to the amino acid activation and tRNA charging activities. Complete removal of the CP1 domain (hmtLeuRS-ΔCP1) resulted in considerable loss of aminoacylation activity, whereas substitution of this domain with the Mmlinker (hmtLeuRS-Mmlinker) could partly assist the enzyme in retaining the aminoacylation activity. Our results
suggested that the existence of a CP1 domain is beneficial for hmtLeuRS to optimize activation and aminoacylation catalytic efficiency, as observed in EcLeuRS (12). We also validated that the MmLinker can functionally compensate for the CP1 domain of LeuRSs in aminoacylation activity, to some extent (13). It is possible that hmtLeuRS still retains this domain to benefit the catalytic function under environmental stress. Likewise, prolyl-tRNA synthetases (ProRSs) show divergence in the editing domain through evolution. For most bacterial ProRSs, a domain (INS) is inserted between the canonical core to perform cis-editing, and in lower eukaryotes, there is a homologous extension of ProRS appended to its N terminus. Previous work showed that in S. cerevisiae, although they have various evolutionary origins, are mainly of bacterial type (41). From an evolutionary point of view, the CP1 domains of bacterial and mitochondrial LeuRSs have the same insertion point and orientation, which are different from archaeal/eukaryal LeuRSs, which may account for the discrepancy in the catalytic activities between mosaic enzymes, according to the origins of the CP1 domain.

AN2690 Resistance of LeuRSs—AN2690 is an antibiotic used to treat leukonychia. Rock et al. (23) isolated S. cerevisiae spontaneous and ethyl-methanesulfonate-induced AN2690-resistant variants and found all the AN2690-resistant mutations were in the CP1 domain or the editing-active site of ScLeuRS. They demonstrated that AN2690 inhibits LeuRS by forming a stable tRNA\(^{\text{AN2690}}\)-AN2690 adduct in the editing site, mediated via tRNA’s A76 and AN2690’s boron atom (23). According to the crystal structure of TtLeuRS with tRNA\(^{\text{Leu}}\) and AN2690 (PDB number 2V0G), the configuration between AMP-AN2690 and the CP1 domain is facilitated by hydrogen bonds to the conserved Thr-rich peptide (Thr\(^{247}\) and Thr\(^{248}\) in TtLeuRS) and a water molecule (23).

Here, we found that the degenerate CP1 domain confers resistance to AN2690 on hmtLeuRS during aminoacylation. Sequence alignment showed that, in addition to alteration to Ala of the counterpart of TtLeuRS Thr\(^{248}\) in hmtLeuRS, hmtLeuRS also diverges in some residues whose mutations led to the insensitivity to AN2690 in ScLeuRS (Fig. 10). Therefore, we hypothesized that the degeneracy of the hmtLeuRS CP1 domain, especially in the binding site with AN2690, inhibits AN2690 from forming an appropriate adduct with A76 of the tRNA. We could not exclude the possibility that the 3’ end of the tRNA cannot shift between the synthetic active site and the defective CP1 domain of hmtLeuRS, as is found in EcLeuRS (where the 3’ end of tRNA translocates between the synthetic active site and the functional CP1 domain of EcLeuRS) (42).

It is notable that human cytosolic LeuRS is readily inhibited by AN2690, which affects the external use of AN2690 in disease treatment. Combined with the previously identified GluLeuRS and AaLeuRS, and hmtLeuRS here, three LeuRSs have been found to be insensitive to AN2690 despite bearing a CP1 domain (22). However, hmtLeuRS is the only enzyme located in an organelle. Indeed, the detailed resistance mechanism of hmtLeuRS to AN2690 remains to be determined.

hmtLeuRS Only Has tRNA-independent Pre-transfer Editing for Nva—Recent studies showed that Met\(^{40}\) in EcLeuRS plays a key part in discriminating Ile; prevention of incorrect Nva incorporation is the major biological editing activity of EcLeuRS (21). Our study determined systematically the catalytic efficiency of hmtLeuRS toward different analogs of Leu. We found that Nva and Val could be mis-activated by hmtLeuRS; however, the presence of tRNA\(^{\text{Leu}}\) does not affect its editing activity. The
data showed that hmtLeuRS has neither tRNA-dependent pre-transfer editing nor post-transfer editing, only retaining tRNA-independent pre-transfer editing for Nva.

The tertiary structure of TtLeuRS-tRNALeu in the post-transfer editing conformation (PDB number 2BYT) showed that Lys302 and Arg346 of TtLeuRS interact with the C74 base and C75 phosphate of tRNA, whereas Thr332 (Thr in the GTG region) makes contact with A76 phosphate (43). Previous studies showed that a proper binding between the CCA end of a tRNA and the editing domain, rather than an active site of editing, is a prerequisite for LeuRS to hydrolyze noncognate adenylate intermediates and perform tRNA-dependent pre-transfer editing (24, 44), which is likely to occur in the aminoacylation active site. The communication between the aminoacylation and CP1 domain could modulate tRNA-dependent pre-transfer editing (45). Sequence alignments showed that hmtLeuRS has deletions in regions corresponding to the TtLeuRS's regions containing Lys302 and mutations in residues corresponding to Tyr332 and Arg346 in TtLeuRS (Fig. 10). Therefore, hmtLeuRS shows divergence in the conserved residues essential for binding tRNA, which might explain why hmtLeuRS possesses no tRNA-dependent pre-transfer editing.

As for hmtLeuRS, in vitro assays showed that it could produce considerable mis-charged product Nva-tRNALeu, indicating that tRNA-independent pre-transfer editing was not sufficient to clear mis-activated Nva. This is consistent with previous results (13, 24), which showed that pre-transfer edit-
CP1 Domain Contributes to Aminoacylation

Our study presents a new viewpoint on the effect of the editing-inactive CP1 domain on the aminoacylation function of hmtLeuRS. We showed that hmtLeuRS cannot function as a single sieve for Nva or Val. It exhibits only tRNA-independent pre-transfer editing for Nva, which is not sufficient to remove mis-activated Nva. In addition, the editing-inactive CP1 domain confers resistance to AN2690 on the enzymes. By fusing with the editing-active CP1 domain from other LeuRSs, hmtLeuRS chimeras regained post-transfer editing activities and displayed aminoacylation fidelity.

Author Contributions—Q. Y., X. L. Z., and E. D. W. designed the experiments, analyzed the data, and wrote the manuscript. M. W. assisted in the gene cloning and expression of mosaic enzymes. Z. P. F., Z. R. R., and Q. Q. J. assisted in obtaining tRNAs. Q. Y. performed all the other experiments. All authors reviewed the results and approved the final version of the manuscript.

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References


Original Research Article

In vitro. For Val, it seems that mis-activated Val cannot be efficiently transferred to tRNA-Leu Ec. In contrast, when hmtLeuRS-Ec CP1-D345A was evaluated in an in vitro translation system with rat liver mitochondria, it was found that the levels of most of them (including Leu, 5.16 mM in mitochondria) were higher than in the cytosol or serum. However, the amount of Nva in any mitochondrial system has not been reported. We hypothesized that normally the higher eukaryotic mitochondria contain Nva at a low concentration such that the mis-activated Nva could be removed completely by tRNA-dependent pre-transfer editing, and tRNA-dependent editing might be redundant. Therefore, hmtLeuRS does not need to retain its tRNA-dependent editing function. However, it is possible that under specific circumstances, Nva would accumulate or be imported into mitochondria from the cytoplasm, where it would damage the quality control in mitochondrial protein synthesis. Otherwise, mitochondria may endure a sufficient to remove mis-activated Nva. In addition, the editing-inactive CP1 domain might be focused in the co- or post-translational steps, and the incorporation of noncognate amino acids might lead to the production of misfolded proteins, which would be rapidly degraded by the mitochondrial proteolytic system (48).

hmtLeuRS Chimeras Gain Post-transfer Editing Function and Translational Accuracy—We found that hmtLeuRS chimeras with a fusion of the CP1 domain from different species gained post-transfer editing activities and fidelity during aminoacylation, just as ScProRS regained post-transfer editing function by replacement of N-terminal domain from the EcProRS INS domain (39). Additionally, the gain of post-editing activity was not dependent on the origin of the fused CP1 domain; both eukaryotic and prokaryotic LeuRSs were functional. EcLeuRS is close to hmtLeuRS in evolution; therefore, we specifically inspected the mis-acylation characteristics of hmtLeuRS-EcCP1 and hmtLeuRS-EcCP1-D345A. hmtLeuRS-EcCP1 prohibited the mis-charging of tRNA with Nva, although hmtLeuRS-EcCP1-D345A catalyzed the formation of a large amount of Nva-tRNA Ec. The editing function of the hmtLeuRS-EcCP1 chimera to Nva also depended on the conserved D345A residue in the CP1 domain. Moreover, hmtLeuRS-EcCP1 and hmtLeuRS-EcCP1-D345A only produced a trace amount of Val-tRNA Ec.

Concluding Remarks—Our study presents a new viewpoint on the effect of the editing-inactive CP1 domain on the aminoacylation of tRNA-Leu Ec in vitro. For Val, it seems that mis-activated Val cannot be efficiently transferred to tRNA-Leu Ec. In contrast, when hmtLeuRS-Ec CP1-D345A was evaluated in an in vitro translation system with rat liver mitochondria, it was found that the levels of most of them (including Leu, 5.16 mM in mitochondria) were higher than in the cytosol or serum. However, the amount of Nva in any mitochondrial system has not been reported. We hypothesized that normally the higher eukaryotic mitochondria contain Nva at a low concentration such that the mis-activated Nva could be removed completely by tRNA-dependent pre-transfer editing, and tRNA-dependent editing might be redundant. Therefore, hmtLeuRS does not need to retain its tRNA-dependent editing function. However, it is possible that under specific circumstances, Nva would accumulate or be imported into mitochondria from the cytoplasm, where it would damage the quality control in mitochondrial protein synthesis. Otherwise, mitochondria may endure a sufficient to remove mis-activated Nva. In addition, the editing-inactive CP1 domain might be focused in the co- or post-translational steps, and the incorporation of noncognate amino acids might lead to the production of misfolded proteins, which would be rapidly degraded by the mitochondrial proteolytic system (48).

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CP1 Domain Contributes to Aminoacylation


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