Peripheral insertion modulates the editing activity of the isolated CP1 domain of leucyl-tRNA synthetase

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A large insertion domain called CP1 (connective peptide 1) present in class Ia aminoacyl-tRNA synthetases is responsible for post-transfer editing. LeuRS (leucyl-tRNA synthetase) from Aquifex aeolicus and Giardia lamblia possess unique 20 and 59 amino acid insertions respectively within the CP1 that are crucial for editing activity. Crystal structures of AaLeuRS-CP1 [2.4 Å (1 Å = 0.1 nm)], GLeuRS-CP1 (2.6 Å) and the insertion deletion mutant AaLeuRS-CP1Δ20 (2.5 Å) were solved to understand the role of these insertions in editing. Both insertions are folded as peripheral motifs located on the opposite side of the proteins from the active-site entrance in the CP1 domain. Docking modelling and site-directed mutagenesis showed that the insertions do not interact with the substrates. Results of molecular dynamics simulations show that the intact CP1 is more dynamic than its mutant devoid of the insertion motif. Taken together, the data show that a peripheral insertion without a substrate-binding site or major structural role in the active site may modulate catalytic function of a protein, probably from protein dynamics regulation in two respective LeuRS CP1s. Further results from proline and glycine mutational analyses intended to reduce or increase protein flexibility are consistent with this hypothesis.

Key words: aminoacyl-tRNA synthetase, Aquifex aeolicus, connective peptide 1 (CP1), Giardia lamblia, post-transfer editing, tRNA.

INTRODUCTION

aaRSs (aminoacyl-tRNA synthetases) provide charged tRNAs for protein synthesis by catalysing the esterification reaction (aminoacylation) of amino acids to their cognate tRNAs [1,2]. The aminoacylation reaction usually occurs in two steps: (i) the amino acids are activated by ATP to form aminoacyl-adenylates (aa-AMP), and (ii) the aminoacyl residues are transferred to the 3′-end of tRNA to yield aminoacyl-tRNAs [3]. High-fidelity aminoacylation is important for cell metabolism; indeed, it has been reported that minor errors during this step could cause accumulation of misfolded (or unfolded) proteins in mouse cells, leading to serious neural degeneration [4].

Recognition of some amino acids that are similar in size, shape and polarity is particularly challenging for aaRSs. For example, IleRS (isoleucyl-tRNA synthetase) must discriminate isoleucine from valine on the basis of a single methyl group [5,6]. ValRS (valyl-tRNA synthetase) mis-activates threonine, which has a hydroxy group that is isosteric to the methyl moiety in valine [7]. LeuRS (leucyl-tRNA synthetase), which is highly homologous with IleRS and ValRS, misactivates threonine, site of the CP1 domain, including the T-rich region (threonine-rich region; TtLeuRS; Thermus thermophilus LeuRS; ValRS, valyl-tRNA synthetase.

Abbreviations used: aaRS, aminoacyl-tRNA synthetase; CP1, connective peptide 1; DTT, dithiothreitol; GST, glutathione transferase; IleRS, isoleucyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; AaLeuRS, Aquifex aeolicus LeuRS; CaLeuRS, Candida albicans LeuRS; EcLeuRS, Escherichia coli LeuRS; GlLeuRS, Giardia lamblia LeuRS; PEG, poly(ethylene glycol); Nva2AA, 2-(L-norvalyl) amino-2-deoxyadenosine; RMSD, root mean square deviation; T-rich region, threonine-rich region; TtLeuRS, Thermus thermophilus LeuRS; VaRS, valyl-tRNA synthetase.

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During evolution, the CP1 domain was recruited into the Rossman-fold aminocacylation domain to perform post-transfer editing. However, most of the isolated CP1 domains of LeuRS are without detectable editing activity [23, 24]. Up to now, only three CP1 domains of LeuRS with editing activity have been isolated from different organisms. The CP1 domain with flanking regions from the deep-rooted bacterium *Aquifex aeolicus* LeuRS (termed *Aa*LeuRS) was isolated as a 331-amino-acid long peptide (AaLeuRS-CP1 [23]). The CP1 domain of LeuRS from the ancient euarkyte *Giardia lamblia* (termed GlLeuRS) was isolated as a 301-amino-acid long peptide (GlLeuRS-CP1[24]). The editing activity of these isolated CP1 domains was crucially dependent on the presence of a unique insertion of 20 and 59 amino acids for *Aa*LeuRS and GlLeuRS respectively. The third CP1 domain that could be isolated with editing capacity was the one from *Escherichia coli* LeuRS (termed EcLeuRS); however, to be active, long flanking sequences from the β-strand linkers had to be added [25]. These studies indicated that the minimalist CP1 domain could hardly catalyse the editing reaction without the presence of a unique internal insertion for *Aa*LeuRS-CP1 or GlLeuRS-CP1, or the flanking sequences for EcLeuRS-CP1. When the unique insertions of *Aa*LeuRS-CP1 or GlLeuRS-CP1 were inserted into a minimalist EcLeuRS-CP1 deprived of flanking sequences, a surprising activation of its editing ability was observed [23, 24].

In order to investigate the role of these unique insertions in editing activity of isolated CP1 domains, the crystal structures of *Aa*LeuRS-CP1, GlLeuRS-CP1 and the insertion deletion mutant *Aa*LeuRS-CP1 Δ20 were determined. In combination with docking models, molecular dynamics simulations and enzymatic-based mutagenesis studies, the results of the present study show that the insertion motif modulates editing activity by affecting the plasticity of the isolated CP1 domain. In addition, the crystal structure of GlLeuRS-CP1 also provides an opportunity to understand the mechanism of AN2690 resistance, a boron-containing antifungal agent that targets the editing site and inhibits both synthetic and editing activities of LeuRS [26].

**EXPERIMENTAL**

**Expression, purification and crystallization of CP1s**

The *Aa*LeuRS-CP1 (residues 228–439) and *Aa*LeuRS-CP1Δ20 (deletion of residues 242–261) were constructed into vector pET30b (Novagen) and expressed in *E. coli* BL21 (DE3) with six histidine residues at the N-terminus. The protein was purified by affinity chromatography on Ni-NTA (Ni²⁺-nitritoltriacetate) Superflow resin (Qiagen), followed by gel-filtration chromatography with Superdex™ 75 (Amersham Biosciences). Purified AaLeuRS-CP1 and AaLeuRS-CP1Δ20 proteins were concentrated to 16 mg/ml and 25 mg/ml respectively for crystallization. The initial crystallization conditions were screened using Crystal Screen kits I and II from Hampton Research. After optimizing the crystallization conditions, good crystals of *Aa*LeuRS-CP1 were obtained by the sitting-drop vapour-diffusion method at 20 °C after 7–10 days under conditions of 16–17% PEG [poly(ethylene glycol)] 8000, 0.1 M sodium cacodylate trihydrate (pH 6.8) and 0.2 M calcium acetate hydrate. *Aa*LeuRS-CP1Δ20 crystals were obtained under conditions of 30% PEG4000, 0.1 M sodium citrate tribasic dihydrate (pH 5.6) and 0.2 M ammonium acetate after 30 days at 20 °C.

GlLeuRS-CP1 (residues 253–561) was concentrated to 8 mg/ml for crystallization. Initial crystallization conditions were screened using Index HT™ kits from Hampton Research; however, no good crystal could be obtained, even after optimizing the crystallization conditions. As GlLeuRS-CP1 protein contains nine cysteine residues that may form disulphide bonds, two surface cysteine to serine mutations (C395S/C533S) were introduced into GlLeuRS-CP1. The resulting engineered protein gave suitable crystals. Crystals of GlLeuRS-CP1 (C395S/C533S) were obtained using the hanging-drop vapour-diffusion method at 20 °C after 10 days under conditions of 2% glycerol, 0.1 M BisTris (pH 5.8) and 2.2 M ammonium sulfate. The structure reported as GlLeuRS-CP1 in the present paper contains the double C395S/C533S substitution.

**Diffraction data collection and processing**

*Aa*LeuRS-CP1 and AaLeuRS-CP1Δ20 crystals were directly mounted on to a nylon loop and flash-cooled into a liquid nitrogen stream (−170 °C). For AaLeuRS-CP1 crystals, paraffin oil (Hampton Research) was used as a cryoprotectant. All crystal diffraction data were collected at the Shanghai Synchrotron Radiation Facility 100 K beamline BL-17U1. All diffraction data were processed using the HKL2000 program package [27]; statistics of the diffraction data analysis are presented in Table 1.

**Crystal structure determination**

The AaLeuRS-CP1Δ20 crystal belongs to space group *I*41. Its structure was solved by the molecular replacement method implemented in the program MOLREP [28] of the CCP4 suite [29] using the structure of the *Thermus thermophilus* LeuRS (termed TtLeuRS; PDB code 1H3N [12]) as the search model. Initial structure refinement was carried out with the program REFMAC5 [31]. Model building was performed manually with the program COOT [32]. The AaLeuRS-CP1 crystal belongs to space group *P*2₁, and was determined by molecular replacement using the structure of AaLeuRS-CP1Δ20 as the starting model in MOLREP. Model building and refinement were carried out as described above.

The GlLeuRS-CP1 crystal belongs to space group *C*2. Molecular replacement was used to solve the structure by MOLREP using the structure of the CP1 domain of *Candida albicans* LeuRS (termed CaLeuRS; PDB code 2WFE) as the search model. Structure refinement was performed using the program REFMACS and PHENIX [33]. Throughout the refinement, 5% of randomly chosen reflections were set aside for free *R* factor monitor. The quality of final models was evaluated by PROCHECK [34]. The statistics of structure refinement and validation are presented in Table 1. All molecular graphics were generated with PyMOL (DeLano Scientific; http://www.pymol.org).

**Construction of CP1 mutants**

All point mutants were constructed following the procedures described in the KOD-plus mutagenesis kit (TOYOBO). AaLeuRS-CP1Δ20/ΔAGAG and AaLeuRS-CP1Δ20/Δ2xAG were also constructed using the KOD-plus mutagenesis kit by adding a fragment encoding AGAG into primers. The genes encoding AaLeuRS-CP1Δ20/ΔG59aa and AaLeuRS-CP1Δ20/ΔGST were constructed as follows. First, restriction sites of SacI and NdeI or BamHI were introduced into AaLeuRS-CP1Δ20 between residues 241 and 262 by the KOD-plus mutagenesis kit. Secondly, the genes encoding GlLeuRS-59aa and *Schistosoma japonicum* GST (glutathione transferase) were amplified by PCR from GlLeuRS-CP1 plasmid and pGEX-4T-1.
vector (GE Healthcare) respectively. After restriction the two DNA fragments were incorporated into AaLeuRS-CP1Δ20.

**Editing activity assays**

Hydrolitic activities of isolated wild-type and mutant AaLeuRS-CP1 for Ile-Aa tRNA<sup>Leu</sup> were measured in reaction mixtures containing 100 mM Tris/HCl (pH 7.8), 30 mM KCl, 12 mM MgCl<sub>2</sub>, 0.5 mM DTT (dithiothreitol) and 1 μM [3H]Ile-Aa tRNA<sup>Leu</sup> at 37°C. Reactions were initiated by adding isolated CP1 domain (5 μM) [23]. Hydrolytic activities of different isolated G./LeuRS-CP1 domains towards [3H]Ile-Ec tRNA<sup>Leu</sup> were determined in a reaction mixture containing 60 mM Tris/HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 2 mM DTT and 1 μM [3H]Ile-Ec tRNA<sup>Leu</sup> at 37°C. Reactions were initiated by adding 5 μM wild-type or mutant G./LeuRS-CP1 [24]. Kinetic parameters for deacylation of mischarged Ile-tRNA<sup>Leu</sup> were determined using 1–50 μM [3H]Ile-tRNA<sup>Leu</sup> and 1 μM CP1 domain.

**Docking model of CP1 with tRNA or AN2690**

The docking model of the G./LeuRS-CP1–tRNA and AaLeuRS-CP1–tRNA complexes were constructed based on the structure of the TtLeuRS–tRNA complex (PDB code 2BYT) in which the 3′-end of the tRNA is bound at the editing active site. The TtLeuRS–tRNA model was docked to AaLeuRS-CP1 and G./LeuRS-CP1, based on superposition of the catalytic core of the CP1 domain. In both docking models, the 3′ terminal CCA of the tRNA acceptor arm was well positioned in the editing pocket, without obvious steric conflict with the surrounding residues of the protein.

**RESULTS**

**Crystal structure of AaLeuRS-CP1**

The final model of AaLeuRS-CP1 (PDB code 3PZ0) was refined to 2.4 Å (1 Å = 0.1 nm) with R<sub>work</sub> and R<sub>free</sub> values of 0.217 and 0.274 respectively (Table 1). The overall structure of AaLeuRS-CP1 together with the known structures of the CP1 domain from prokaryotic TtLeuRS and EcLeuRS (PDB codes 1H3N and 2AJG respectively [12,20]) are shown in Figures 1 and 2(A). The structure of AaLeuRS-CP1 is a globular β-barrel surrounded by α-helices (Figure 2A). A distinguishing feature of AaLeuRS-CP1 is the extra insertion motif (coloured magenta in Figure 2A). The structure has a total of eight α-helices and seven β-strands, which include a conserved catalytic core [19] and three insertions. The conserved catalytic core shared by the CP1 domain from LeuRS, ValRS and IleRS is coloured deep blue in Figure 2(A). The secondary structure elements of the conserved catalytic core are in order β1–β2–α1–β3–β4–β5–β6–α2–α3–β7 (Figure 2A). Two additional insertions (pale cyan in Figure 2A) are found beside the catalytic core, designated 12b and 14b, according to a previous report [19]. The I2b insertion is composed of three helices inserted between β3 and β4 of the catalytic core. The I4b insertion is a long loop located between helix α2 and α3. In the I2b insertion, residues 316–328 (corresponding to 289–302 in TtLeuRS and 287–300 in EcLeuRS) are in different conformations compared with those in TtLeuRS, whether free or in complex with tRNA.

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**Table 1 Data collection and refinement statistics**

Values in parentheses are for highest-resolution shell

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The secondary structure elements of AaLeuRS-CP1 and GlLeuRS-CP1 are presented, the strictly conserved residues are boxed with a black background. The insertion motifs of AaLeuRS-CP1 and GlLeuRS-CP1 are marked out in a grey background. The T-rich region and the ‘GTG loop’ are marked as T-rich and GTG respectively, and the conserved aspartate residue is indicated with a star.

A.a. LeuRS, AaLeuRS; T.t. LeuRS, TtLeuRS (PDB code 1OBC [21]); E.c. LeuRS, EcLeuRS (PDB code 1AJH); H.s. LeuRS, Homo sapiens cytosolic LeuRS (PDB code 2WFD); C.a. LeuRS, C. albicans cytosolic LeuRS (PDB code 2WFE); P.h. LeuRS, Pyrococcus horikoshii LeuRS (PDB code 1WKB); and G.l. LeuRS, GlLeuRS. aa, amino acid.

[12,17]. The I2b insertion contacts the 3′ end of tRNA in the post-transfer-editing conformation [17]. The editing active site of AaLeuRS-CP1 (Figure 2B) is located at the bottom of the catalytic main domain, whereas the 20-amino-acid insertion motif is on the top of the main domain. The conserved editing active site is composed of the T-rich region, the ‘GTG loop’ and the strictly conserved Asp373 (indicated by a star in the sequence alignment of Figure 1). When superimposed with the structure of TtLeuRS-CP1 (PDB code 1OBC [21]) bound with the post-transfer editing analogue Nva2AA [17], the conformations of the editing active residues in AaLeuRS-CP1 are extremely consistent with those in TtLeuRS (Figure 2B). Between strands β2 and β3 is found the T-rich region (Figure 2A). Usually, the sequence is ‘TRPDT’ in prokaryotic LeuRSs and ‘TLRPET’ in eukaryotic LeuRSs. The first strictly conserved threonine residue (Thr268) is located at the end of strand β2; it stabilizes the catalytic intermediate and is critical for editing activity (Figure 2B). The other conserved threonine residue (Thr373) is equivalent to Thr345 in EcLeuRS and is located in the small helix between strands β2 and β3 (Figure 2B); it controls the allowed size of the amino acid side chain [35,36]. The side chain of Arg270 forms a salt bridge with Asp272. Arg270 is conserved in every LeuRS and the residue at 272 is always an amino acid with a negative charge, suggesting that the salt bridge should exist in other LeuRS structures, where it could contribute in some way to the conformation and editing activity of the T-rich region. The conserved catalytic Asp373 (Asp347 in TtLeuRS and Asp345 in EcLeuRS, marked with a star in Figure 1), which interacts with the editing substrate (Figure 2B), is in the middle of helix α2. The position of this aspartate residue is extremely conserved in all of the known LeuRS structures [37] and its mutation to alanine abolishes the editing activity of the CP1 domain. The ‘GTG loop’, involved in binding of Ade76 from tRNA, is located between strands β5 and β6 (Figure 2A). The ‘GTG loop’ is located in a flexible region (Figure 2B) as suggested by the different conformations observed in the structures of various LeuRSs.

The secondary structure elements of the conserved catalytic core and the conformation of the active sites in AaLeuRS-CP1 are strictly conserved with those in EcLeuRS-CP1 and TtLeuRS-CP1. The major structural difference in AaLeuRS-CP1 is the presence of a 20 amino acid insertion motif which is between strand β1 and β2 of the catalytic core (Figure 2A) and is essential for editing. This peripheral insertion motif comprises two short helices and a long loop is located on the opposite side of the proteins from the active-site entrance. The average temperature factors (B-factor) of this insertion motif are much higher than the main domain of CP1, indicating that the 20 amino acid insertion is a more flexible region.

**Crystal structure of GlLeuRS-CP1**

To extend our investigation of the CP1 insertion motif, a similar study was performed on GlLeuRS-CP1, which exhibits a comparable, but larger, insertion of 59 amino acids [24]. The crystal belongs to the space group C2. Six protein molecules were
identified in the asymmetric unit. The final structure (PDB code 3PZ6) was refined against 2.6 Å resolution data to a crystallographic R_{work} = 0.215 and R_{free} = 0.271 respectively. The present model covers amino acid residues 259–557. The 59-amino-acid insertion partially lacked electron density, suggesting a flexible conformation. The six protein molecules could be divided into two groups according to the conformation of the 59-amino-acid insertion. In form A, residues 281–296 in the 59-amino-acid insertion were missing in the electron density map. In form B, up to 30 residues from 276 to 305 were totally invisible (GICP1_B in Figure 2C).

The ribbon diagram of GlLeuRS-CP1 is shown in Figure 2(C). Form A has dimensions of 49 Å × 40 Å × 61 Å and is composed of the main domain with the conserved catalytic core and the flexible 59-amino-acid insertion motif (in magenta). The structure of the main domain is quite similar to those of other known eukaryotic and archaean LeuRSs (Figure 2C; PDB codes 2WFD, 2WFE and 1WKB [18,19]); despite their rather low sequence similarity (GlLeuRS-CP1 shares 38%, 33% and 29% sequence identity with the CP1 domain of human cytosolic, C. albicans cytosolic and Pyrococcus horikoshii LeuRS respectively). It is composed of a conserved catalytic core (in deep blue) and four specific insertions. The topology of the catalytic core is very similar to the AaLeuRS-CP1 structure depicted above with the secondary structure ordered β1-β2-α1-β3-β4-β5-β6-α2-α3-β7 (Figures 1 and 2C). Important elements of the active site are also conserved and are highlighted in red in Figure 2(C) and identified below the sequence in Figure 1. In GlLeuRS-CP1 the 59-amino-acid insertion occurs between the catalytic core strands β1 and β2. This corresponds to I1ae in other eukaryotic LeuRS-CP1 (as depicted in Figure 2C; I1ae is a helix in HcLeuRS and CaLeuRS and a loop in PhLeuRS). The structures of the three other eukaryotic specific insertions (I2ae, I3ae and I4ae; coloured pink in Figure 2C) are well conserved between GlLeuRS-CP1 and human cytosolic and CaLeuRS-CP1. I2ae occurs between the catalytic core strands β3 and β4 and is a 48-residue motif formed as β-β-α-β-α. In GlLeuRS-CP1, I3ae is found after the conserved catalytic core helix α2. I4ae is inserted before the conserved catalytic core helix α3, and comprises two long α-helices and a loop. The loop connecting the two helices is partly invisible in the electron-density map (Figure 2C). The second α-helix is pointing towards the active site, which makes the active site pocket smaller than in prokaryotic LeuRS-CP1. This was also observed for eukaryotic HcLeuRS and CaLeuRS [19]. Moreover, the large side chain of residue Tyr515 in this helix (Supplementary Figure S1 at http://www.BiochemJ.org/bj/440/bj4400217add.htm) reduces the size of the active-site pocket of GlLeuRS-CP1 relative to pockets in HcLeuRS-CP1 and CaLeuRS-CP1.

The 59-amino-acid insertion which confers editing activity is an isolated motif stretching out from the main domain. It is inserted between strands β1 and β2 of the catalytic core and in this respect is similar to the 20-amino-acid motif in AaLeuRS-CP1. The peripheral orientation of the 59-amino-acid insertion motif is also similar to that observed in AaLeuRS-CP1. In form A GlLeuRS-CP1, the visible part of the 59-amino-acid insertion is a 26 Å long hook that is almost comparable in length with
the catalytic main domain, whereas in form B GI/LeuRS-CP1, up to 30 amino acids of this insertion are invisible, suggesting a flexible conformation. In summary, both structures of AaLeuRS-CP1 and GI/LeuRS-CP1 are composed of a main catalytic domain on which an appended flexible insertion motif is observed. The insertion motif, which is required for editing activity, is located on the opposite side of the protein from the active-site entrance.

The peripheral insertion motif is crucial for editing in CP1 and does not change the structure of the catalytic domain

It was proposed that the unique insertion may play a role in stabilizing the whole CP1 structure [23,24]; however, the crystal structures of AaLeuRS-CP1 and GI/LeuRS-CP1 show that this unique insertion motif is peripherally oriented and not part of the editing catalytic core. To exclude the possible influence of flanking sequences on the editing activity of the previously long isolated CP1 domain from AaLeuRS (residues 125–456) [23], we tested the deacylation activity of the minimal AaLeuRS-CP1 (residues 228–439) used in the present study. Although the two Zn$^{2+}$-binding motifs were removed, the minimalist AaLeuRS-CP1 (residues 228–439) still possessed the ability to hydrolyse mis-charged Ile-tRNA$^{Leu}$ (Figure 3A), suggesting that editing activity is an intrinsic property of the AaLeuRS CP1 domain and is not conferred by extra appended flanking sequences, as in EcLeuRS [25]. When the idiosyncratic 20-amino-acid insertion was deleted from AaLeuRS-CP1 (hereafter named AaLeuRS-CP1Δ20), mis-acylated Ile-tRNA$^{Leu}$ could not be deacylated (Figure 3A), consistent with the previous result of Zhao et al. [23] that the 20-amino-acid insertion controls the editing ability of the isolated CP1 domain.

To further understand why removal of the 20-amino-acid insertion abolished editing activity, we determined the structure of AaLeuRS-CP1Δ20. The crystal structure of AaLeuRS-CP1Δ20 was refined to 2.5 Å with an R$_{work}$ of 0.215 and an R$_{free}$ of 0.266 (PDB code 3PZ5). Two highly similar AaLeuRS-CP1Δ20 protein molecules were identified in the asymmetric unit, with an RMSD (root mean square deviation) of 0.17 Å when all Ca atoms were superimposed. The overall structure of AaLeuRS-CP1Δ20 is very similar to the available CP1 structures from EcLeuRS and TtLeuRS (PDB codes 1H3N and 2AJG) (Figure 3B), and almost identical with the main domain of AaLeuRS-CP1. Interestingly, when the structure of AaLeuRS-CP1Δ20 was superimposed on that of AaLeuRS-CP1 (Figure 3C) it appeared that deletion of the insertion induced only minor changes in the structure. The AaLeuRS-CP1Δ20 almost overlapped with the main domain of AaLeuRS-CP1 with an RMSD of 0.72Å when all Ca atoms were superimposed. The orientation of key residues associated with post-transfer editing, including the T-rich region, Asp$^{275}$ and the GTG loop (Figures 3B and 3C), were coincident with those found in the entire AaLeuRS CP1 domain. This might explain why deletion of this 20-amino-acid insertion in the native full-length enzyme did not affect its post-transfer editing ability [23].

Taken together, the results show that deletion of the 20-amino-acid insertion abolishes the editing activity of the isolated domain even though it does not alter the structure of the CP1 domain. In fact, deletion of the 20-amino-acid insertion induces no structural change in the AaLeuRS-CP1 catalytic core, suggesting that a probable role for this motif is to work as an independent module that controls the catalytic function through some unexplained regulatory mechanism.

The insertion motif does not directly interact with tRNA in modelling

It was hypothesized that the 20-amino-acid insertion within AaLeuRS, which is rich in lysine and arginine residues, might stabilize interactions between the isolated CP1 domain and the mischarged tRNA$^{Leu}$ substrate to further modulate editing activity [23]. To check whether the insertion peptide might directly interact with tRNA substrates, the structures of AaLeuRS-CP1 and GI/LeuRS-CP1 were docked to the existing TtLeuRS–tRNA complex with the 3′-end of tRNA in the CP1 domain in a post-transfer editing conformation (PDB code 2BYT [24]). The resulting model of AaLeuRS-CP1 with tRNA (Figure 4A) shows that the 3′-end of tRNA$^{Leu}$ interacts with Phe$^{267}$, Thr$^{268}$, Thr$^{269}$ and Arg$^{270}$ in the T-rich region and with residues in the GTG loop region. Similar interactions were described in the TtLeuRS–tRNA complex [17]. In the AaLeuRS-CP1–tRNA model, nucleotides Cyt74 and Cyt75 are at bonding distance of residues Thr$^{267}$, Arg$^{272}$, Thr$^{273}$, Met$^{274}$, Glu$^{323}$ and Lys$^{326}$ in the 12b region. In the docking model, the 20-amino-acid insertion is too far from the tRNA to be able to directly interact with it. In the model of GILeuRS-CP1 with tRNA$^{Leu}$, Ade76 could also interact with the T-rich region and the GTG loop, and Cyt74 and Cyt75 could interact with the flexible α-helix of the I4ae region (Figure 4B). As in AaLeuRS-CP1, the visible part of the 59-amino-acid insertion within GILeuRS-CP1 is far from tRNA.

Collectively, the docking models of AaLeuRS-CP1 and GI/LeuRS-CP1 with tRNA$^{Leu}$ show that Ade76 may interact with the conserved T-rich region and the GTG loop region in the editing core. Additional interactions could exist between Cyt74 and Cyt75 of the tRNA 3′-end and the 12b and 14ae regions in the AaLeuRS and GI/LeuRS CP1 domains respectively. The docking models clearly exclude the possibility that the tRNA interacts directly with the insertion motif in either the AaLeuRS or GI/LeuRS CP1 domains in the structure captured in crystals.

Replacement of the unique insertion with some unrelated sequences preserves editing activity

The insertion motifs of AaLeuRS-CP1 and GI/LeuRS-CP1 exhibit inherent flexibility, in particular, the long 59-amino-acid loop in
**Figure 4** Docking models and editing activity of insertion-substituted AaLeuRS-CP1s

(A) Docking model of AaLeuRS-CP1 with tRNA, the model was performed using the structure of TcLeuRS-tRNA (PDB code 2BYT). (B) Docking model of GtLeuRS-CP1 with tRNA. Possible interaction sites are coloured orange and red (within the active site) and the insertion motif is coloured magenta. (C) Decacylation of 1 μM Ile-ectRNA[AGAG] by 5 μM AaLeuRS-CP1 (○) or mutated derivatives: AaLeuRS-CP1Δ20/Δ20/GST (○); AaLeuRS-CP1Δ20/Δ20/GtLeuRS59Aa (△); AaLeuRS-CP1Δ20/Δ20/AGAG (■); or AaLeuRS-CP1Δ20/Δ20/2xAGAG (□), with AaLeuRS-CP1Δ20 (●) as a negative control.

Gl/GtLeuRS-CP1 appears to be very flexible and bears several basic residues in the middle. Hence, it still remains a possibility that insertion motifs could contact the aminoacyl-tRNA substrate in conformations different from those captured by crystal packing to regulate editing activity. We subsequently cloned different insertions into AaLeuRS-CP1Δ20, including the 59-amino-acid insertion from Gl/GtLeuRS, the simple tetrapeptide AGAG insertion composed of small and flexible alanine/glycine residues, the octapeptide insertion of two AGAG repeats, and even an unrelated fusion protein GST. The editing activity is showing in Figure 4(C). All of the insertion mutants into AaLeuRS-CP1Δ20 exhibited editing activity, and the activities were even slightly higher than that of wild-type AaLeuRS-CP1 (Figure 4C). The results suggest that all of the insertions introduced, even the simple AGAG insertion, could functionally compensate the original 20-amino-acid insertion of AaLeuRS-CP1 in the editing activity. As the AGAG insertion or the GST protein did not contain an element which could assist in binding the non-cognate aminoacyl-tRNA substrates, the possibility of these insertions directly contacting substrates to further modulate the editing activity was excluded. The observation that unrelated peptides can impart editing activity to the isolated CP1 suggests that they may modify the intrinsic dynamics of the CP1 domain, thereby modulating the editing activity.

**Molecular dynamics simulations of AaLeuRS-CP1 and AaLeuRS-CP1Δ20**

To investigate the protein dynamics of the CP1 domain, a molecular dynamics simulation was implemented. We carried out the simulation for AaLeuRS-CP1 and AaLeuRS-CP1Δ20 with GROMACS 4.0.7 [38] employing OPLS-AA all-atom force field [39]. We ran the simulation from our crystal structures for 20 ns after minimization. The results (Figure 5A) of an all atom RMSD show that the AaLeuRS-CP1 possesses much more fluctuant RMSD than AaLeuRS-CP1Δ20, which suggests that the AaLeuRS-CP1 is more dynamic than its insertion deletion. Root mean square fluctuations (http://manual.gromacs.org/online/g_rmsf.html) of the last 2 ns of simulation provide information on the equilibrium states on this trajectory. These fluctuations were converted into the thermal B-factors (the Debye–Waller factor). The average all atom and Ca B-factors of the catalytic site including the T-rich region, GTG loop and Asp73 are listed in Figure 5(B). The results show that the B-factors of all the three catalytic parts of AaLeuRS-CP1 are higher than those in AaLeuRS-CP1Δ20, which suggests a higher flexibility of the catalytic site when CP1 contains the insertion motif. Based on the sum of these results, we hypothesize that the insertion motifs increase the dynamics of the CP1 domain thereby modulating editing.

Proline mutation intended to reduce protein flexibility decreases the editing activity of the isolated CP1

According to the CP1 structures, the insertion motif is connected to the T-rich region, the key element for post-transfer editing, through a conserved β-strand (β2 from Ile266 to Thr268 in AaLeuRS-CP1, β2 from Val330 to Thr336 in Gl/GtLeuRS-CP1; Figures 1 and 2). This connective strand was chosen to modify the protein flexibility of the CP1 domain to test our hypothesis. In AaLeuRS-CP1, residues Ile266 and Val268 were separately substituted by proline residues in order to restrict movement of the β-strand linker. The I264P mutation, located before the middle of the β-sheet, preserved approximately half of the editing activity of native AaLeuRS-CP1 (Figure 6A). The V266P mutation exhibited a more severely decreased post-transfer editing capability (Figure 6A). The results described above suggest that the closer the substitution of proline to the active site, the greater the effect on post-transfer editing. The combination of I264P and V266P abolished deacylation activity with mischarged tRNA (Figure 6A). We also performed alanine

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substitution for Ile\textsuperscript{264} and Val\textsuperscript{266}. Isoalted CP1 mutants of I264A, V266A and I264A/V266A were constructed and tested; however, their hydrolytic activities were the same as wild-type CP1 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/440/bj4400217add.htm). Since proline substitutions are known to provide rigidity to protein, these data indicate that the structural flexibility of the \(\beta\)-strand, but not the side chain of the amino acid residues at 264 and 266, modulates post-transfer editing of AaLeuRS-CP1.

We also performed a similar mutational analysis on the homologous \(\beta\)-strand of the isolated GlLeuRS-CP1. Similarly, mutants of the isolated GlLeuRS-CP1 such as L333P and L333P/A335P significantly reduced or abolished post-transfer editing activity (Figure 6B), whereas an L333A mutation increased editing activity (Figure 6B). To exclude the possibility that the proline mutations were disrupting the folding of the CP1 domains (which could explain the loss of editing activity), the CP1 mutants were examined by CD spectroscopy. There were no noticeable differences of the CD spectra between the proline mutants and the corresponding wild-type CP1, indicating that the CP1 mutants retained the original structure of wild-type CP1 (Supplementary Figure S3 at http://www.BiochemJ.org/bj/440/bj4400217add.htm).

In summary, proline, but not alanine, mutations in both AaLeuRS-CP1 and GlLeuRS-CP1 reduce the editing activity, suggesting that the flexibility of the connective \(\beta\)-strand linker connecting the insertion motif to the editing active site plays a pivotal role in post-transfer catalysis by the isolated editing domain. The concomitant loss of protein flexibility and editing activity associated with proline substitution is consistent with our former hypothesis.

Recovery of the deacylation activity by glycine substitution mutants

We hypothesize that protein dynamics is important for editing by isolated CP1. If this is true, it is possible that the CP1 domain without insertion could regain editing activity by introduction of protein flexibility. Since structural flexibility of the connective \(\beta\)-strand was shown to be important for catalysis by both CP1s, we introduced glycine substitutions into this strand of inactive AaLeuRS-CP1\(\Delta 20\). Editing assays showed that AaLeuRS-CP1\(\Delta 20\) with a V266G or F267G mutation could deacylate Ile-tRNA\textsuperscript{Leu} with a catalytic efficiency comparable with the wild-type AaLeuRS-CP1 (Figure 6C). The data show that flexibility attributable to a glycine residue in the \(\beta\)-strand can lead to recovery of editing activity by AaLeuRS-CP1\(\Delta 20\), further confirming that flexibility of the connective \(\beta\)-strand to the T-rich region is important for catalytic activity of the CP1 domain. Kinetic studies showed that \(K_m\) and \(k_{cat}\) values for mis-charged Ile-tRNA\textsuperscript{Leu} by AaLeuRS-CP1\(\Delta 20\) and this is consistent with the former proline mutational analyses intended to reduce flexibility in AaLeuRS-CP1. The results further support our hypothesis that the insertion motifs provide dynamics to the CP1 domain and modulate its editing activity.

DISCUSSION

The insertion motif modulates the editing activity of CP1 without changing its structure

The CP1 domains from AaLeuRS and GlLeuRS were previously isolated as active domains [23,24]. The editing activity was crucially dependent on an insertion motif found in the two isolated domains. To understand its structural basis, we solved crystal structures of both CP1 domains. The overall structure of AaLeuRS-CP1 is composed of a catalytic main domain which is very similar to other known prokaryotic LeuRS-CP1 domains, and a flexible 20-amino-acid insertion motif, located on the opposite side from the active site entrance. This motif is essential...
for the editing activity of *AaLeuRS-CP1* [23]. Its structure is more flexible than the main domain, as indicated by a higher average B factor. The main domain structure of *GlLeuRS-CP1* exhibits a typical eukaryotic-like CP1 structure. Similarly, in *GlLeuRS-CP1*, the 59-amino-acid motif is inserted at the same position in the editing core. The insertion motif in *GlLeuRS-CP1* is extremely flexible and only partially visible in the electron-density map. In both structures, the 20-amino-acid and 59-amino-acid insertions are peripherally oriented discrete motifs and do not modify the topology of the catalytic main domain when compared with other structurally known CP1 domains. These data suggest that the insertion motif may work as an independent element controlling the hydrolytic function rather than modulating the overall conformation of the CP1 domain. Therefore, to investigate the role of the insertion motif, the structure of the deletion mutant *AaLeuRS-CP1Δ20* was solved. Although the isolated CP1 domain devoid of the 20-amino-acid motif is completely inactive in editing, its structure is nearly identical with the main domain of *AaLeuRS-CP1*. Taken together, the structural and enzymatic properties of the three CP1 structures suggest that the insertion motif is a catalysis modulator element of the CP1 domain. Importantly, both insertion motifs of *AaLeuRS-CP1* and *GlLeuRS-CP1* are inserted at the same position of the catalytic core and both are connected to the T-rich region through a flexible β-strand.

**Peripheral insertion motifs modulate the editing activity without interacting with the substrates**

By docking tRNA to the CP1 structures, we excluded the possibility of a direct interaction between the insertion motif and the tRNA substrate. However, it remains a possibility that the flexible insertion motifs interact with the editing substrates in conformations distinct from those captured in the crystals. We further showed that the loss of editing from the 20-amino-acid deletion could be restored by several alternative sequences, including the 59-amino-acid insertion from *GlLeuRS*, simple tetra- and octa-peptides bearing alanine and glycine, and even the protein GST. The AG peptides or the GST protein are unlikely to bind the mischarged tRNA substrates, therefore we exclude direct contact with substrates as a mechanism for modulating editing activity. The ability of unrelated sequences to impart editing activity to isolated CP1 suggests that their presence alters the overall structure or dynamics of the CP1 domain. The hydrolysis of the correctly charged Leu-tRNA<sub>Lew</sub> by wild-type CP1s and all their mutants used in the present study was checked; the results showed that none of them could deacetylate Leu-tRNA<sub>Lew</sub> (Supplementary Figure S4 at http://www.BiochemJ.org/bj/440/bj4400217add.htm), suggesting that the peripheral insertions in CP1s do not affect the specificity of enzyme for substrate.

**The peripheral insertion motifs may accelerate catalysis by increasing protein dynamics**

Individual proteins are in constant motion rather than static, and dynamic proteins play important roles in biological processes, such as signal transduction, protein biological interactions or enzyme catalysis [40]. In a way, the functions of proteins are governed by their dynamic character, and it has been shown that the intrinsic plasticity of some enzymes is a key element for catalysis [41]. Interestingly, previous studies suggest that protein motion is not only localized to the active site but also to a wider dynamic network [42]. In the present study, the flexible insertion motifs which do not directly interact with substrates could modulate the editing activity of the isolated CP1 without changing the structure of the catalytic core. Molecular dynamics simulations suggest that the insertion motif could make the CP1 domain, and in particular the active site, more dynamic. Therefore it is possible that the flexible insertions enhance the editing activity of the active site by increasing the plasticity of the overall CP1 domain. We propose that the isolated CP1 domain of *AaLeuRS* and *GlLeuRS* is too rigid to catalyse editing without the insertion motif. Greater dynamic flexibility of the editing active site is observed when specific 20 amino acid or 59 amino acid peptides from *AaLeuRS* and *GlLeuRS* respectively are added to the isolated CP1 domain. Similarly, it was shown that addition of flanking sequences to the minimal CP1 domain could increase the editing activity of the isolated CP1 domain [25]. In both cases, adding flexible peptides (insertion motif in the first case and flanking peptides in the second case) improved editing activity, suggesting that a certain structural plasticity is required to activate editing activity of the CP1 domain. In *AaLeuRS-CP1* and *GlLeuRS-CP1*, the unique insertions were linked to the catalytic T-rich region by a conserved β-strand. To modulate the flexibility of this strand, two residues were substituted with more rigid proline residues. The proline mutations decreased post-transfer editing activity of the *AaLeuRS-CP1* and *GlLeuRS-CP1*, whereas alanine mutations at the same positions did not alter the hydrolytic activity. These results suggest that flexibility of the connective β-strand is an important modulator of the editing activity of *GlLeuRS-CP1* and *AaLeuRS-CP1*. In contrast, when the flexibility of the connective β-strand was increased by selective substitution with glycine residues, editing activity of the *AaLeuRS-CP1Δ20* was recovered. Taken together, the data are consistent with our hypothesis that the insertion motif modulates catalysis by increasing the flexibility of the CP1 domain.

**Insertion motifs may be relics from primitive freestanding editing domains**

Extra domain insertion has happened widely in the 20 aaRSs, which often results in new functions for the enzyme [43]. It has even been suggested that the insertion rate is higher in the aaRS family than in other protein families [44], which makes the investigation of these insertion domains more interesting. Here we have shown that addition of an insertion motif in ancient prokaryotic and eukaryotic LeuRSs can regulate editing activity. These highly dynamic domains may confer increased flexibility to the editing active site so that substrates can be accommodated in their different energetic states.

Remarkably, the insertions studied here are both found in old and primitive organisms, and only in a subset of enzymes. Therefore they may be relics of an ancestor of the CP1 domain which may have existed as a freestanding protein before its insertion into the Rossmann-fold synthetic domain. In this context, the role of the insertion motif could have been crucial to the hydrolytic function of the freestanding protein. The catalytic efficiency of minimalistic *AaLeuRS-CP1* is comparable with the long isolated CP1s [23]; however, it is several hundredfold lower than that of full-length *AaLeuRS* [23]. Deletion of the unique insertion does not cause any decrease in the editing activity of full-length *LeuRS* [23,24] suggesting that the unique insertion is not necessary for editing after the freestanding protein has been inserted into the Rossmann-fold. Following this event the insertion motif may have been secondarily lost. Its trivial role in the editing function of full-length *LeuRS* [23,24] suggests the editing in whole enzyme is a more complex network, depending not only on the CP1 domain, but also on other elements of the enzyme and tRNA.
Solving CP1 structures helps to understand antibiotic resistance mechanism

Previously, the editing domain from *Saccharomyces cerevisiae* LeuRS (termed ScLeuRS) was shown to be the target of the antifungal agent AN2690 [26]. The boron atom of AN2690 forms a covalent bond with the 2- and 3-oxygen atoms of tRNA Ade76 which traps RNA-Leu in the editing site of LeuRS and prevents turnover, thus inhibiting both synthetic and editing activities of LeuRS [26]. AN2690 has a dominant inhibitory effect on a wide range of LeuRSs from prokaryotes and eukaryotes. Interestingly, *G*LeuRS shows complete resistance to AN2690 [45]. As *G. lamblia* is one of the most prevalent parasitic protozoa causing giardiasis disease with high incidence [46], structural investigations on *G*LeuRS-CP1 may be helpful to further design antibiotics against giardiasis. As *G*LeuRS-CP1 has no binding affinity for AN2690, a complex structural investigation could not be performed and the compound was docked on to the *G*LeuRS-CP1 structure, according to the structural data of the TrLeuRS-tRNALeu–AN2690 complex [26]. The docking model shows a sterically trapped 2.2 Å respectively (Supplementary Figure S1). This result suggests that AN2690 cannot enter the editing site of *G*LeuRS-CP1 and/or there is no space for its binding. Indeed, enlarging the editing active site by introducing a D444A mutation induced AN2690 sensitivity, possibly by improving its binding with the CP1 [45]. Conversely, a D444E mutant with a larger amino acid side chain remained resistant to AN2690 [45]. In summary, the structure of the editing domain shows that AN2690 resistance of *G*LeuRS may result from poor binding due to steric clash with the side groups of key amino acid residues in the editing active site.

**AUTHOR CONTRIBUTION**

En-Duo Wang designed the research. Ru-Juan Liu performed the structural work. Min Tan performed the enzymatic activity studies. Dao-Hai Du performed some gene cloning. Bei-Si Xu performed the molecular dynamics simulations. Ru-Juan Liu, Gilbert Eriani and En-Duo Wang analysed the data and wrote the paper.

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**REFERENCES**

Editing by isolated CP1 of LeuRS


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SUPPLEMENTARY ONLINE DATA
Peripheral insertion modulates the editing activity of the isolated CP1
domain of leucyl-tRNA synthetase
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MATERIALS AND METHODS

tRNA substrate preparation
A. aeolicus tRNAleuGAG (Aa tRNAleu) and E. coli tRNAleuGAG (Ec tRNAleu) with accepting activities of 1400 and 1500 pmol/A260 respectively, were prepared from an overproduction strain constructed in our laboratory [1]. Mischarged [3H]Ile-Ec tRNAleu and [3H]Ile-Aa tRNAleu were prepared using the E. coli LeuRS editing-defective Y330D mutant [2].

CD spectroscopy analysis
CP1 protein samples [0.2 mg/ml in 10 mM potassium phosphate buffer (pH 6.8)] were analysed on a Jasco J-715 spectropolarimeter with nitrogen purge at room temperature (25 °C). A 1-mm path-length cuvette was used, and spectra were accumulated over five scans.

Figure S1 Docking model of GiLeuRS-CP1 with AN2690
The model is based on the structure of the TtLeuRS-tRNA–AN2690 complex (PDB code 2V0G) in which the 3′-end of the tRNA is in the editing site (A and B). The side chains of Tyr515 and Arg538 clash with AN2690 at 1.6 Å and 2.2 Å distances respectively. (B) The surface of the GiLeuRS-CP1 active pocket and the steric clash with AN2690.

Figure S2 Deacylation activities of AaLeuRS-CP1 and mutated derivatives
Deacylation of 1 μM Ile-Ec tRNAleu by 5 μM AaLeuRS-CP1 (C) or mutated derivatives AaLeuRS-CP1-I264A ( ), AaLeuRS-CP1-V266A ( ) and AaLeuRS-CP1-I264A/V266A ( ), with no enzyme ( ) as a negative control.

Figure S3 CD spectra of LeuRS-CP1
(A) CD spectra for AaLeuRS-CP1 and its proline mutants. (B) CD spectra for GiLeuRS-CP1 and corresponding proline mutants.

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Figure S4  Hydrolysis of Leu-tRNA<sub>Leu</sub> by CP1s and their mutants

(A) Deacylation of 2 μM Leu-AatRNA<sup>Leu</sup> by 5 μM AaLeuRS-CP1 (●); AaLeuRS-CP1Δ20 (○); AaLeuRS-CP1-I264P (▼); AaLeuRS-CP1-V266P (□) and AaLeuRS-CP1-I264P/V266P (■); and 5 nM EcLeuRS-T252A (▲) as positive control and spontaneous hydrolysis (▽) as a negative control. (B) Hydrolysis of 2 μM Leu-AatRNA<sup>Leu</sup> by 5 μM AaLeuRS-CP1Δ20 relative mutants which rescue its post-transfer editing activity. Different symbols represent spontaneous hydrolysis (●); AaLeuRS-CP1Δ20-V266G (▲) and AaLeuRS-CP1Δ20-T267G (○); AaLeuRS-CP1Δ20/V●GleuRS59Aa (■), /−VAGAG (□), /−V2xAGAG (▲) and /-VGST (▽); and 5 nM EcLeuRS-T252A (○). (C) Hydrolysis of 2 μM Leu-EctRNA<sup>Leu</sup> by 5 μM G1LeuRS-CP1 (○) and mutants derivatives: G1LeuRS-CP1-L333P/A335P (▲); G1LeuRS-CP1-L333P (□) and G1LeuRS-CP1-L333A (▼) with spontaneous hydrolysis (●) as negative control and 5 nM EcLeuRS-T252A (□) as a positive control.

REFERENCES


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