Interdomain communication modulates the tRNA-dependent pre-transfer editing of leucyl-tRNA synthetase

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EcLeuRS [Escherichia coli LeuRS (leucyl-tRNA synthetase)] has evolved both tRNA-dependent pre- and post-transfer editing capabilities to ensure catalytic specificity. Both editing functions rely on the entry of the tRNA CCA tail into the editing domain of the LeuRS enzyme, which, according to X-ray crystal structural studies, leads to a dynamic disordered orientation of the interface between the synthetic and editing domains. The results of the present study show that this tRNA-triggered conformational rearrangement leads to interdomain communication between the editing and synthetic domains through their interface, and this communication mechanism modulates the activity of tRNA-dependent pre-transfer editing. Furthermore, tRNA-dependent editing reaction inhibits misactivating non-cognate amino acids from the synthetic active site. These results also suggested a novel quality control mechanism of EcLeuRS which is achieved through the co-ordination between the synthetic and editing domains.

Key words: domain communication, leucyl-tRNA synthetase (LeuRS), pre-transfer editing, quality control, tRNA.

INTRODUCTION

aaRSs (aminoacyl-tRNA synthetases) establish the genetic code by catalysing the covalent attachment of amino acids to their cognate tRNAs, which harbour the specific triplet anticodon [1,2]. The aminoacylation reaction carried out by aaRS is in two steps. First, the amino acid is condensed with ATP to yield an aa-AMP (aminoacyl-adenylate) intermediate. The aminoacyl moiety is then transferred from AMP to the 3'-end of the cognate tRNA to form aa-tRNA (aminoacylated tRNA) [3]. The 20 aaRSs can be grouped into two classes of 10 members each on the basis of conserved sequences and characteristic structural motifs [4,5].

aaRSs are modular proteins with individual domains exhibiting different functions. It has been proposed that the ancestral aaRSs contained only the catalytic domain responsible for adenylate formation and binding to the primitive tRNAs [6]. Later in evolution, new domains were appended to or inserted into the catalytic core, increasing the efficiency and accuracy of the aminoacylation process [7]. The overall architecture of extant aaRSs consists primarily of two domains. The synthetic domain is responsible for amino acid activation and tRNA aminoacylation, whereas the tRNA-binding domain is responsible for selection and binding of the cognate tRNA [3]. For certain aaRSs, the inherent inability of the active site to discriminate among similar amino acids may lead to misacylation of non-cognate amino acids isosteric to the cognate substrate, thus mischarging tRNA and subsequently incorporating the wrong amino acid at a specific codon during translation [8]. To maintain catalytic fidelity, some aaRSs have recruited an editing domain to eliminate the misacylated products, and this additional error-correcting mechanism is known as editing or proofreading [8,9]. The CP1 (connective peptide 1) domain of LeuRS (leucyl-tRNA synthetase), IleRS (isoleucyl-tRNA synthetase) and ValRS (valyl-tRNA synthetase) is a representative editing domain [10–14]. In addition to the endogenous editing activity carried out by the editing domain of aaRSs, there are a few examples of freestanding editing domains encoded by various genomes [8,15–19]. For instances, YbaK, a homologue of the editing domain of ProRS (proline-tRNA synthetase), clears mischarged tRNApro [15,16]. The widely distributed AlaXp, which is homologous to the AlaRS (alanyl-tRNA synthetase) editing domain, specifically hydrolyses Ser- and Gly-tRNAAla [17,19]. Both endogenous and freestanding editing functions have proven to be essential for cell function and viability [20–23].

Efficient aminoacylation of tRNA by aaRSs requires communication between the tRNA-binding and aminoacylation catalytic domains [24]. The tRNA-binding domain interacts with the anticodon-containing arm of the tRNA, and by an induced-fit mechanism, contacts with the anticodon to activate formation of a robust transition state at the aminoacylation active site at a long distance. This induced-fit-based activation is thought to occur through domain-domain signalling, and such long-range signalling has been well reported for CysRS (cysteinyl-tRNA synthetase), MetRS (methionyl-tRNA synthetase), TyrRS (tyrosyl-tRNA synthetase), ArgRS (arginyl-tRNA synthetase) and GlnRS (glutaminyl-tRNA synthetase) [25–29]. Moreover, there is also evidence in LeuRS and IleRS enzymes that the tRNA-binding domain improves the efficiency and specificity of editing reactions catalysed by the editing domain [11,30,31], indicating that long-range communication occurs between these domains. However, there is no evidence suggesting that a direct communication mechanism exists between the aminoacylation and editing domains, and these two catalytic domains have been thought to function independently.

A recent crystal structure study of EcLeuRS (Escherichia coli LeuRS) reveals that the synthetic and editing domains...
co-ordinate to facilitate the intermolecular translocation of the tRNA CCA tail for the aminoacylation reaction [32]. When the tRNA CCA tail binds to the editing domain, the conformation required for tRNA-dependent pre-transfer editing [33], part of the interface between the synthetic and editing domains showed weak interpretable electron density, indicating that the interface region is in dynamic motion [32,34,35]. Furthermore, a MD (molecular dynamics) simulation study has demonstrated that the two sides of the interface between synthetic and editing domains are in a significant coupling motion [36]. All of these results suggest that the editing and synthetic domains co-operate during tRNA-dependent pre-transfer editing. In the present study, we find that the interface between the editing and synthetic domains is critical for tRNA-dependent pre-transfer editing of EcLeuRS and that mutation within this region can introduce pleiotropic effects on this function. Moreover, when EcLeuRS was maintained in the editing conformation by insertion of the tRNA CCA tail into the editing active site, amino acid activation by the synthetic active site was inhibited. These investigations, together with the previous crystal structure and MD studies, strongly suggest that there is interdomain communication between the editing and synthetic domains through their interface, which modulates tRNA-dependent pre-transfer editing reaction. We also propose a novel quality control mechanism using this interdomain communication mechanism.

EXPERIMENTAL

Preparation of enzymes and RNA substrates

EcLeuRS and its mutants were overproduced in E. coli BL21 (DE3) cells with a N-terminal His6 tag and purified by Ni-NTA (Ni2+–nitrilotriacetic acid) chromatography, as described previously [10]. The genes encoding the various mutations were constructed by using the KOD Plus Mutagenesis kit (Toyobo Life Science). The primer sequences for constructing the mutants were: EcLeuRS-E184R forward, 5′-CGAgaagatccgctggtt-3′ and reverse, 5′-aaacgttgaagagccdgcagagcag-3′; EcLeuRS-E184R forward, 5′-GAaagatccgctggtt-3′ and reverse, 5′-ttaaacgttgaagagccdgcagagcag-3′; and EcLeuRS-G226R forward, 5′-GAaagatccgctggtt-3′ and reverse, 5′-ttaaacgttgaagagccdgcagagcag-3′ (the mutated codons are in capitals). For single-site mutation, the construct containing WT (wild-type) EcLeuRS was used as template. For EcLeuRS-E184R/T252R, the template was the EcLeuRS-T252R construct. All mutants were confirmed by DNA sequencing. E. coli tRNALeu(3′end) (EctRNAleu) with an accepting activity of 1400 pmol/A260 was isolated from an E. coli overproduction strain constructed in our laboratory [37]. Overexpression and purification of EctRNAleu also followed our previous procedures [37]. [3H]Ile-EctRNAleu was obtained using the EcLeuRS-Y330D mutant, which lost tRNA-dependent editing functions [33].

ATP/PPi exchange, RNA charging and deacylation

The amino acid activation of EcLeuRS was measured by an ATP/PPi exchange reaction performed at 37°C in a reaction mixture containing 100 mM Hepes (pH 7.8), 10 mM MgCl2, 10 mM KF, 4 mM ATP, 2 mM [32P]pyrophosphate (PerkinElmer), 5 mM leucine or 15 mM non-cognate amino acid Nva (norvaline), and 10 mM enzyme. AN2690 was a boron-containing antifungal that may be calculated by 

\[I = \frac{[\text{ATP}]_0 - [\text{ATP}]_t}{0.5[I]_0}[\text{ATP}]
\]

where [ATP]0 is the initial concentration of ATP, [ATP]t is the concentration of ATP at time t, and k2 is the pseudo-first-order rate constant for the hydrolysis of aa-AMP. The intercept (t) of the plot of [ATP] against time is defined as 

\[\text{t} = \frac{[\text{ATP}]_0 - [\text{ATP}]_t}{0.5[I]_0}[\text{ATP}]
\]

The incorporation of enzyme was measured as described previously [36]. The amino acid activation of EcLeuRS was assessed at 37°C in a reaction mixture containing 100 mM Tris/HCl (pH 7.8), 30 mM KCl, 12 mM MgCl2, 0.5 mM DTT (dithiothreitol), 4 mM ATP, 20 μM EctRNAleu, 40 μM [3H]leucine (15 Ci/mmol), 0 or 100 μM AN2690, and 20 nM EcLeuRS [10,33]. The misacylation assays were performed in a similar system, except that 40 μM [3H]isoleucine (30 Ci/mmol) and 1 μM enzymes were used. The hydrolytic editing assays of EcLeuRS and the mutants were performed at 37°C in 100 mM Tris/HCl (pH 7.5), 30 mM KCl, 12 mM MgCl2, 0.5 mM DTT and 1 μM [3H]Ile-tRNAleu (300 μCi/μmol), and the reactions were initiated with 5 nM enzyme. For the kinetic assay, the concentration of enzyme was 1 nM with [3H]Ile-tRNAleu ranging from 0.2 to 10 μM.

AMP formation

AMP formation by EcLeuRS was measured as described previously [33]. The reaction mixture contained 100 mM Tris/HCl (pH 7.8), 30 mM KCl, 12 mM MgCl2, 5 mM DTT, 5 units/ml pyrophosphatase (Roche), 3 mM ATP, 20 nM [α-32P]ATP (3000 Ci/mmole, Amersham Bioscience) and 15 mM Na2, in the presence or absence of 5 μM tRNAleu. The reaction was initiated by the addition of 0.2 μM EcLeuRS or its mutants and incubated at 37°C. Aliquots (1.5 μl) were quenched in 6 μl of 200 mM sodium acetate (pH 5.0). Quenched aliquots (1.5 μl each) were spotted in duplicate on PEI-cellulose (polyethyleneimine–cellulose) plates (Merck) prewashed with water. Separation of aa-32PAMP, [32P]AMP and [32P]ATP was performed by developing TLC plates in 0.1 M ammonium acetate and 5% acetic acid. The plates were visualized by phosphorimaging and the data were analysed using Multi Gauge V3.0 software (Fuji Film). The grey densities of the [32P]AMP spots were compared to the grey density of the input [32P]ATP (time 0 in each TLC plate) to calculate the concentrations of generated AMP. The amount of generated AMP was plotted against time to fit a polynomial linear equation. The observed AMP formation rate (kobs) was obtained from the slope of the curve.

Active site titration

The titration was performed according to method of Fersht et al. [39]. In the amino acid activation reaction, when the initial concentration of ATP ([ATP]0) was much higher than that of EcLeuRS ([E]0), the number of moles of aa-AMP/mole of EcLeuRS, that is the number of active sites (n), may be deduced from the following equation: 

\[[\text{ATP}]_0 = [\text{ATP}]_t + n[E]_0 - n[E]_0 k_2 t \]

where [ATP]0 is the concentration of ATP at time t and k2 is the pseudo-first-order rate constant for the hydrolysis of aa-AMP. The intercept (t) of the plot of [ATP] against t is defined as 

\[[\text{ATP}]_0 - [\text{ATP}]_t = n[E]_0 - n[E]_0 k_2 t \]

The titration was performed as follows: 20 μM [γ-32P]ATP (20 μCi/ml), 12 mM MgCl2, 100 mM Hepes (pH 7.5), 1 mM leucine and 10 units/ml yeast inorganic pyrophosphatase were incubated at 37°C. Before adding enzymes, a 15 μl aliquot of the mixture was withdrawn and added into 200 μl of 7% perchloric acid and 8% activated charcoal in water to determine the zero time radioactivity of ATP for calculation of the relationship between [ATP] and the c.p.m. of ATP. The reaction was initiated by adding enzymes to a concentration of 5 μM ([E]0). Aliquots (15 μl) were taken and quenched at various time intervals (t) as described above. The quenched samples were filtered through a Whatman GF/C glass fibre filter, washed and dried. The [γ-32P]ATP adsorbed in the charcoal was counted to obtain the [ATP],

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values. The number of active sites was calculated through the above equation.

**Non-enzymatic hydrolysis of Nva-adenylate**

The rate of non-enzymatic hydrolysis of adenylate was measured by a chase experiment wherein a large excess of unlabelled ATP was added to the reaction mixtures following initiation of Nva–AMP synthesis [40]. The excess of unlabelled ATP induces the release of the non-cognate aa-AMP from the active site into solution where its spontaneous hydrolysis is monitored on TLC plates. A solution of 1 μM EcLeuRS was first incubated with 15 mM Nva and 100 μM [α-32P]ATP for 5 min at 37 °C to prepare Nva–[32P]AMP. Then, unlabelled ATP (120 mM = 1200-fold molar excess of unlabelled ATP) was added and the hydrolysis activity was quenched at various time points (0.5–10 min) by mixing 2 μl of the reaction mixture with 6 μl of 200 mM sodium acetate (pH 5.0) and 0.1% SDS. Separation of Nva–[32P]AMP and [32P]AMP by TLC was then performed and the reactions were quantified as described above. The rate constants for the non-enzymatic hydrolysis reactions were obtained by plotting the concentration of Nva–[32P]AMP against time and fitting the data to a first-order decay curve.

**RESULTS**

**The E184R substitution specifically inhibits tRNA-dependent pre-transfer editing**

The mechanism of tRNA-dependent pre-transfer editing remains controversial [40]. Our previous work showed that this editing function of EcLeuRS depends on the entry of the tRNA 3' terminal into the CP1 domain [33]. In addition, X-ray crystal studies, together with MD simulation research, revealed the synthetic and editing domain were in coupled dynamic motion when the tRNA CCA tail bound the CP1 domain [32,34–36]. On the basis of these findings, we speculated that the interface between the aminoacylation and CP1 domains plays a role in tRNA-dependent pre-transfer editing. A mutagenesis screening was then performed on the interface between the synthetic and editing domains to characterize the key element of this region involving tRNA-dependent pre-transfer editing.

Glu184, a residue within the synthetic domain, is located at the interface between the aminoacylation and CP1 domains (Figure 1). We changed this negatively charged residue to either a small alanine (E184A) or to a positively charged arginine (E184R), and both mutants performed the activities of amino acid activation, aminoacylation and deacylation of mischarged tRNAs as well as the native enzyme (Figures 2A and 2B, and Supplementary Figure S1 at http://www.biochemj.org/bj/449/bj4490123add.htm). These results were consistent with the peripheral location and solvent-exposed orientation of Glu184, far from the active site of both the editing and synthetic domains (Figure 1A). In the TCL-based AMP formation assay, which reflects total editing activity, the E184R substitution significantly decreased tRNA-dependent AMP formation in the presence of Nva and the observed AMP formation rate \( k_{\text{obs}} \) was 1.8 s\(^{-1}\) (Figures 2C and 2D, and Table 1). On the other hand, the \( k_{\text{obs}} \) value of EcLeuRS-E184A was 3.6 s\(^{-1}\) (Figures 2C and 2D, and Table 1), which was similar to that of the WT EcLeuRS \( k_{\text{obs}} = 3.3 \text{ s}^{-1}; \text{Table 1} \). Theoretically, AMP formation in the presence of non-cognate Nva could be affected by the following processes: (i) the formation of aa-tRNA in the synthetic active site; (ii) translocation of aa-tRNA from the synthetic active site to the editing centre; (iii) hydrolysis of aa-tRNA at the editing site; (iv) the spontaneous hydrolysis of aa-AMP after release into solution; and (v) hydrolysis of aa-AMP by the aaRS. Formation of cognate leucyl-tRNA\(^\text{aa}\) also goes through processes (i) and (ii), and EcLeuRS-E184R exhibited no altered aminoacylation activity from the WT enzyme (Figure 2A). This result implied that processes (i) and (ii) were not affected by the E184R substitution. The activity of hydrolysis of mischarged tRNA\(^\text{aa}\) by EcLeuRS-E184R was also not disturbed (Figure 2B and Table 2). The rate for spontaneous hydrolysis of Nva–AMP in reaction buffer was 0.00021 s\(^{-1}\) (Supplementary Figure S2 at http://www.biochemj.org/bj/449/bj4490123add.htm), such a rate was so low that it was negligible compared with total AMP formation. Therefore the reason for the decreasing AMP formation by the E184R substitution was caused by the enzymatic hydrolysis of Nva–AMP. Non-cognate Nva–AMP could be cleared in the absence or presence of tRNA.

Taking into account the fact that E184R mutation had little effect on the tRNA-independent enzymatic hydrolysis of Nva–AMP (Table 1), the E184R replacement specifically inhibited tRNA-dependent hydrolysis of Nva–AMP, which was also termed tRNA-dependent pre-transfer editing.

According to our previous work, tRNA-dependent pre-transfer editing relies on the binding of the tRNA CCA tail into the CP1 domain and the Y330D mutation blocked the entry of tRNA CCA-tail into the editing domain, leading to abolished tRNA-dependent pre-transfer editing and antagonism to AN2690 [33]. We then wanted to know whether the reason for the decreased tRNA-dependent pre-transfer editing by the E184R replacement was due to altered binding of the tRNA CCA tail into the
Table 1  Observed rate constants for AMP synthesis at 37°C for EcLeuRS and its mutants

The rates were determined according to AMP formation in the TLC assay, as described in the Experimental section. All rates are means ± S.D. for three trials.

<table>
<thead>
<tr>
<th>LeuRS</th>
<th>tRNA</th>
<th>AMP formation [k_{obs} (s^{-1})]</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>–</td>
<td>0.32 ± 0.039</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.3 ± 0.21</td>
</tr>
<tr>
<td>E184A</td>
<td>–</td>
<td>0.36 ± 0.052</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.6 ± 0.45</td>
</tr>
<tr>
<td>E184R</td>
<td>–</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.8 ± 0.35</td>
</tr>
<tr>
<td>T252R</td>
<td>–</td>
<td>0.33 ± 0.028</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.5 ± 0.32</td>
</tr>
<tr>
<td>E184R/T252R</td>
<td>–</td>
<td>0.26 ± 0.045</td>
</tr>
<tr>
<td>R185E</td>
<td>–</td>
<td>0.36 ± 0.066</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.28 ± 0.045</td>
</tr>
<tr>
<td>R286E</td>
<td>–</td>
<td>0.31 ± 0.034</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.4 ± 0.5</td>
</tr>
</tbody>
</table>

CP1 domain. Two additional processes also required precise interaction between tRNA CCA tail and the CP1 domain: (i) deacylation of mischarged tRNA; and (ii) trapping the tRNA CCA tail in the CP1 domain by AN2690. The E184R mutation did not alter the activity of hydrolysing mischarged tRNA (Figure 2B and Table 2). In addition, EcLeuRS-E184R retained the same sensitivity to AN2690 as WT EcLeuRS (Supplementary Figure S3 at http://www.biochemj.org/bj/449/bj4490123add.htm). These results suggested that the E1484R replacement did not influence the binding of the tRNA CCA-tail into the CP1 domain, which corresponded to the orientation of Glu^{184}, located in the synthetic domain and far from the tRNA CCA-binding site of the CP1 domain (Figure 1B). We also carried out a comprehensive mutagenesis study of Glu^{184} to investigate its role in tRNA-dependent pre-transfer editing. All of the Glu^{184} mutations, including to leucine, tryptophan, glutamine, histidine, tyrosine and lysine, had little effect on this editing activity (Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490123add.htm), suggesting that the reason for the blocking of the tRNA-dependent pre-transfer editing activity by the E184R substitution was due to the strong positive charge of the arginine residue (pK_{a} = 12.48).

Table 2  Apparent kinetic parameters for hydrolytic activity towards mischarged [3H]Ile-tRNA^{Ile} at 37°C for EcLeuRS and its mutants

Kinetics were determined using the deacylation assay described under the Experimental section in the presence of 0.2–10 μM Ile-tRNA^{Ile}. All rates are means ± S.D. for three trials.

<table>
<thead>
<tr>
<th>LeuRS</th>
<th>K_{m} (μM)</th>
<th>k_{cat} (s^{-1})</th>
<th>k_{cat}/K_{m} (s^{-1}·μM^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.1 ± 0.28</td>
<td>7.0 ± 1.1</td>
<td>3.3</td>
</tr>
<tr>
<td>E184R</td>
<td>2.5 ± 0.38</td>
<td>8.8 ± 0.91</td>
<td>3.5</td>
</tr>
<tr>
<td>R185E</td>
<td>2.0 ± 0.25</td>
<td>7.6 ± 1.2</td>
<td>3.8</td>
</tr>
<tr>
<td>R286E</td>
<td>2.4 ± 0.32</td>
<td>7.4 ± 0.84</td>
<td>3.1</td>
</tr>
</tbody>
</table>

E184R co-operates with T252R to block both tRNA-dependent editing pathways

In our previous work we identified a mutation in the CP1 domain (T252R) that selectively shuts down the post-transfer editing pathway while preserving the tRNA-dependent pre-transfer editing capability [33]. In the present study we engineered an E184R substitution within the synthetic domain, which specifically reduced the tRNA-dependent editing activity (see

Figure 2  Aminoacylation and editing activity of EcLeuRS and its mutants

(A) Aminoacylation of 20 μM EcLeuRNA^{Ile} by 20 nM WT EcLeuRS ( ), 20 nM EcLeuRS-E184A (C) or 20 nM EcLeuRS-E184R ( ). (B) Deacylation of 1 μM Ile-EcLeuRNA^{Ile} by 5 nM EcLeuRS in a pH 7.5 reaction buffer ( ), EcLeuRS-E184A (C) or EcLeuRS-E184R ( ), with no enzyme ( ) as negative control. (C) TLC-based AMP formation assay of EcLeuRS-E184A (right-hand gel) or EcLeuRS-E184R (left-hand gel) in the presence of 5 μM tRNA^{Leu} and 15 mM Nva. (D) The concentration of generated AMP in (C) was plotted against time to fit a polynomial linear equation. The slope of the curve is the k_{obs} value for AMP formation given in Table 1. ( ), EcLeuRS-E184A; ( ), EcLeuRS-E184R. Results are means ± S.D. from three trials.
Figure 3  Enzymatic activities of EcLeuRS-E184R/T252R

The enzymes tested in (A) and (B) were the WT EcLeuRS (●) and EcLeuRS-E184R/T252R (○). (A) Aminoacylation of 20 μM EcRNA218 by 20 nM enzyme. (B) Deacylation of 1 μM Ile-EcRNA218 by 5 μM enzyme and no enzyme control (▼). (C) AMP formation assay of EcLeuRS-E184R/T252R in the absence (left-hand gel) and presence (right-hand gel) of 5 μM RNA218 and 15 mM Nva. (D) The concentration of generated AMP in (C) was plotted against time to fit a polynomial linear equation. The slope of the curve is the $k_{\text{obs}}$ value for AMP formation given in Table 1. ●, Without tRNA; ○, with tRNA. Results are means ± S.D. from three trials.

above). We then combined these two single mutations in a double-site mutant, EcLeuRS-E184R/T252R, and its amino acid activation and tRNA aminoacylation activities were found to be similar to those of EcLeuRS (Supplementary Figure S1 and Figure 3A). As expected, the double mutant could not deacetylate the non-cognate Ile-tRNA218 (Figure 3B) and produced mischarged tRNA218 (Supplementary Figure S4 at http://www.biochemj.org/bj/449/bj4490123add.htm) due to the presence of the T252R substitution [33]. In the TLC-based editing assay with non-cognate Nva, the tRNA-independent observed AMP formation rate ($k_{\text{obs}}$) for EcLeuRS-E184R/T252R was 0.28 s$^{-1}$, which was similar to the rates for the two single-site mutants (0.26 and 0.33 s$^{-1}$ respectively; Figures 3C and 3D, and Table 1). tRNA addition raised $k_{\text{obs}}$ to 0.59 s$^{-1}$ (Figures 3C and 3D, and Table 1), which was much lower than that for either EcLeuRS-E184R ($k_{\text{obs}} = 1.8$ s$^{-1}$) or EcLeuRS-T252R ($k_{\text{obs}} = 1.4$ s$^{-1}$; Table 1). These results showed that E184R and T252R co-operated to eliminate both the tRNA-dependent pre- and post-transfer editing activity of EcLeuRS.

Taken together, both of the tRNA-dependent editing functions (pre- and post-) can be blocked by the combination of two single mutations in different domains, each of which selectively prohibited only one of the two tRNA-dependent editing functions. These results indicated that tRNA-dependent pre- and post-transfer editing were independent processes carried out by different catalytic sites within EcLeuRS.

R185E and R286E replacement significantly enhances tRNA-dependent pre-transfer editing activity

In addition to Glu184 we also characterized two other residues (Arg185 and Arg286) at the interface between the synthetic and CP1 domains, which were also involved in tRNA-dependent pre-transfer editing. Arg185 is in the editing domain, whereas Arg286 is located in the editing domain (Figure 1) [32]. Both residues were replaced by amino acids of opposite charge (glutamic acid). However, neither EcLeuRS-R185E nor EcLeuRS-R286E altered the amino acid activation and deacylation activities towards mischarged tRNAs (Figures 4A and 4B, and Table 2), which was consistent with the peripheral locations of Arg185 and Arg286 far from the active sites of both synthetic and editing domains (Figure 1). On the other hand, both mutants exhibited decreased aminoacylation activity (Figure 4C). More interestingly, the total editing activity measured by the AMP formation of EcLeuRS-R185E and EcLeuRS-R286E was significantly increased. The tRNA-dependent $k_{\text{cat}}$ values of EcLeuRS-R185E and EcLeuRS-R286E were 8.2 and 6.4 s$^{-1}$ respectively (Figure 4D and Table 1), which were higher than that of the WT EcLeuRS ($k_{\text{cat}} = 3.3$ s$^{-1}$; Table 1). tRNA stimulated both the tRNA-dependent pre- and the post-transfer editing pathway to rapidly accumulate AMP in the presence of non-cognate Nva. The post-transfer editing cycle included the formation and the consecutive deacylation of misacylated tRNA, and this editing pathway increased the $k_{\text{obs}}$ value of AMP accumulation by 1.9 s$^{-1}$ in the TLC-based editing assay for the WT EcLeuRS [33]. Steady-state kinetic studies showed that the $k_{\text{cat}}$ value for hydrolysing mischarged tRNA by EcLeuRS was 7.0 s$^{-1}$ (Table 2), which was far beyond the AMP formation rate by the post-transfer editing pathway. These results implied that the rate-limiting step for AMP accumulation due to post-transfer editing was tRNA mischarging. Both EcLeuRS-R185E and EcLeuRS-R286E decreased the aminoacylation efficiency, which, theoretically, should equivalently reduce tRNA mischarging. This in turn should result in a declined contribution of post-transfer editing to cumulative AMP production. Therefore both EcLeuRS-R185E and EcLeuRS-R286E significantly increased tRNA-dependent pre-transfer editing, leading to the enhanced total editing activity observed. WT EcLeuRS could efficiently misactivate non-cognate Nva with a similar $k_{\text{cat}}$ value to that for cognate leucine (Supplementary Table S2 at http://www.biochemj.org/bj/449/bj4490123add.htm). This rate (63.1 s$^{-1}$; Supplementary Table S2) was much higher than that of the AMP formation by pre-transfer editing (approximately 1.2 s$^{-1}$). Therefore the limiting step in producing AMP by pre-transfer editing was the hydrolysis of Nva–AMP. We proposed that R185E or R286E replacement accelerated the hydrolysis of Nva–AMP through an unknown mechanism. In addition, both EcLeuRS-R185E and EcLeuRS-R286E exhibited no increased hydrolysing mischarged tRNA activity (Figure 4B).
and Table 2) and preserved the same sensitivity to AN2690 as the WT enzyme (Supplementary Figure S3). We propose that the reason for the increased tRNA-dependent pre-transfer editing by these mutants was not due to affecting the binding of the tRNA CCA end into the CP1 domain.

Two mutations, located on different sides of the interface between the synthetic and CP1 domains, brought about similar effects on tRNA-dependent pre-transfer editing. These results confirmed the hypothesis that the interface between the synthetic and editing domains played a critical role in tRNA-dependent pre-transfer editing. As all of these perturbing mutations were peripherally oriented and far from the catalytic active sites (Figure 1A), we speculate that the molecular basis by which they modulated tRNA-dependent pre-transfer editing was through interdomain communication between the synthetic and editing domains.

**The editing reaction inhibits amino acid activation by the synthetic active site**

The mutagenesis studies described above suggested an interdomain communication between the synthetic and editing domains of EcLeuRS. We therefore investigated this interdomain communication in the WT enzyme by adding uncharged tRNA\textsubscript{Leu}\textsuperscript{tRNA}\textsubscript{tRNA} to the amino acid activation assay. The activity of activating cognate leucine in the presence of tRNA was about 80% of that without tRNA (Figures 5A and 5C). However, misactivation of non-cognate Nva decreased approximately 60% in the presence of tRNA, compared with that in the absence of tRNA (Figures 5B and 5C). When both Nva and tRNA were present, the WT EcLeuRS could activate both tRNA-dependent pre- and post-transfer editing to guarantee catalytic fidelity. However, tRNA only stimulated the aminoacylation reaction when the amino acid substrate was the cognate leucine. These results indicated that the editing reaction that occurs inhibited amino acid activation activity by the active site of the synthetic domain.

Two possibilities remained that might explain the inhibition of Nva activation by the tRNA-dependent editing reaction. First, the active site for tRNA-dependent pre-transfer editing could overlap with the synthetic active site. In this situation, the synthetic active site was recruited to perform pre-transfer editing in the presence of both tRNA and Nva. As a consequence, fewer Nva molecules would be activated. Alternatively, the editing reaction could lead to intramolecular signalling that modulates the synthetic active site. To distinguish between these two possibilities, AN2690 was introduced to maintain WT EcLeuRS in the editing conformation. Previous research had shown that AN2690 can trap the tRNA CCA tail within the CP1 domain [38], thereby mimicking the tRNA-dependent editing conformation [33]. In addition, AN2690 can selectively block the post-transfer editing capability of the WT EcLeuRS, while having little effect on tRNA-dependent pre-transfer editing [33]. AN2690 itself had no effect on amino acid activation (Supplementary Figure S5 at http://www.biochemj.org/bj/449/bj4490123add.htm). However, in the presence of both tRNA and AN2690, both leucine activation and Nva misactivation were severely inhibited, with a decrease of 2- and 4-fold respectively (Figure 5). Considering the fact that the WT EcLeuRS could not edit its cognate leucine, these results strongly supported the hypothesis that the editing reaction triggered an intramolecular signalling mechanism modulating the synthetic active site, which was dependent on the entry of the tRNA CCA tail into the CP1 domain. The fact that Nva misactivation was more sensitive to AN2690 (Figure 5) also raised the possibility that tRNA-dependent pre-transfer editing occurred within the aminoacylation activation domain, perhaps overlapping with the catalytic site.

**DISCUSSION**

**Communication between the aminoacylation and CP1 domains modulates tRNA-dependent pre-transfer editing**

The pre-transfer editing reaction, which hydrolyses the misaminoacyl-adenylate intermediate, can be carried out by LeuRS in the absence of tRNA, and the synthetic domain
A novel quality control mechanism of leucyl-tRNA synthetase

was thought to be responsible for this reaction [41,42]. tRNA stimulates this editing activity, which is therefore termed ‘tRNA-dependent pre-transfer editing’. However, the mechanism underlying this tRNA-triggered process remains controversial [40]. Our previous results show that tRNA-dependent pre-transfer editing relies on the entry of the tRNA CCA tail into the editing domain [33], which, according to crystal structure and MD simulation studies, brings the interface of the synthetic and CP1 domains into coupled motion [32,36]. All of these findings indicate that the interface between the CP1 and synthetic domains is involved in tRNA-dependent pre-transfer editing.

In the present study, after a mutagenesis screening within the interface between the synthetic and editing domains, we identified residue Glu184 within this region as being involved in tRNA-dependent pre-transfer editing. Substitution of Glu184 with the strongest positively charged residue, arginine (p\(K_a\) = 12.48), selectively inhibits tRNA-dependent pre-transfer editing without affecting other catalytic processes (Figure 2 and Table 2). However, mutations of this residue to other amino acids, including lysine which also possesses a positive-charged side chain (p\(K_a\) = 10.53), have little effect on tRNA-dependent pre-transfer editing (Supplementary Table 1), indicating that Glu184 itself is not involved in the active core of this function. As tRNA-dependent pre-transfer is a tRNA-triggered process, the enzyme must undergo conformational rearrangement in order to rapidly hydrolyse the non-cognate aa-AMP. In addition, crystal structures of EcLeuRS have revealed that the Glu184-containing region exhibits dynamic movement in the editing conformation, resulting in weak uninterpretable electron density [32]. We propose that the E184R mutation perturbs the conformational rearrangement required for tRNA-dependent pre-transfer editing through its strongly positive electric charge, leading to an inhibited tRNA-dependent pre-transfer editing activity. Moreover, two mutations (R185E and R286E) located on either side of the interface between the synthetic and editing domains, increase the total editing activity by enhancing tRNA-dependent pre-transfer editing (Figure 4D and Table 2). To our knowledge, this is the first demonstration that mutations can enhance the pre-transfer editing activity of an aaRS. Also, an active site titration assay showed that the altered editing activity of mutants tested was not due to the different concentration of active enzyme (Supplementary Figure S6 at http://www.biochemj.org/bj/449/bj4490123add.htm).

The fact that mutations within the interface between the CP1 and synthetic domains can induce pleiotropic effects on tRNA-dependent pre-transfer editing activity strongly supports our initial hypothesis that there is interdomain communication between the CP1 and synthetic domains during tRNA-dependent pre-transfer editing that modulates this activity. In addition to mutagenesis studies, when using AN290 to trap the tRNA CCA tail within the editing domain, which forces the enzyme to mimic the editing conformation, amino acid activation by the synthetic active site was found to be severely inhibited (Figure 5). This result suggests that the interaction between the CP1 and aminoacylation domains mediated by tRNA triggers an intramolecular signalling mechanism regulating the activity of the synthetic active site. Moreover, this hypothesis is confirmed by the recent crystal structure study of EcLeuRS, which revealed that the binding of the tRNA CCA tail into the CP1 domain leads to the synthetic catalytic site being in an open relaxed conformation even in the presence of leucine or an LeuAMS analogue of Leu-AMP [32]. We also propose that the open relaxed conformation of the synthetic active site triggered by tRNA facilitates the hydrolysis of the misactivated Nva–AMP.

A novel mechanism to improve the fidelity of EcLeuRS

Translation quality control, which comprises several sequential molecular recognition events, is critical for normal cell function [8]. The formation of aa-tRNAs by aaRSs is the first step in translation. However, half of the aaRSs cannot discriminate between amino acids similar to their cognate substrate and misactivates these amino acids. To maintain the catalytic specificity required for accurate translation, aaRSs have evolved a multistage proofreading mechanism that includes pre- and post-transfer editing [8]. Pre-transfer editing hydrolyses misactivated amino acids, whereas a discrete editing domain recruited by ancestral aaRS during evolution performs the post-transfer editing function of deacylating mischarged tRNAs. Besides the intrinsic editing capability of aaRSs, there are also freestanding trans-editing factors homologous to the editing domains of aaRSs and associated with clearing mischarged tRNAs [15–19]. The results of the present study suggest a novel quality control
mechanism that operates through interdomain communication within LeuRS. According to this proposed mechanism, misactivation of non-cognate amino acids stimulates the editing reaction with the assistance of tRNA. In response, tRNA-dependent editing reaction initiates interdomain communication: entry of the tRNA CCA tail into the CP1 domain triggers conformational rearrangement that is transmitted to the synthetic active site to inhibit activation of non-cognate amino acids. Such a cycle comprises a negative feedback between editing and amino acid activation. The editing reaction removes the present misactivated amino acid, whereas the negative feedback helps to reduce the further formation of misactivated amino acids. Both processes co-operate to maintain the fidelity of LeuRS. We speculate that this negative feedback through interdomain communication between synthetic and domains provides a new quality control mechanism of LeuRS.

AUTHOR CONTRIBUTION
En-Duo Wang, Min Tan and Bin Zhu designed the research. Min Tan performed the experiments. Ru-Juan Liu performed the structural analysis. Xin Chen and Xiao-Long Zhou analysed the data. Min Tan and En-Duo Wang wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Interdomain communication modulates the tRNA-dependent pre-transfer editing of leucyl-tRNA synthetase

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Figure S1 Amino acid activation by EcLeuRS and its mutants

Leucine (5 mM) activation assay using 10 nM EcLeuRS (●), EcLeuRS-E184A (○), EcLeuRS-E184R (▼) or EcLeuRS-E184R/T252R (□) enzymes. Results are means ± S.D. from three trials.

Figure S2 The spontaneous hydrolysis of Nva–AMP

(A) Non-enzymatic hydrolysis of Nva–AMP measured in solution. The assay was performed following the protocol described in the Experimental section of the main text. The data were fitted to the equation \( y = y_0 + A \cdot e^{-kt} \), where \( y \) is the concentration of Nva–AMP at different times, \( y_0 \) is the initial concentration of Nva–AMP and \( k \) is the observed spontaneous hydrolysis rate of Nva–AMP. (B) Kinetic parameters of the non-enzymatic hydrolysis of Nva–AMP measured in solution with EcLeuRS at 37°C. Results are means ± S.D. for at least three independent experiments.

Figure S3 The effect of AN2690 on the aminoacylation activity of EcLeuRS and its mutants

For each mutant, the initial aminoacylation activity in the presence of 100 μM AN2690 was normalized by that in the absence of tRNA. Results are means ± S.D for three trials.

Figure S4 Misaminoacylation by the WT EcLeuRS and EcLeuRS-E184R/T252R

Mischarging 20 μM E. coli tRNA leucine with isoleucine by 1 μM EcLeuRS (●) and EcLeuRS-E184R/T252R (□). Results are means ± S.D. for three trials.

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Figure S5  The effect of AN2690 on amino acid activation by the WT EcLeuRS

Amino acid activation by 10 nM WT EcLeuRS in different conditions: leucine without AN2690 (●); leucine with 100 μM AN2690 (○); Nva without AN2690 (▲); Nva with 100 μM AN2690 (▲); and 100 μM AN2690 only (■). Results are means ± S.D. from three trials.

Figure S6  Active site titration assay for EcLeuRS and its mutants

The active site titration was performed by measuring ATP exhaustion in the formation of leucyl adenylate at 37 °C. The enzymes used were WT EcLeuRS (A), EcLeuRS-E184R (B), EcLeuRS-E184R/T252R (C), EcLeuRS-R185E (D) and EcLeuRS-R286E (E). The number of active sites was calculated by the equation $n = ([ATP]_0 - I)/[E]_0$, where $n$ is the number of active sites, $[ATP]_0$ is the initial concentration of ATP and $[E]_0$ is the initial concentration of the enzyme, and is given in (F).
Table S1  The observed rate constants for AMP synthesis at 37°C for EcLeuRS and its mutants

The rates were determined using the AMP formation in the TLC assay described in the Experimental section. All rates are means ± S.D. for three trials.

<table>
<thead>
<tr>
<th></th>
<th>tRNA</th>
<th>AMP formation rate k_{obs} (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcLRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>+</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>3.3 ± 0.21</td>
</tr>
<tr>
<td>E184Y</td>
<td>+</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>3.2 ± 0.43</td>
</tr>
<tr>
<td>E184K</td>
<td>+</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>3.3 ± 0.48</td>
</tr>
<tr>
<td>E184H</td>
<td>+</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>3.5 ± 0.41</td>
</tr>
<tr>
<td>E184L</td>
<td>+</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>4.1 ± 0.32</td>
</tr>
<tr>
<td>E184W</td>
<td>+</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>3.9 ± 0.43</td>
</tr>
<tr>
<td>E184Q</td>
<td>+</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>3.6 ± 0.28</td>
</tr>
</tbody>
</table>

Table S2  Kinetic constants of the WT EcLeuRS in amino acid activation reaction

Results are means ± S.D. for three independent ATP/PPi exchange determinations as described in the Experimental section. The concentration of leucine ranged from 0.05 to 0.4 mM and for Nva the concentration gradient was 0.5 to 50 mM.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>K_m (mM)</th>
<th>k_{cat} (s^{-1})</th>
<th>k_{cat}/K_m (s^{-1}·mM^{-1})</th>
<th>k_{cat}/K_m (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>0.039 ± 0.004</td>
<td>73.7 ± 9.1</td>
<td>1890</td>
<td>1</td>
</tr>
<tr>
<td>Nva</td>
<td>3.11 ± 0.412</td>
<td>63.1 ± 6.8</td>
<td>20.3</td>
<td>1.1×10^{-2}</td>
</tr>
</tbody>
</table>