The CP2 Domain of Leucyl-tRNA Synthetase Is Crucial for Amino Acid Activation and Post-transfer Editing*

Xiao-Long Zhou, Bin Zhu, and En-Duo Wang

From the State Key Laboratory of Molecular Biology and Graduate School of the Chinese Academy of Sciences, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China

Leucyl-tRNA synthetase (LeuRS) has an insertion domain, called connective peptide 2 (CP2), either directly preceding or following the editing domain (CP1 domain), depending on the species. The global structures of the CP2 domains from all LeuRSs are similar. Although the CP1 domain has been extensively explored to be responsible for hydrolysis of mischarged tRNA<sup>Leu</sup>, the role of the CP2 domain remains undefined. In the present work, deletion of the CP2 domain of <i>Giardia lamblia</i> LeuRS (GILeuRS) showed that the CP2 domain is indispensable for amino acid activation and post-transfer editing and that it contributes to LeuRS-tRNA<sup>Leu</sup> binding affinity. In addition, its functions are conserved in both eukaryotic/archaeal and prokaryotic LeuRSs from <i>G. lamblia</i>, <i>Pyrococcus horikoshii</i> (<i>Ph</i>LeuRS), and <i>Escherichia coli</i> (EcLeuRS). Alanine scanning and site-directed mutagenesis assays of the CP2 domain identified several residues that are crucial for its various functions. Data from the chimeric mutants, which replaced the CP2 domain of GILeuRS with either PhLeuRS or EcLeuRS, showed that the CP2 domain of PhLeuRS but not that of EcLeuRS can partially restore amino acid activation and post-transfer editing functions, suggesting that the functions of the CP2 domain are dependent on its location in the primary sequence of LeuRS.

Aminoacyl-tRNA synthetases (aaRSs)<sup>2</sup> catalyze the esterification of their cognate amino acids at the 3'-end of their cognate tRNAs in a two-step reaction: the synthesis of an aminoacyl-adenylate (aa-AMP) as an activated intermediate from an amino acid and ATP, and the subsequent transfer of the aminoacyl-adenylate (aa-AMP) to the ribosome, and the attached amino acid is incorporated into the protein.

The family of aaRSs is divided into two structurally distinct and apparently unrelated classes, which are considered to have evolved from two different ancestors, based on completely distinct folds of the aminoacylation domains (2). The class I aaRSs contain two signature peptides, HIGH and KMSKS, located in the characteristic nucleotide binding fold (Rossmann fold) of the active site domain for ATP binding and amino acid activation. The catalytic domain is interrupted by two major inserts, which are designated as connective peptide 1 (CP1) and connective peptide 2 (CP2) (3).

Leucyl-tRNA synthetase (LeuRS) belongs to subclass Ia of the group of aaRSs with cysteinyl-, isoleucyl-, methionyl-, and valinyl-tRNA synthetases (CysRS, IleRS, MetRS, and ValRS, respectively) (2). These aaRSs in class Ia share a common α-helical anticodon-binding domain. LeuRS is responsible for Leu-tRNA<sup>Leu</sup> synthesis and has an aminoacylation catalytic core defined by a Rossmann fold (4). LeuRS, IleRS, and ValRS (LIV-aaRSs) edit their mistakes via a hydrolytic site within the CP1 domain, which is inserted into the catalytic Rossmann fold (4–7). The CP1 domain hydrolyzes mischarged aminoacyl-tRNAs (post-transfer editing) or misformed aa-AMPs (pre-transfer editing), which is deduced from the inability of LeuRS to effectively distinguish isosteric sets of amino acids that are structurally similar (i.e. Leu, Nov, Ile, and Met) (4–7). The isolated CP1 domains of <i>Aquifex aeolicus</i> LeuRS (<i>Aa</i>LeuRS), <i>Escherichia coli</i> IleRS (EcIleRS), and <i>Bacillus stearothermophilus</i> ValRS (BsValRS) all have editing functions (8, 9). In addition, the CP2 domain exists in all three of the above mentioned aaRSs (10). The CP2 domain of prokaryotic and eukaryotic/archaeal LeuRSs consists of 32 and 36 amino acid residues, respectively (10). The CP2 domain of <i>Pyrococcus horikoshii</i> LeuRS (<i>Ph</i>LeuRS) is inserted between the second and third β-strands of the CP core and consists of a pair of antiparallel α-helices and a connecting β-strand with the overall shape of “U” (10). Tertiary structure of LeuRS-tRNA<sup>Leu</sup>, IleRS-tRNA<sup>Ile</sup>, and ValRS-tRNA<sup>Val</sup> complexes in the post-transfer editing conformation suggested that the CP2 domain is spatially close to the acceptor stem of tRNA (Fig. 2, A and B) (6, 11, 12). However, the specific role of CP2 on amino acid activation, aminoacylation, and editing activities of LeuRS remains unknown.

<i>Giardia lamblia</i> is a unicellular eukaryote, among the most ancient eukaryotes, and causes prevalent giardiasis. LeuRS from <i>G. lamblia</i> (GILeuRS) consists of 1173 amino acid residues and has been obtained by gene expression in our labora-

---

* This work was funded by the National Key Basic Research Foundation of China (Grant 2006CB910301), the Natural Science Foundation of China (Grant 30670463), and the Committee of Science and Technology in Shanghai (Grant 06JC14076). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviation used are: aaRS, aminoacyl-tRNA synthetase; CP1, connective peptide 1; CP2, connective peptide 2; IleRS, isoleucyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; aa-AMP, aminoacyl-adenylate; DTT, dithiothreitol; Ni-NTA, nickel-nitrilotriacetic acid.
The CP2 Domain of Leucyl-tRNA Synthetase

Construction of Various LeuRSs and Their Mutants

The CP2 domain of GILeuRS is represented by a dotted line, while the CP2 domain of PhLeuRS or EcLeuRS is represented by a dashed line.

5’-AGTTTATCTAGATCGTGACACCGTGTCAGCGTGTTCTTTTGGT-3’ and PhCP2-R(XapI), 5’-TGGAAAAATTTTGTCCAGAGACCGAGGCTAGTTTGAAGCTATAATAGATTGGCTT-3’ were constructed in our laboratory.

Materials—L-Leucine, dithiothreitol (DTT), NTP, 5’-GMP, tRNA\textsubscript{Leu}, and tRNALeu\textsubscript{AAG} were obtained from Sigma. [L-3H]Leucine, [L-3H]isoleucine, and tetrasodium [32P]pyrophosphate were obtained from Amersham Biosciences (England). GF/C filters were purchased from Whatman (Germany).

EXPERIMENTAL PROCEDURES

Construction of Various LeuRSs and Their Mutants—The plasmids containing the genes encoding GILeuRS, EcLeuRS, PhLeuRS, pET28a(+)–glleuS,\textsuperscript{3} pET30a(+)–eclleuS (7), and pET28a(+)–phleuS (9) were constructed in our laboratory. Deletion and single-point mutants within their CP2 domains were constructed according to the protocol provided by the KOD-Plus-Mutagenesis kit (TOYOBO, Japan). As for the construction of the genes encoding GILeuRS–PhCP2 and GILeuRS–EcCP2, four long oligonucleotide primers were designed as follows: for GILeuRS–PhCP2: PhCP2–F(XapI), 5’-GATGAAAATTATTGTCCAGAGACCGAGGCTAGTTTGAAGCTATAATAGATTGGCTT-3’ and PhCP2-R(XapI), 5’-TGGAAAAATTTTGTCCAGAGACCGAGGCTAGTTTGAAGCTATAATAGATTGGCTT-3’. GILeuRS–EcCP2 was constructed as follows (Fig. 1). First, the 5’ DNA fragment encoding GILeuRS–PhCP2 was obtained by using primer combination of the forward primer,\textsuperscript{3} PhCP2–F(XapI), and the reverse primer.\textsuperscript{3} Second, the 5’ DNA fragment and the 3’ DNA fragment were cleaved by NcoI and XapI, respectively, and were simultaneously ligated into pET28a(+) cleaved by NcoI and BamHI. GILeuRS–EcCP2 was constructed according to the same method except that different primers and restriction enzymes were used. All sequences of the recombinant plasmids were confirmed by DNA sequencing.

Preparation of Various tRNA\textsubscript{Leu}\textsuperscript{AAG}—G. lamblia tRNA\textsubscript{Leu}(AAG) was obtained by T7 RNA polymerase transcription as described previously (17). Seven complementary and overlapping oligonucleotides encoding the T7 promoter, the gene, and its complementary chain were chemically synthesized by Invitrogen. Oligonucleotides were phosphorylated by T4 polynucleotide kinase, hybridized, and ligated by T4 DNA ligase into pUC19 between EcoRI and BamHI to produce pUC19-GtRNA\textsubscript{Leu}. The in vitro T7 RNA polymerase transcription was carried out as described previously, and the accepting activity of GtRNA\textsubscript{Leu} was 660 pmol/A260 (17). The P. horikoshii tRNA\textsubscript{Leu} (PhtRNA\textsubscript{Leu}) gene was cloned by the same method, and PhtRNA\textsubscript{Leu} was obtained by in vitro T7 RNA polymerase transcription also with an accepting activity of 510 pmol/A260 (17). E. coli tRNA\textsubscript{Leu} (EctRNA\textsubscript{Leu}) was obtained from an overproduction strain in vivo in our laboratory, and its accepting activity was over 1300 pmol/A260 (16).

Purification of Proteins—E. coli BL21-Codon Plus (DE3)-RIL cells (Stratagene) were transformed with the plasmids containing the genes encoding GILeuRS, EcLeuRS, PhLeuRS and their

\textsuperscript{3} X.-L. Zhou, P. Yao, L.-L. Ruan, B. Zhu, J. Luo, L.-H. Qu, and E.-D. Wang, submitted for publication.
The CP2 Domain of Leucyl-tRNA Synthetase

CP2 deletion and single-point mutants to overproduce the above LeuRSs and their mutants, respectively. A single colony of transformants was chosen and cultured in 500 ml of 2×YT medium (8 g of tryptone, 5 g of yeast extract, and 2.5 g of NaCl in 500 ml of sterile water) at 37 °C. When the cells were grown to mid-log phase ($A_{600} = 0.6$), isopropyl-1-thio-$\beta$-galactopyranoside was added to a final concentration of 0.5 mM, and cultivation continued for 6 h at 22 °C. The cells were collected by centrifugation at 5000 rpm for 20 min at 4 °C and washed twice with buffer A (10 mM imidazole, 300 mM NaCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 50 mM NaH$_2$PO$_4$, pH 8.0). The purification was carried out by affinity chromatography on Ni-NTA Superflow according to the manufacturer's protocol (Qiagen). Wet cells from 500 ml of culture were suspended in 10 ml of buffer A and sonicated on ice. The lysates were centrifuged at 10,000 rpm for 30 min. The supernatant was then ultra centrifuged at 4.6 $\times$ 10$^4$ rpm for 1 h to remove the debris and insoluble fractions. The supernatant was gently mixed with 1.5 ml of Ni-NTA Superflow resin for 1 h. The mixture was loaded onto a minicolumn for gravity flow chromatography. The resin was then washed with 30 ml of buffer B (20 mM imidazole, 300 mM NaCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM NaH$_2$PO$_4$, pH 8.0) to remove nonspecific binding contaminants. Then the enzyme was eluted in sequence with 7 ml of buffer C (50 mM imidazole, 300 mM NaCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 50 mM NaH$_2$PO$_4$, pH 8.0), and 8 ml of buffer D (250 mM imidazole, 300 mM NaCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 50 mM NaH$_2$PO$_4$, pH 8.0). The eluted fractions were pooled, dialyzed, and concentrated by an Amicon Ultra-15 filter (Millipore, 30-kDa molecular mass cutoff), gently mixed with an equal volume of 100% ethanol. The pads were dried under a heat lamp. The radioactivities of the precipitates were quantified by a scintillation counter (Beckman Coulter).

Determination of Aminoacylation and Misaminoacylation—Leucylation of tRNA$^{Leu}$ or isoleucylation of tRNA$^{Leu}$ was carried out in 60 μl of reaction mixture containing 60 mM Tris-HCl (pH 8.2), 10 mM MgCl$_2$, 2 mM DTT, 4 mM ATP, 20 μM $[^3H]$Leu or $[^3H]$Ile, 40 μM tRNA$^{Leu}$, and 50 nM GtLeuRS or its editing-defective mutants at 45 °C. Aliquots of 11 μl of reaction solution were removed at specific time points, quenched on Whatman filter pads, and equilibrated with 5% trichloroacetic acid. The pads were washed three times for 15 min each with cold 5% trichloroacetic acid and then three times for 10 min each with 100% ethanol. The pads were dried under a heat lamp. The radioactivities of the precipitates were quantified by a scintillation counter (Beckman Coulter).

Hydrolytic Editing Assay—The $[^3H]$Ile-tRNA$^{Leu}$ decylation assays were carried out at 37 °C in 60 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 2 mM DTT, 2 μM $[^3H]$Ile-tRNA$^{Leu}$ (from Gt-, Ec-, and PhtRNA$^{Leu}$, respectively, as indicated), and 20 nM each LeuRS or mutant, separately. Aliquots of 11 μl of reaction solution were removed at specific time points, quenched on Whatman filter pads, washed, and analyzed as described above.

Determination of $K_D$ by Tryptophan Fluorescence Quenching—Equilibrium titrations were performed at room temperature with 0.1 μM of enzyme or the mutant in 60 mM Tris-HCl (pH 8.2), 10 mM MgCl$_2$, and 2 mM DTT. Tryptophan fluorescence was excited at 295 nm. An emission wavelength of 338 nm was used to quantify binding after correction for dilution and for the inner filter effect. Control solutions of bovine serum albumin or tryptophan were performed to show that there was no fluorescence response to tRNA. The $K_D$ values were determined by fitting fluorescence intensity change data versus tRNA concentration using Originpro 7.5 software.

RESULTS

Deletion of CP2 Domain Abolished Leucine Activation and Post-transfer Editing—G. lamblia contains a eukaryotic/archaeal LeuRS with 1173 amino acid residues. The tertiary structures of PhLeuRS-tRNA$^{Leu}$ and TtLeuRS-tRNA$^{Leu}$ complexes show that the CP2 domains are very close to the acceptor stem of tRNA$^{Leu}$ (Fig. 2, A and B). For example, the distance between PhLeuRS-CP2 main chain and tRNA$^{Leu}$ acceptor stem...
The CP2 Domain of Leucyl-tRNA Synthetase

phosphate backbone is ~9 Å (7.64 and 9.84 Å marked in Fig. 2A); that of TtLeuRS-CP2 main chain and tRNA\textsubscript{Leu} acceptor stem phosphate backbone is ~7 Å (5.91 and 7.20 Å marked in Fig. 2B). By sequence alignment of various LeuRSs, GlLeuRS is 39.2% homologous to PhLeuRS and 25.4% homologous to TtLeuRS. The CP2 domain of GlLeuRS (GlLeuRS-CP2) contains 37 amino acid residues from Tyr-581 to Arg-617, and that of EcLeuRS (EcLeuRS-CP2) contains 32 residues from Lys-194 to Gly-225, according to the structures of PhLeuRS and TtLeuRS (Fig. 2C).

To investigate the effect of GlLeuRS-CP2 on various activities, the mutant GlLeuRS-CP2, which replaced GlLeuRS-CP2 with three Ala residues, was constructed. Based on the crystal structure of PhLeuRS (10), three Ala residues would function as a linker to maintain the approximate distance between the second and the third β-strands of the CP core. GlLeuRS-CP2 with an N-terminal 6-His tag was stably produced in an E. coli strain containing its gene and purified to over 90% homogeneity by Ni-NTA affinity chromatography (data not shown). The activation, aminoacylation, and post-transfer editing activities of GlLeuRS-CP2 were assayed. GlLeuRS-CP2 did not activate cognate leucine at all in the ATP-PP\textsubscript{i} exchange assay (supplemental Fig. S1A), indicating that the CP2 domain plays an indispensable role in amino acid activation. The mutant could not leucylate tRNA\textsubscript{Leu}, as expected (data not shown). Compared with the post-transfer editing ability of GlLeuRS, the mutant could not catalyze Ile-tRNA\textsubscript{Leu} hydrolysis either, and its editing activity was absolutely lost (supplemental Fig. S1B).

The CP2 Domain Contributed to LeuRS-tRNA\textsubscript{Leu} Binding Affinity—Because of the inability of GlLeuRS-CP2 to activate leucine, the contribution of GlLeuRS-CP2 to the binding of tRNA\textsubscript{Leu} could not be measured by aminoacylation kinetics. Intrinsic tryptophan equilibrium fluorescence was carried out to test the role of GlLeuRS-CP2 in the binding affinity for tRNA\textsubscript{Leu}.

The excitation and emission wavelengths were 295 nm and 338 nm, respectively. Fluorescence quenching of the enzyme by tRNA\textsubscript{Leu} titration was measured, and the $K_{D}$ values of GlLeuRS and its mutant with tRNA\textsubscript{Leu} were calculated. For GlLeuRS and

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Primary and tertiary structure of the LeuRS CP2 domain. Crystal structures of PhLeuRS-tRNA\textsubscript{Leu} complex in the aminoacylation conformation (A) (22) and TtLeuRS-tRNA\textsubscript{Leu} in the post-transfer editing conformation (B) (11). The CP2 domains are colored in magenta, while the other parts of the LeuRSs are in green. tRNA is shown as an orange ribbon. The right parts of A and B are pictures with a closer look showing the relative positions and distances between the CP2 main chain and tRNA\textsubscript{Leu} phosphate backbone. In A, 7.64 and 9.84 Å are marked, whereas in B, 5.91 and 7.20 Å are marked. C, primary sequence alignment of the CP2 domain from eukaryotic/archaeal and prokaryotic LeuRSs. Conserved and homologous residues are highlighted in black and gray, respectively. The three CP2 domains used in this study are indicated with their location in their respective LeuRSs. The amino acids of GlLeuRS in which site-directed mutagenesis was performed are highlighted by an arrow. The abbreviations of the organisms are as follows: Gi, Giardia lamblia; Sc, Saccharomyces cerevisiae; Hs, Homo sapiens; Ph, Pyrococcus horikoshii; Pa, Pyrococcus abyssi; Mj, Methanococcus jannaschii; Tt, Thermus thermophilus; Bs, Bacillus subtilis; Ec, Escherichia coli; and Aa, Aquifex aeolicus.}
\end{figure}
GlLeuRS-ΔCP2, the $K_D$ values were 0.19 μM (±0.02 μM) and 0.37 μM (±0.02 μM), respectively, indicating that the GlLeuRS-ΔCP2 has looser binding with tRNALeu and GlLeuRS-CP2 should contribute to optimal tRNALeu binding (Fig. 3).

Important Residues of GlLeuRS-CP2 Were Identified by Alanine Scanning—Sequence alignment from several eukaryotic/archaeal LeuRSs showed that residues in GlLeuRS-CP2 are not strictly conserved (Fig. 2C). To find the crucial residues for the functions of GlLeuRS-CP2, some conserved and/or semi-conserved amino acid residues (Tyr-581, Trp-586, Lys-587, Asp-588, Asp-603, Lys-606, and Phe-609) were selected and mutated to Ala (Fig. 2C). These single-point mutants were named as GlLeuRS-Y581A, GlLeuRS-W586A, GlLeuRS-K587A, GlLeuRS-D588A, GlLeuRS-D603A, GlLeuRS-K606A, and GlLeuRS-F609A, respectively. Their amino acid activation, aminoacylation, and post-transfer editing activities were assayed and are shown in Fig. 4. Compared with GlLeuRS, GlLeuRS-D603A had similar amino acid activation, aminoacylation, and post-transfer editing activities, indicating that Asp-603 is not a critical residue for the functions of the enzyme. These results were consistent with the fact that some other eukaryotic/archaeal LeuRSs have this aspartic acid replaced with glutamic acid or proline (Fig. 2C).

GlLeuRS-K587A and GlLeuRS-D588A displayed lower amino acid activation and aminoacylation activities than GlLeuRS. In addition, GlLeuRS-K587A had a decreased post-transfer editing activity; however, GlLeuRS-D588A had a similar editing activity as GlLeuRS. The kinetic parameters of the ATP-PPi exchange reaction showed that GlLeuRS-K587A and GlLeuRS-D588A displayed similar $K_m$ values for both leucine and ATP, but the $k_{cat}$ was about half of that of GlLeuRS, so the relative catalytic efficiency ($k_{cat}/K_m$) was about half of that of GlLeuRS (Table 1 and supplemental Table S1). These results suggest that these two residues are important but not indispensable for GlLeuRS-CP2 (Fig. 4, A–C). GlLeuRS-Y581A, GlLeuRS-W586A, and GlLeuRS-K606A lost their amino acid activation, aminoacylation, and post-transfer editing activities completely. GlLeuRS-F609A lost its synthetic activity, and its post-transfer editing activity decreased drastically (Fig. 4, A–C). These data indicated that Tyr-581, Trp-586, Lys-606,
and Phe-609 are the crucial residues for both the synthetic and editing functions of GiLeuRS.

Functional Analysis of Pivotal Residues Was Performed by Site-directed Mutagenesis—The effect of side chains of these crucial residues on the activities was analyzed by further site-directed mutagenesis studies. The side chain of Tyr-581 is composed of a hydroxyl group attached to a benzene ring. We mutated it into serine and phenylalanine to keep either the hydroxyl group or the benzene ring of the side chain, respectively. Tyr-581 was also mutated to glutamic acid to see the effect of a negative charge and to lysine to see the effect of a positive charge. GiLeuRS-Y581S, GiLeuRS-Y581E, and GiLeuRS-Y581K were all unable to activate leucine by the ATP-PP\textsubscript{i} exchange assay, and they all had no post-transfer editing activity. Although GiLeuRS-Y581F had some detectable but significantly reduced leucine activation activity, its post-transfer editing activity was similar to that of GiLeuRS (Fig. 5, A and B). Two hydrophobic and absolutely conserved residues, Trp-586 and Phe-609, in the CP2 domains of eukaryotic/archaeal LeuRSs were mutated to glutamic acid and lysine, respectively. All four mutants lost leucine activation activity (Fig. 5, C and G). Their post-transfer editing activities were either lost or were very weak (Fig. 5, D and H). These results indicate that both Trp-586 and Phe-609 play crucial roles in interacting with mischarged tRNA in the post-transfer editing complex formation. This role may be accompanied by a hydrophobic interaction; all of the mutants with glutamic acid, lysine, and alanine at these residues abolished or impaired editing activity (Figs. 4C, 5D, and 5H). Lys-606 is a semi-conserved residue and is replaced with arginine in other eukaryotic/archaeal LeuRSs (Fig. 2C). Its conservation might indicate that its positive charge is necessary for its function. The two mutants GiLeuRS-K606R and GiLeuRS-K606E, which replaced Lys-606 with positively charged arginine and negatively charged glutamic acid, had no difference in leucine activation and post-transfer editing activities compared with GiLeuRS (Fig. 5, E and F). In addition, the mutant GiLeuRS-K606L with a large side chain and the mutant GiLeuRS-K606D with a negative charge had similar leucine activation and post-transfer editing activities as GiLeuRS (Fig. 5, E and F). Likewise, their \( k_{\text{cat}} \) and \( k_{\text{m}} \) values for both ATP and leucine, and the relative \( k_{\text{cat}}/k_{\text{m}} \) in the ATP-PP\textsubscript{i} exchange reaction were similar to those of GiLeuRS (Table 1 and supplemental Table S1). Because the mutant GiLeuRS-K606A with the smallest side chain was inactive, the above results suggest that a given size but not electric charge of the side chain of the residue at position 606 may be important for leucine activation and post-transfer editing activities.

Role of CP2 Domain Was Conserved in LeuRSs from Different Species—The CP2 domain has a different insertion point in LeuRSs from prokaryotes (before the CP1 domain) and eukaryotes/archaea (after the CP1 domain) to adapt to different tRNA recognition modes (10). Its sequence is more conserved in prokaryotic LeuRSs than in eukaryotic LeuRSs, albeit they show similar global structures (Fig. 2). Additionally, the CP2 domains from prokaryotic LeuRSs display significant sequence differences from those from eukaryotic LeuRSs (Fig. 2C). Whether the function of the CP2 domain is conserved despite its insertion point and sequence difference was studied. Based on the CP2 domain crystal structures of TtLeuRS and PhLeuRS (4, 10), EcLeuRS-\( \Delta \)CP2, which replaced the CP2 domain of EcLeuRS (prokaryotic) by five Ala residues, and PhLeuRS-\( \Delta \)CP2, which substituted three Ala residues for the CP2 domain of PhLeuRS (archaeal), were constructed. The distance of the two ends of the CP2 domain of TtLeuRS is 16.22 Å, whereas that of PhLeuRS is 8.22 Å; we hypothesized that these Ala linker peptides would maintain the approximate distances between

### TABLE 1

| Substrate | GiLeuRS K587A | D588A | D601A | K606R | K606E | K606L | K606D |
|-----------|---------------|-------|-------|-------|-------|-------|-------|
| ATP       | 0.43          | 0.57  | 0.91  | 0.98  | 0.90  | 0.85  | 0.86  |
| Leu       | 0.42          | 0.37  | 0.94  | 0.99  | 0.99  | 0.98  | 0.98  |

This table shows the relative \( k_{\text{cat}}/k_{\text{m}} \) values for ATP and Leu for various mutants from GiLeuRS in the ATP-PP\textsubscript{i} exchange reaction.
the two ends. These proteins were purified to over 90% homogeneity by affinity chromatography using an N-terminal 6-His tag. The leucine activation, aminoacylation, and hydrolysis of mischarged tRNA\textsuperscript{Leu}\textsuperscript{Leu} activities of the two native LeuRSs and the above deletion mutants were measured and are shown in supplemental Fig. S2. As compared with EcLeuRS and PhLeuRS, EcLeuRS-ACP2 (supplemental Fig. S2, A and B) and PhLeuRS-ACP2 (supplemental Fig. S2, C and D), like GLLeuRS-ACP2, lost their leucine activation, aminoacylation (data not shown), and post-transfer editing activities. The relatively lower post-transfer editing activity of PhLeuRS was assayed at 37 °C, which is not the optimal temperature for PhLeuRS (10). These results show that the role of the CP2 domain in amino acid activation and post-transfer editing is conserved among various LeuRSs from different species, although the CP2 domains are located at various positions and have different sequences among prokaryotic and eukaryotic/archaeal LeuRSs.

The CP2 Domain from PhLeuRS but Not EcLeuRS Can Substitute That of GLLeuRS—To understand whether the role of the CP2 domain is dependent on its conserved conformation or divergent location in the primary sequence of different species of LeuRSs, the CP2 domain of GLLeuRS was substituted by the CP2 domains of PhLeuRS (from Tyr-469 to Asp-504) (10) and EcLeuRS (from Lys-194 to Gly-225), and the chimeric mutants were named GLLeuRS-PhCP2 and GLLeuRS-EcCP2, respectively. Both proteins were overproduced stably and efficiently in E. coli transformants containing their genes and purified by N-terminal six-His affinity chromatography.

The ATP-PP\textsubscript{i} exchange, aminoacylation, and hydrolysis of Ile-tRNA\textsuperscript{Leu} data showed that GLLeuRS-PhCP2 had these three activities (Fig. 6, A–C), although the three activities of the chimeric GLLeuRS with the CP2 domain of PhLeuRS decreased significantly as compared with those of GLLeuRS. However, GLLeuRS-EcCP2 lost amino acid activation, aminoacylation, and post-transfer editing activities (Fig. 6, A–C). These results suggest that Leu-AMP formation and enzyme-tRNA interaction are possibly very sensitive to the different position of the CP2 domain rather than its similar global domain structure. The different location of EcLeuRS-CP2 in the chimeric GLLeuRS-EcCP2 from its natural position in the EcLeuRS may account for the functional absence of GLLeuRS-EcCP2.

DISCUSSION

The presence of CP2 domains in LIV-RSs with similar lengths and conformations indicates that the CP2 domain may have emerged before the divergence of these three related aaRSs. The CP2 domain is composed of two antiparallel \( \alpha \)-helices and a connecting \( \beta \)-strand, but its sequence between bacteria and eukaryotic/archaeal LeuRSs is not conserved. Meanwhile, the CP2 domains of LeuRSs from different groups have different insertion points into the aminoacylation active site, which is derived from a distinct editing domain (the CP1 domain) insertion point and orientation patterns (10).

Until now, the function of the CP2 domain of class I aaRSs was only studied in Acidithiobacillus ferrooxidans tryptophanyl-tRNA synthetase (TrpRS) (21). A deletion mutant in the CP2 domain of A. ferrooxidans TrpRS retained in vitro activity and showed no effect on amino acid activation, suggesting that the conformation of the activation domain of TrpRS was not distorted. However, the CP2 domain deletion mutant of A. ferrooxidans TrpRS had a higher \( K_m \) value for cognate tRNA\textsuperscript{Trp}, showing that the CP2 domain is involved in tRNA binding (21). In the LIV-RSs system, although x-ray structures of \( T.\ thermophilus \) ValRS-tRNA\textsuperscript{Val} (6), \( T.\ thermophilus \) LeuRS-tRNA\textsuperscript{Leu} (11), and \( S.\ aureus \) IleRS-tRNA\textsuperscript{Ile} (12) complexes in the post-transfer editing conformation and \( P.\ horikoshii \) LeuRS-tRNA\textsuperscript{Leu} (22) in the aminoacylation conformation all show that the CP2 domain and the tRNA acceptor stem are
The CP2 Domain of Leucyl-tRNA Synthetase

adenylate analogue (Leu-AMS) also showed that the residues in the CP2 domain are not in direct contact with Leu-AMS (4). Therefore, we suggest that the CP2 domain plays its pivotal role in leucine activation by modulation of the exact conformation of the active site through indirect residue interaction between the CP2 domain and active site.

Tyr-581 is of great importance for amino acid activation and post-transfer editing. When Tyr was changed to Phe, the mutant had significantly decreased amino acid activation activity but similar post-transfer editing activity compared with wild-type GlLeuRS. However, when Tyr was replaced with Ser, Glu, and Lys, the amino acid activation and editing activities of the mutants were abolished completely. Therefore, we suggest that the hydroxyl group of Tyr-581 is crucial for amino acid activation. The benzene ring may function as a linker to support the terminal hydroxyl group to reach into the catalytic core during amino acid activation; because the mutant GlLeuRS-Y581S, with the hydroxyl group and without the benzene ring, is unable to activate amino acid. Meanwhile, the benzene ring of Tyr-581 is of great importance for post-transfer editing. We speculate that the benzene ring might insert between two stacking base pairs of the acceptor stem or form hydrophobic interactions with bases of the acceptor stem, based on the proximity of the CP2 domain and the acceptor stem revealed by the co-crystal structure of TrpValRS-tRNAVal (6), TrpLeuRS-tRNALeu (11), Ssal-tRNAile (12), and PhLeuRS-trNALeu (22). Trp-586 and Phe-609 might function through hydrophobic interactions with other residues of the activation domain and mischarged tRNA. As for Lys-606, it was surprisingly found that it could be replaced by four amino acid residues with a large side chain of varying polarity (Leu, Glu, Arg, and Asp). However, when Lys-606 was substituted with Ala, the mutant lost its synthetic and editing activities completely. The size of the side chain of residue 606 may be important to its activities; however, the detailed chemical mechanism remains unclear.

After removal of the CP2 domain, the dissociation constant of GlLeuRS-ΔCP2-tRNALeu was elevated 2-fold, indicating that the CP2 domain contributed to LeuRS-tRNALeu binding affinity. This is consistent with the CP2 domain of A. ferrooxidans TrpRS (21). The deletion mutant was also unable to hydrolyze mischarged tRNA. Combined with a series of co-crystal structures of TrLeuRS-tRNALeu, SsalRS-tRNAile, and TrValRS-tRNAVal in the post-transfer editing conformation (6, 11, 12), our results suggested that GlLeuRS-ΔCP2 may be defective in orienting the 3′-end of the mischarged tRNALeu into the editing active site located in the CP1 domain, because the interaction between the acceptor stem of mischarged tRNA and LeuRS is disturbed.

REFERENCES
1. Ibba, M., and Soll, D. (2000) Annu. Rev. Biochem. 69, 617–650
2. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) Nature 347, 203–206
3. Starzyk, R. M. Webster, T. A., and Schimmel, P. (1987) Science 237, 1614–1618
4. Cusack, S., Yaremchuk, A., and Tukalo, M. (2000) EMBO J. 19, 2351–2361
5. Nureki, O., Vassylyev, D., Tateno, M., Shimada, A., Nakama, T., Fukai, S., Konno, M., Hendrickson, T., Schimmel, P., and Yokoyama, S. (1998) Science 280, 578–582
The CP2 Domain of Leucyl-tRNA Synthetase

6. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylev, D. G., and Yokoyama, S. (2000) Cell 103, 793–803
7. Chen, J. F., Guo, N. N., Li, T., Wang, E. D., and Wang, Y. L. (2000) Biochemistry 39, 6726–6731
8. Lin, L., Hale, S. P., and Schimmel, P. (1996) Nature 384, 33–34
9. Zhao, M. W., Zhu, B., Hao, R., Xu, M. G., Eriani, G., and Wang, E. D. (2005) EMBO J. 24, 1430–1439
10. Fukunaga, R., and Yokoyama, S. (2005) J. Mol. Biol. 346, 57–71
11. Tukalo, M., Yaremchuk, A., Fukunaga, R., Yokoyama, S., and Cusack, S. (2005) Nat. Struct. Mol. Biol. 12, 923–930
12. Silvian, L. F., Wang, J., and Steitz, T. A. (1999) Science 285, 1074–1077
13. Deleted in proof
14. Deleted in proof
15. Li, Y., Wang, E. D., and Wang, Y. L. (1998) Protein Expr. Purif. 16, 355–358
16. Li, Y., Wang, E. D., and Wang, Y. L. (1998) Sci. China (Ser C) 41, 225–231
17. Li, Y., Chen, J. F., Wang, E. D., and Wang, Y. L. (1999) Sci China (Ser C) 42, 185–190
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
19. Xu, M. G., Chen, J. F., Martin, F., Zhao, M. W., Eriani, G., and Wang, E. D. (2002) J. Biol. Chem. 277, 41590–41596
20. Xu, M. G., Li, J., Du, X., and Wang, E. D. (2004) Biochem. Biophys. Res. Commun. 318, 11–16
21. Zuñiga, R., Salazar, J., Canales, M., and Orellana, O. (2002) FEBS Lett. 532, 387–390
22. Fukunaga, R., and Yokoyama, S. (2005) Nat. Struct. Mol. Biol. 12, 915–922