THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 52, pp. 36608–36616, December 26, 2008

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

Received for publication, September 2, 2008, and in revised form, October 23, 2008 Published, JBC Papers in Press, October 27, 2008, DOI 10.1074/jbc.M806745200

Xiao-Long Zhou, Bin Zhu, and En-Duo Wang[1]

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 $tRNA^{Leu}$, the role of the CP2 domain remains undefined. In the present work, deletion of the CP2 domain of *Giardia lamblia* LeuRS (*Gl*LeuRS) showed that the CP2 domain is indispensable for amino acid activation and post-transfer editing and that it contributes to LeuRS-$tRNA^{Leu}$ binding affinity. In addition, its functions are conserved in both eukaryotic/archaeal and prokaryotic LeuRSs from *G. lamblia*, *Pyrococcus horikoshii* (*Ph*LeuRS), and *Escherichia coli* (*Ec*LeuRS). 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 *Gl*LeuRS with either *Ph*LeuRS or *Ec*LeuRS, showed that the CP2 domain of *Ph*LeuRS but not that of *Ec*LeuRS 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)[2] 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 moiety to the 3′ terminus of the cognate tRNA to yield the aminoacyl-tRNA (1). Correctly charged tRNA is transferred 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-$tRNA^{Leu}$ synthesis and has an aminoacylation catalytic core defined by a Rossmann fold (4). LeuRS, IleRS, and ValRS (LIVRSs) 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 *Aquifex aeolicus* LeuRS (*Aa*LeuRS), *Escherichia coli* IleRS (*Ec*IleRS), and *Bacillus stearothermophilus* ValRS (*Bs*ValRS) 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 *Pyrococcus horikoshii* LeuRS (*Ph*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-$tRNA^{Leu}$, IleRS-$tRNA^{Ile}$, and ValRS-$tRNA^{Val}$ 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.

Giardia lamblia is a unicellular eukaryote, among the most ancient eukaryotes, and causes prevalent giardiasis. LeuRS from *G. lamblia* (*Gl*LeuRS) 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.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

[1] To whom correspondence should be addressed: 320 Yue Yang Road, Shanghai 200031, China. Tel.: 86-21-5492-1241; Fax: 86-21-5492-1011; E-mail: edwang@sibs.ac.cn.

[2] The abbreviations 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.

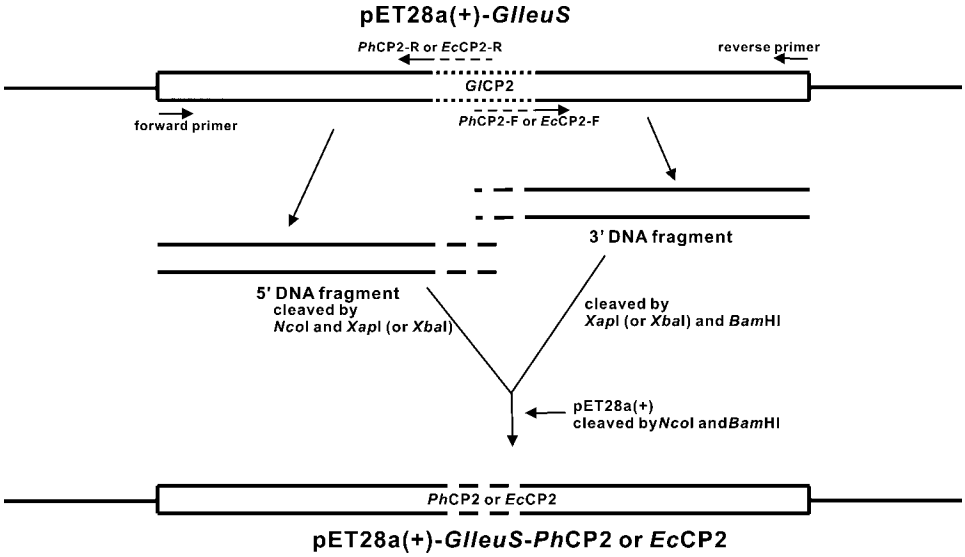

FIGURE 1. **Representative diagram of pET28a(+)-*GlleuS-Ph*CP2 or pET28a(+)-*GlleuS-Ec*CP2 construction.** The CP2 domain of *Gl*LeuRS is represented by a *dotted line*, while the CP2 domain of *Ph*LeuRS or *Ec*LeuRS is represented by a *dashed line*.

tory. Its properties, the function of CP1, and the role of a unique 49-amino acid motif within CP1 have been studied.[3] Here, we studied the function of the CP2 domain in various LeuRSs from *G. lamblia* (eukaryote), *E. coli* (bacterium), and *P. horikoshii* (archaebacterium) (*Gl*LeuRS, *Ec*LeuRS, and *Ph*LeuRS) by site-directed mutagenesis and performing leucine activation, aminoacylation, and post-transfer editing assays of these mutants.

EXPERIMENTAL PROCEDURES

Materials—L-Leucine, dithiothreitol (DTT), NTP, 5′-GMP, tetrasodium pyrophosphate, inorganic pyrophosphate, ATP, Tris-HCl, magnesium chloride, sodium chloride, and activated charcoal were purchased from Sigma. [L-^{3}H]Leucine, [L-^{3}H]isoleucine, and tetrasodium [^{32}P]pyrophosphate were obtained from Amersham Biosciences (England). GF/C filters were purchased from Whatman (Germany). *Pfu* DNA polymerase, a DNA fragment rapid purification kit, and a plasmid extraction kit were purchased from the Biotech Co. T4 ligase and restriction endonucleases were obtained from MBI Fermentas. Ni^{2+}-NTA Superflow was purchased from Qiagen. Pyrobest DNA polymerase and the dNTP mixture were obtained from Takara (Japan). Oligonucleotide primers were synthesized by Invitrogen. The pET28a(+) and pUC19 vectors were acquired from Novagen. T7 RNA polymerase was purified from an overproduction strain in our laboratory (15). *E. coli* $tRNA^{Leu}$ was isolated from an *E. coli* overproduction strain in our laboratory (16).

Construction of Various LeuRSs and Their Mutants—The plasmids containing the genes encoding *Gl*LeuRS, *Ec*LeuRS, *Ph*LeuRS, pET28a(+)-*glleuS*,[3] pET30a(+)-*ecleuS* (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 *Gl*LeuRS-*Ph*CP2 and *Gl*LeuRS-*Ec*CP2, four long oligonucleotide primers were designed as follows: for *Gl*LeuRS-*Ph*CP2: *Ph*CP2-F(XapI), 5′-GATGAAAATTTTGCCAGAGACCAGGAGGGCTCAGTTTGAAGCTATAATAGATTGGCTTGACGAATGGGCTGCCTCTCGTTCTTTTGGTTTGGG-3′ and *Ph*CP2-R(XapI), 5′-TGGCAAAATTTTCATCCTTTCAAGTGCTTTTCTTGCTTTTTCTTTCCACTCTGGATTTCCATAATCGAGGTACCACTGATCAGCTGCAGCAACG-3′; for *Gl*LeuRS-*Ec*CP2: *Ec*CP2-F(XbaI), 5′-ACGATCTAGATAAACTGGATCACTGGCCAGACACCGTTAAAACCATGCAGCGTAACTGGATCGGTGAATGGGCTGCCTCTCGTTCTTTTGGTTTGGG-3′ and *Ec*CP2-R(XbaI), 5′-AGTTTATCTAGATCGTTGAGCAGCTCGTCAGCGTAAGCAGTGATTTTATCGAGGTACCACTGATCAGCTGCAGCAACG-3′. *Gl*LeuRS-*Ph*CP2 was constructed as follows (Fig. 1). First, the 5′ DNA fragment encoding *Gl*LeuRS-*Ph*CP2 was obtained by using a primer combination of the forward primer,[3] *Ph*CP2-F(XapI), and pET28a(+)-*glleuS* as the template. The 3′ DNA fragment encoding *Gl*LeuRS-*Ph*CP2 was obtained using the same method with the primer combination of *Ph*CP2-R(XapI), and the reverse primer.[3] Second, the 5′ DNA fragment and the 3′ DNA fragment were cleaved by NcoI and XapI, and XapI and BamHI, respectively, and were simultaneously ligated into pET28a(+) cleaved by NcoI and BamHI. *Gl*LeuRS-*Ec*CP2 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 $tRNA^{Leu}$s—G. lamblia $tRNA^{Leu}$(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-*Gl*$tRNA^{Leu}$. The *in vitro* T7 RNA polymerase transcription was carried out as described previously, and the accepting activity of *Gl*$tRNA^{Leu}$ was 660 pmol/A260 (17). The *P. horikoshii* $tRNA^{Leu}$ (*Ph*$tRNA^{Leu}$) gene was cloned by the same method, and *Ph*$tRNA^{Leu}$ was obtained by *in vitro* T7 RNA polymerase transcription also with an accepting activity of 510 pmol/A260 (17). *E. coli* $tRNA^{Leu}$ (*Ec*$tRNA^{Leu}$) 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 *Gl*LeuRS, *Ec*LeuRS, *Ph*LeuRS and their

[3] X.-L. Zhou, P. Yao, L.-L. Ruan, B. Zhu, J. Luo, L.-H. Qu, and E.-D. Wang, submitted for publication.

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-β-D-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_2PO_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 ultracentrifuged at 4.6×10^4 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_2PO_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_2PO_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_2PO_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 glycerol, and stored at −20 °C. All purification steps were carried out at 4 °C. All deletion mutants are properly folded and display little change in the secondary structure compared with respective wild-type LeuRS by CD analysis (data not shown). Protein concentrations were determined by the Bradford method (18). The amount of active enzyme in the preparation was ~90% by active site titration (19).

Preparation of Mischarged tRNA^{Leu}s—[^{3}H]Ile-tRNA^{Leu} from *Gl*tRNA^{Leu} was prepared by incubating a reaction mixture containing 60 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 20 μM [^{3}H]Ile, 10 μM *Gl*tRNA^{Leu}, and 4 μM editing-defective *Gl*LeuRS-D444A at 45 °C for 30 min.[3] [^{3}H]Ile-tRNA^{Leu} from *Ph*tRNA^{Leu} was prepared by incubating a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM ATP, 40 μM [^{3}H]Ile, 10 μM *Ph*tRNA^{Leu}, and 2 μM editing-defective *Ph*LeuRS-D332A at 65 °C for 30 min (10). [^{3}H]Ile-tRNA^{Leu} from *Ec*tRNA^{Leu} was prepared by incubating a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM $MgCl_2$, 4 mM ATP, 0.1 mM EDTA, 0.5 mM DTT, 20 μM *Ec*tRNA^{Leu}, 20 μM [^{3}H]Ile, and 5 μM editing-defective *Ec*LeuRS-T252E at 37 °C for 30 min (20). The mischarged tRNAs were purified by repeated phenol/chloroform extractions, followed by ethanol precipitation.

Assay of ATP-PP$_i$ Exchange Reaction—The rate of the first reaction step of various LeuRSs, the formation of aa-AMP, was assayed by the ATP-PP$_i$ exchange reaction. The rates of the ATP-PP$_i$ exchange reactions catalyzed by *Gl*LeuRS, *Ec*LeuRS, and their mutants were carried out in 65 μl of reaction mixture containing 60 mM Tris-HCl (pH 8.2 or pH 7.8), 10 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 1 mM Leu, 2 mM tetrasodium [^{32}P]pyrophosphate, and 20 nM enzyme at 45 °C or 37 °C. Those by *Ph*LeuRS and *Ph*LeuRS-ΔCP2 were performed in 65 μl of reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 1 mM Leu, 2 mM tetrasodium [^{32}P]pyrophosphate, and 50 nM enzymes at 65 °C. A 15-μl aliquot of reaction mixture was removed every 5 min and added to 200 μl of quenching solution containing 2% activated charcoal, 3.5% $HClO_4$, and 50 mM tetrasodium pyrophosphate, and then mixed by vortex for 20 s. The solution was filtered through a Whatman GF/C filter, followed by washing with 20 ml of 10 mM tetrasodium pyrophosphate solution and 10 ml of 100% ethanol. The filters were dried and [^{32}P]ATP was counted 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 [^{3}H]Leu or [^{3}H]Ile, 40 μM tRNA^{Leu}, and 50 nM *Gl*LeuRS 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 [^{3}H]Ile-tRNA^{Leu} deacylation assays were carried out at 37 °C in 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 2 μM [^{3}H]Ile-tRNA^{Leu} (from *Gl*-, *Ec*-, and *Ph*tRNA^{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.[3] The tertiary structures of *Ph*LeuRS-tRNA^{Leu} and *Tt*LeuRS-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 *Ph*LeuRS-CP2 main chain and tRNA^{Leu} acceptor stem

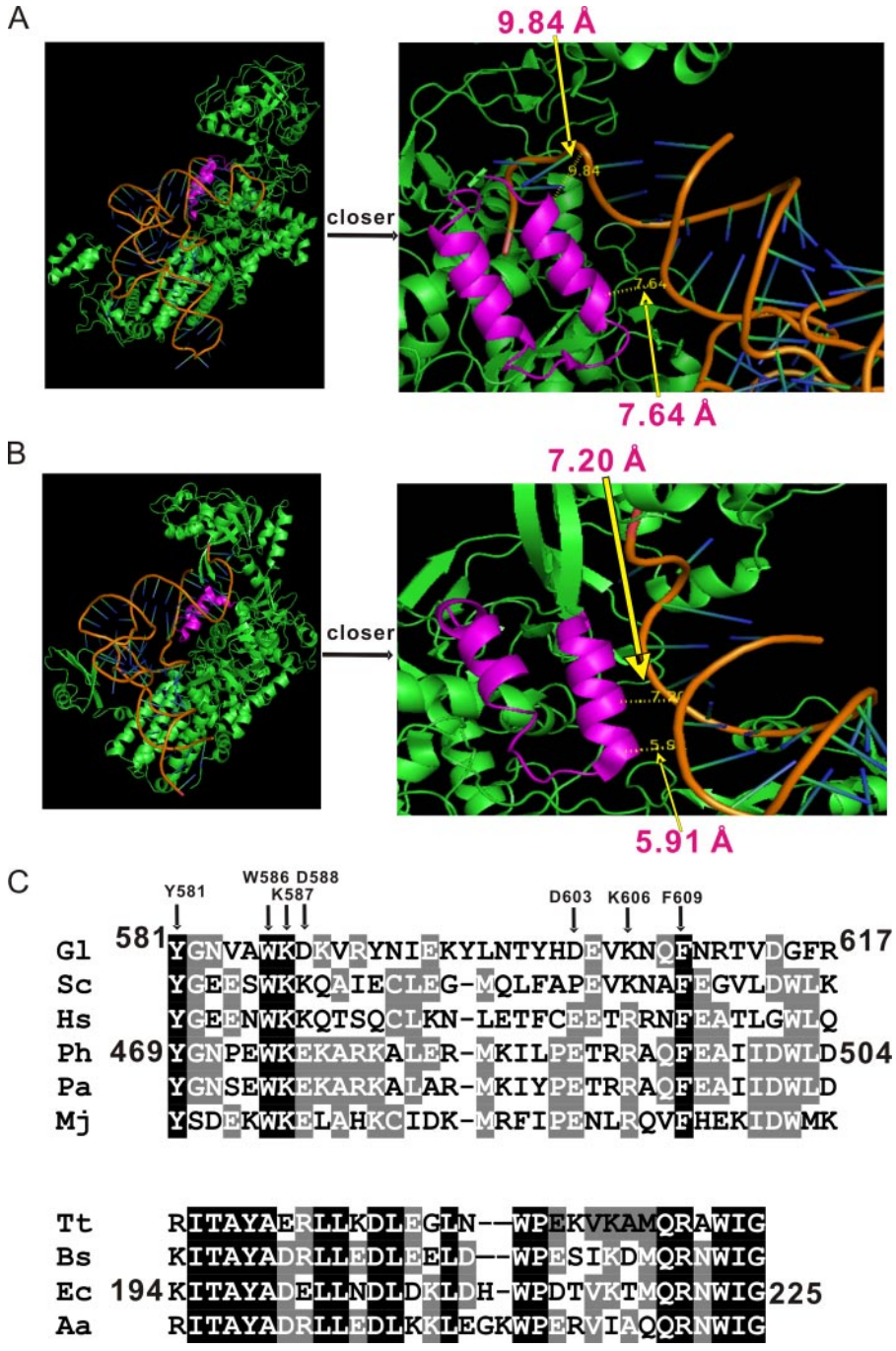

FIGURE 2. **Primary and tertiary structure of the LeuRS CP2 domain.** Crystal structures of *Ph*LeuRS-tRNA^{Leu} complex in the aminoacylation conformation (*A*) (22) and *Tt*LeuRS-tRNA^{Leu} in the post-transfer editing conformation (*B*) (11). The CP2 domains are colored in *magenta*, while the other 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^{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 *Gl*LeuRS in which site-directed mutagenesis was performed are highlighted by an *arrow*. The abbreviations of the organisms are as follows: *Gl*, *Giardia lamblia*; *Sc*, *Saccharomyces cerevisiae*; *Hs*, *Homo sapiens*; *Ph*, *Pyrococcus horikoshii*; *Pa*, *Pyrococcus abyssi*; *Mj*, *Methanococcus jannaschii*; *Tt*, *Thurmus thermophilus*; *Bs*, *Bacillus subtilis*; *Ec*, *Escherichia coli*; and *Aa*, *Aquifex aeolicus*.

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

To investigate the effect of *Gl*LeuRS-CP2 on various activities, the mutant *Gl*LeuRS-ΔCP2, which replaced *Gl*LeuRS-CP2 with three Ala residues, was constructed. Based on the crystal structure of *Ph*LeuRS (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. *Gl*LeuRS-ΔCP2 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 *Gl*LeuRS-ΔCP2 were assayed. *Gl*LeuRS-ΔCP2 did not activate cognate leucine at all in the ATP-PP$_i$ exchange assay (supplemental Fig. S1*A*), indicating that the CP2 domain plays an indispensable role in amino acid activation. The mutant could not leucylate tRNA^{Leu}, as expected (data not shown). Compared with the post-transfer editing ability of *Gl*LeuRS, the mutant could not catalyze Ile-tRNA^{Leu} hydrolysis either, and its editing activity was absolutely lost (supplemental Fig. S1*B*).

The CP2 Domain Contributed to LeuRS-tRNA^{Leu} Binding Affinity—Because of the inability of *Gl*LeuRS-ΔCP2 to activate leucine, the contribution of *Gl*LeuRS-CP2 to the binding of tRNA^{Leu} could not be measured by aminoacylation kinetics. Intrinsic tryptophan equilibrium fluorescence was carried out to test the role of *Gl*LeuRS-CP2 in the binding affinity for tRNA^{Leu}. The excitation and emission wavelengths were 295 nm and 338 nm, respectively. Fluorescence quenching of the enzyme by tRNA^{Leu} titration was measured, and the K_D values of *Gl*LeuRS and its mutant with tRNA^{Leu} were calculated. For *Gl*LeuRS and

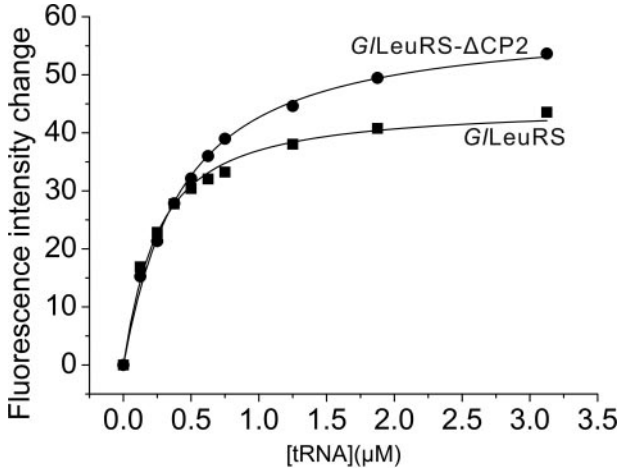

FIGURE 3. **Quenching of intrinsic tryptophan fluorescence of the wild-type *Gl*LeuRS (■) and its CP2 deletion mutant (●) through $tRNA^{Leu}$ titration.** The concentration of tRNA was varied from 0 to 3 μM. The fluorescence intensity is given as an arbitrary unit, and the emission at 338 nm was quantified. Analysis of the wild-type *Gl*LeuRS and its CP2 deletion mutant fluorescence intensity change *versus* tRNA concentration gave the K_D values of 0.19 ± 0.02 μM and 0.37 ± 0.02 μM, respectively.

*Gl*LeuRS-ΔCP2, the K_D values were 0.19 μM (±0.02 μM) and 0.37 μM (±0.02 μM), respectively, indicating that the *Gl*LeuRS-ΔCP2 has looser binding with $tRNA^{Leu}$ and *Gl*LeuRS-CP2 should contribute to optimal $tRNA^{Leu}$ binding (Fig. 3).

Important Residues of GlLeuRS-CP2 Were Identified by Alanine Scanning—Sequence alignment from several eukaryotic/archaeal LeuRSs showed that residues in *Gl*LeuRS-CP2 are not strictly conserved (Fig. 2*C*). To find the crucial residues for the functions of *Gl*LeuRS-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. 2*C*). These single-point mutants were named as *Gl*LeuRS-Y581A, *Gl*LeuRS-W586A, *Gl*LeuRS-K587A, *Gl*LeuRS-D588A, *Gl*LeuRS-D603A, *Gl*LeuRS-K606A, and *Gl*LeuRS-F609A, respectively. Their amino acid activation, aminoacylation, and post-transfer editing activities were assayed and are shown in Fig. 4. Compared with *Gl*LeuRS, *Gl*LeuRS-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. 2*C*). *Gl*LeuRS-K587A and *Gl*LeuRS-D588A displayed lower amino acid activation and aminoacylation activities than *Gl*LeuRS. In addition, *Gl*LeuRS-K587A had a decreased post-transfer editing activity; however, *Gl*LeuRS-D588A had a similar editing activity as *Gl*LeuRS. The kinetic parameters of the ATP-PP_i exchange reaction showed that *Gl*LeuRS-K587A and *Gl*LeuRS-D588A displayed similar K_m values for both leucine and ATP, but the k_{cat} was about half of that of *Gl*LeuRS, so the relative catalytic efficiency (k_{cat}/K_m) was about half of that of *Gl*LeuRS (Table 1 and supplemental Table S1). These results suggest that these two residues are important but not indispensable for *Gl*LeuRS-CP2 (Fig. 4, *A*–*C*). *Gl*LeuRS-Y581A, *Gl*LeuRS-W586A, and *Gl*LeuRS-K606A lost their amino acid activation, aminoacylation, and post-transfer editing activities completely. *Gl*LeuRS-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,

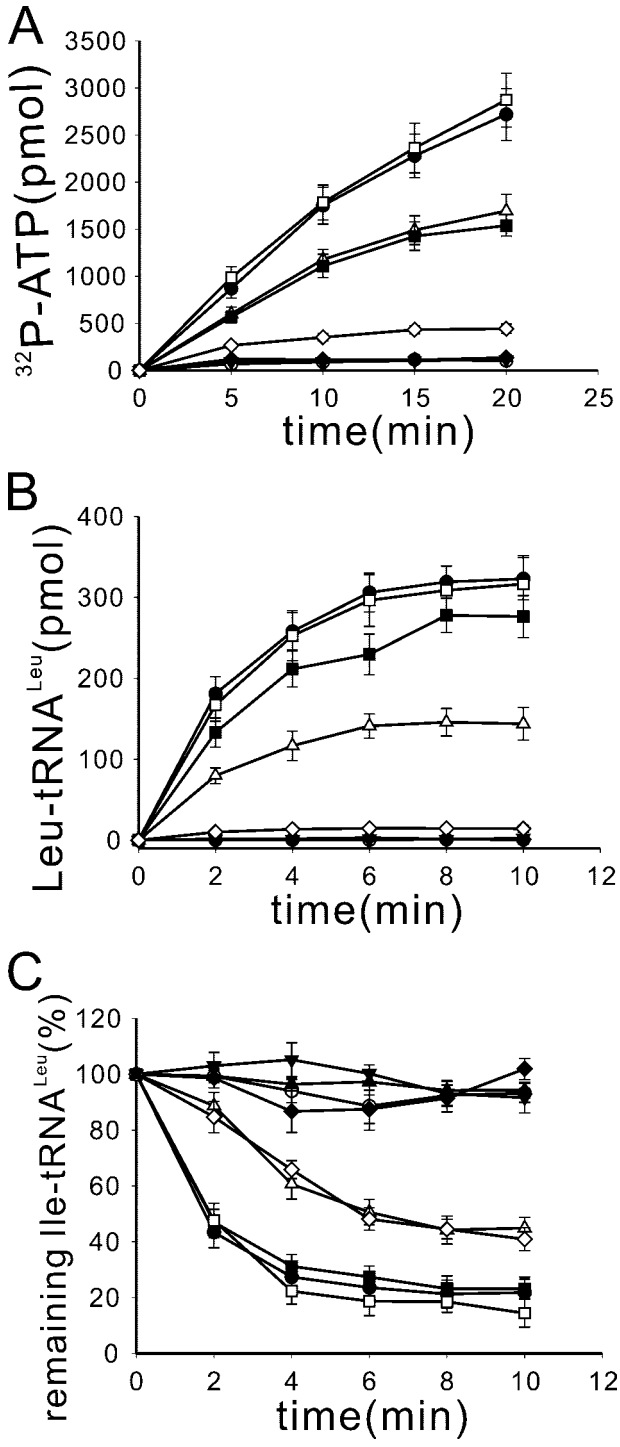

FIGURE 4. **Enzymatic activities of wild-type *Gl*LeuRS and its mutants in the alanine scanning assay.** *A*, the ATP-PP_i exchange reaction was carried out using 1 mM Leu and 20 nM enzyme. *B*, the aminoacylation assay was carried out using 50 nM *Gl*LeuRS or its mutants. *C*, post-transfer editing was performed using 2 μM [^{3}H]Ile-$tRNA^{Leu}$ from *Gl*$tRNA^{Leu}$ and 20 nM *Gl*LeuRS or its mutants. *Symbols* are *Gl*LeuRS (●), *Gl*LeuRS-Y581A (○), *Gl*LeuRS-W586A (▼), *Gl*LeuRS-K587A (△), *Gl*LeuRS-D588A (■), *Gl*LeuRS-D603A (□), *Gl*LeuRS-K606A (◆), and *Gl*LeuRS-F609A (◇). Control (spontaneous hydrolysis) (▲) was performed in the absence of enzyme.

TABLE 1
Relative k_{cat}/K_m of various mutants from *Gl*LeuRS in ATP-PP_i exchange reaction

Substrate	*Gl*LeuRS	K587A	D588A	D603A	K606R	K606E	K606L	K606D
				k_{cat}/K_m				
ATP	1	0.43	0.57	0.91	0.98	0.90	0.85	0.86
Leu	1	0.42	0.37	0.94	0.99	0.99	0.98	0.83

and Phe-609 are the crucial residues for both the synthetic and editing functions of *Gl*LeuRS.

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. *Gl*LeuRS-Y581S, *Gl*LeuRS-Y581E, and *Gl*LeuRS-Y581K were all unable to activate leucine by the ATP-PP_i exchange assay, and they all had no post-transfer editing activity. Although *Gl*LeuRS-Y581F had some detectable but significantly reduced leucine activation activity, its post-transfer editing activity was similar to that of *Gl*LeuRS (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. 4*C*, 5*D*, and 5*H*). Lys-606 is a semi-conserved residue and is replaced with arginine in other eukaryotic/archaeal LeuRSs (Fig. 2*C*). Its conservation might indicate that its positive charge is necessary for its function. The two mutants *Gl*LeuRS-K606R and *Gl*LeuRS-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 *Gl*LeuRS (Fig. 5, *E* and *F*). In addition, the mutant *Gl*LeuRS-K606L with a large side chain and the mutant *Gl*LeuRS-K606D with a negative charge had similar leucine activation and post-transfer editing activities as *Gl*LeuRS (Fig. 5, *E* and *F*). Likewise, their k_{cat} and K_m values for both ATP and leucine, and the relative k_{cat}/K_m in the ATP-PP_i exchange reaction were similar to those of *Gl*LeuRS (Table 1 and supplemental Table S1). Because the mutant *Gl*LeuRS-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

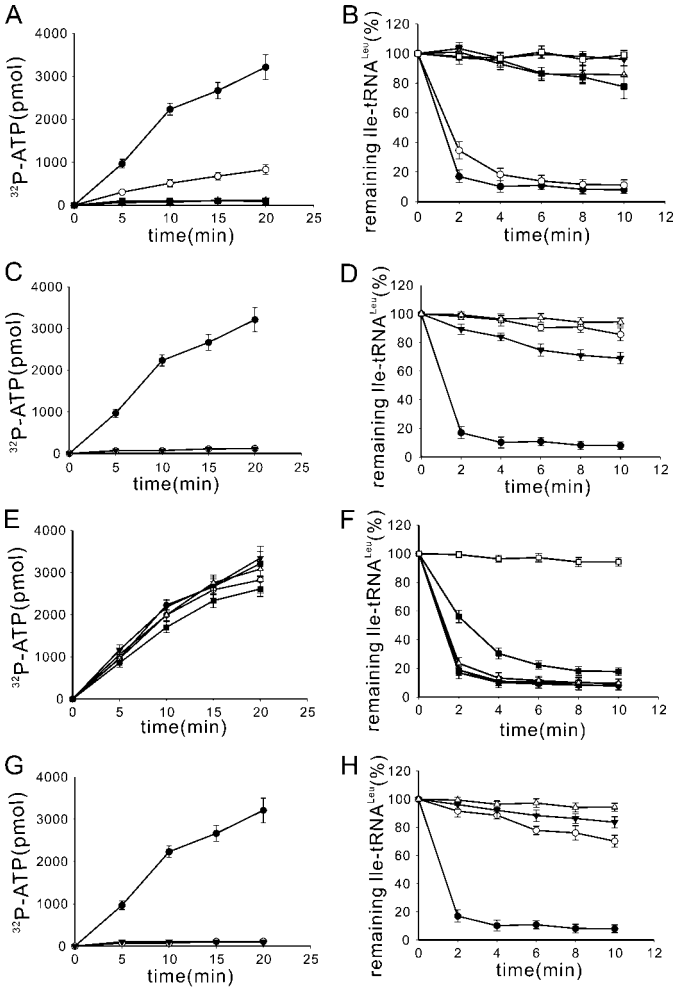

FIGURE 5. **Enzymatic activities of mutants on Tyr-581, Trp-586, Lys-606, and Phe-609.** The ATP-PP_i exchange reaction was carried out using 1 mM Leu, and 20 nM mutated enzyme on Tyr-581 (*A*), Trp-586 (*C*), Lys-606 (*E*), and Phe-609 (*G*); post-transfer editing was performed using 2 μM [^{3}H]Ile-tRNA^{Leu} from *Gl*tRNA^{Leu}, and 20 nM mutated enzyme on Tyr-581 (*B*), Trp-586 (*D*), Lys-606 (*F*), and Phe-609 (*H*). In *A* and *B*, *symbols* are *Gl*LeuRS-Y581F (○), *Gl*LeuRS-Y581S (▼), *Gl*LeuRS-Y581E (△), *Gl*LeuRS-Y581K (■), and hydrolytic control (□); in *C* and *D*, *symbols* are *Gl*LeuRS-W586E (○), *Gl*LeuRS-W586K (▼), and hydrolytic control (△); in *E* and *F*, *symbols* are *Gl*LeuRS-K606E (○), *Gl*LeuRS-K606R (▼), *Gl*LeuRS-K606L (△), *Gl*LeuRS-K606D (■), and hydrolytic control (□); in *G* and *H*, *symbols* are *Gl*LeuRS-F609E (○), *Gl*LeuRS-F6096K (▼), and hydrolytic control (△). Wild-type *Gl*LeuRS is represented by ● in all figures.

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. 2*C*). 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 *Tt*LeuRS and *Ph*LeuRS (4, 10), *Ec*LeuRS-ΔCP2, which replaced the CP2 domain of *Ec*LeuRS (prokaryotic) by five Ala residues, and *Ph*LeuRS-ΔCP2, which substituted three Ala residues for the CP2 domain of *Ph*LeuRS (archaeal), were constructed. The distance of the two ends of the CP2 domain of *Tt*LeuRS is 16.22 Å, whereas that of *Ph*LeuRS is 8.22 Å; we hypothesized that these Ala linker peptides would maintain the approximate distances between

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 $tRNA^{Leu}$ activities of the two native LeuRSs and the above deletion mutants were measured and are shown in supplemental Fig. S2. As compared with *Ec*LeuRS and *Ph*LeuRS, *Ec*LeuRS-ΔCP2 (supplemental Fig. S2, *A* and *B*) and *Ph*LeuRS-ΔCP2 (supplemental Fig. S2, *C* and *D*), like *Gl*LeuRS-ΔCP2, lost their leucine activation, aminoacylation (data not shown), and post-transfer editing activities. The relatively lower post-transfer editing activity of *Ph*LeuRS was assayed at 37 °C, which is not the optimal temperature for *Ph*LeuRS (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 *Gl*LeuRS was substituted by the CP2 domains of *Ph*LeuRS (from Tyr-469 to Asp-504) (10) and *Ec*LeuRS (from Lys-194 to Gly-225), and the chimeric mutants were named *Gl*LeuRS-*Ph*CP2 and *Gl*LeuRS-*Ec*CP2, 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_i exchange, aminoacylation, and hydrolysis of Ile-$tRNA^{Leu}$ data showed that *Gl*LeuRS-*Ph*CP2 had these three activities (Fig. 6, *A*–*C*), although the three activities of the chimeric *Gl*LeuRS with the CP2 domain of *Ph*LeuRS decreased significantly as compared with those of *Gl*LeuRS. However, *Gl*LeuRS-*Ec*CP2 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 *Ec*LeuRS-CP2 in the chimeric *Gl*LeuRS-*Ec*CP2 from its natural position in the *Ec*LeuRS may account for the functional absence of *Gl*LeuRS-*Ec*CP2.

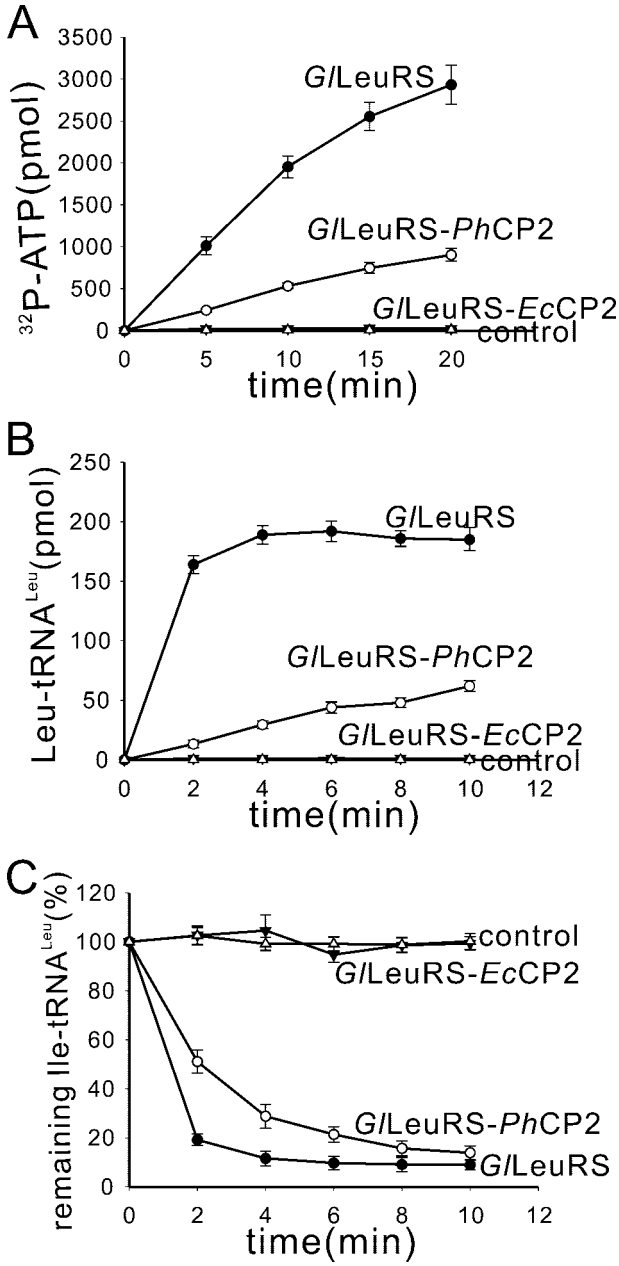

FIGURE 6. **Enzymatic activities of two chimeric enzymes, *Gl*LeuRS-*Ph*CP2 and *Gl*LeuRS-*Ec*CP2.** *A*, the ATP-PP_i exchange reaction was carried out using 1 mM Leu and 20 nM enzyme. *B*, the aminoacylation assay was carried out using 50 nM enzyme. *C*, post-transfer editing was performed using 2 μM [^{3}H]Ile-$tRNA^{Leu}$ from *Gl*$tRNA^{Leu}$ and 20 nM enzyme. Wild-type *Gl*LeuRS, *Gl*LeuRS-*Ph*CP2, and *Gl*LeuRS-*Ec*CP2 are represented by ●, ○, and ▼. Controls without leucine in *A*, without enzyme in *B*, and of spontaneous hydrolysis in *C* are represented by △.

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 α-helices and a connecting β-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* and *in vivo* 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 $tRNA^{Trp}$, showing that the CP2 domain is involved in tRNA binding (21). In the LIV-RSs system, although x-ray structures of *T. thermophilus* ValRS-$tRNA^{Val}$ (6), *T. thermophilus* LeuRS-$tRNA^{Leu}$ (11), and *S. aureus* IleRS-$tRNA^{Ile}$ (12) complexes in the post-transfer editing conformation and *P. horikoshii* LeuRS-$tRNA^{Leu}$ (22) in the aminoacylation conformation all show that the CP2 domain and the tRNA acceptor stem are

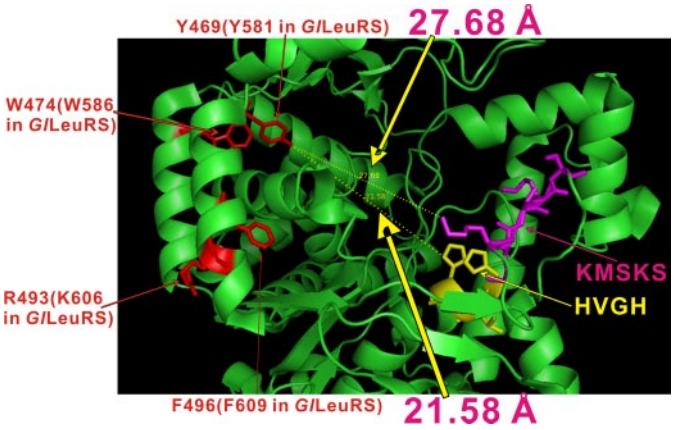

FIGURE 7. **Crystal structure of Rossmann fold of *Ph*LeuRS (10) showing positions of the crucial residues in the CP2 domain relative to the two class I aaRS signature sequences, HVGH and KMSKS (1).** The four residues corresponding to Tyr-581, Trp-586, Lys-606, and Phe-609 of *Gl*LeuRS (Tyr-469, Trp-474, Arg-493, and Phe-496 in *Ph*LeuRS) are colored in *red*, whereas HVGH and KMSKS are highlighted in *yellow* and *magenta*, respectively. The distances between the side hydroxyl group of Tyr-469 and the NH group of the second histidine of "HVGH" (21.58 Å) and the ϵ-NH_3 group of the first lysine of "KMSKS" (27.68 Å) are marked. Other parts of *Ph*LeuRS, including the anticodon binding domain, the CP1 domain, and the C-terminal domain, etc. were omitted for clarity.

close to each other (a little less than 10 Å between the CP2 main chain and tRNA acceptor stem phosphate backbone in *Ph*LeuRS-$tRNA^{Leu}$ and *Tt*LeuRS-$tRNA^{Leu}$ complexes) (Fig. 2, *A* and *B*), the direct interaction between CP2 and the acceptor stem of tRNA was not indicated, and the specific role of CP2 has not been reported.

Our results showed that the CP2 domain of *Gl*LeuRS, *Ph*LeuRS, or *Ec*LeuRS significantly affects amino acid activation and post-transfer editing. This is consistent with the fact that this domain in LeuRSs is located close to the Rossmann fold, and its connecting β-strand forms a β-sheet with the other four β-strands in the Rossmann fold (10). By analyzing the effect of alanine scanning and single-point mutations within CP2 on amino acid activation and post-transfer editing activities, the crucial residues were identified that played multiple roles during the entire precise $tRNA^{Leu}$-charging process. Based on the structure of the archaeal *Ph*LeuRS (10), the CP2 domain is at the opposite side of the two signature sequences of class I aaRSs, HIGH and KMSKS, in the Rossmann fold, which have been shown to be responsible for ATP binding and stabilization of the transition state during catalysis (1) (Fig. 7). The crucial residues Tyr-581, Trp-586, Lys-606, and Phe-609 of *Gl*LeuRS (Tyr-469, Trp-474, Arg-493, and Phe-496 in *Ph*LeuRS) all penetrated into the catalytic core; however, it is a long distance from these residues to HIGH and KMSKS (for example, the side hydroxyl group of Tyr-469 of *Ph*LeuRS is 21.58 and 27.68 Å from the NH group of the second histidine of "HVGH" and the ϵ-NH_3 group of the first lysine of "KMSKS," respectively) (Fig. 7). Thus, it is unlikely that these residues in the CP2 domain directly bind leucine and ATP during leucine activation. This is consistent with our kinetic data that the activity-retaining mutants on Lys-587, Asp-588, Asp-603, and Lys-606 displayed no difference in K_m values for leucine and ATP from those of *Gl*LeuRS (supplemental Table S1). Consistently, the crystal structure of *Tt*LeuRS complexed with a Leu-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 *Gl*LeuRS. 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 *Gl*LeuRS-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 *Tt*ValRS-$tRNA^{Val}$ (6), *Tt*LeuRS-$tRNA^{Leu}$ (11), *Sa*Ile-$tRNA^{Ile}$ (12), and *Ph*LeuRS-$tRNA^{Leu}$ (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 *Gl*LeuRS-ΔCP2-$tRNA^{Leu}$ was elevated 2-fold, indicating that the CP2 domain contributed to LeuRS-$tRNA^{Leu}$ 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 *Tt*LeuRS-$tRNA^{Leu}$, *Sa*IleRS-$tRNA^{Ile}$, and *Tt*ValRS-$tRNA^{Val}$ in the post-transfer editing conformation (6, 11, 12), our results suggested that *Gl*LeuRS-ΔCP2 may be defective in orienting the 3′-end of the mischarged $tRNA^{Leu}$ 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 Söll, 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

6. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylyev, 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. Zúñiga, 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