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Groups on the side chain of T252 in *Escherichia coli* leucyl-tRNA synthetase are important for discrimination of amino acids and cell viability☆

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Abstract

Leucyl-tRNA synthetase (LeuRS) catalyzes the leucylation of $tRNA^{Leu}$. To maintain the fidelity of protein biosynthesis, LeuRS also catalyzes the editing reaction. In the present work, highly conserved T252 in the T-rich region within CP1 domain of *Escherichia coli* LeuRS was mutated to G, D, or E. Steady-state kinetic of aminoacylation, and combined editing assays indicated that not only the size of the amino acid but also the absence of hydrogen bonds between T252 and adjacent molecules may affect the editing. It is further confirmed by in vivo experiments using the temperature-sensitive strain KL231 (Δ*leuS*), which revealed the arrested growth of bacterial cells bearing mutants with highly impaired editing activity in the presence of leucine analog.

Keywords: Aminoacyl-tRNA synthetase; Editing; Temperature-sensitive strain

In protein biosynthesis, each of the 20 aminoacyl-tRNA synthetases (aaRSs) matches an amino acid with its cognate tRNA, thus ensuring the accuracy of the protein biosynthesis [1]. The aminoacylation of tRNA is a two-step reaction: first the amino acid and ATP form an aminoacyl-AMP (activation), and then the aminoacyl moiety is transferred to the 3′-terminal adenosine of its cognate tRNA (aminoacylation) [1]. The amino acid discrimination by aaRS is achieved through two sifting steps: amino acids larger than the cognate substrate are rejected by a "coarse sieve," while the reaction products (misactivated amino acids or misaminoacylated tRNAs) with amino acids similar to the cognate substrate will go through a "fine sieve" for hydrolytic editing [2]. Crystallographic, biochemical, and genetic results demonstrated that the editing site of isoleucyl-tRNA synthetase (IleRS) [3,4], valyl-tRNA synthetase (ValRS) [5], or leucyl-tRNA synthetase (LeuRS) [6,7] is distinct from the synthetic site. The synthetic active site consists of a classic Rossmann fold (an ATP binding domain), containing two signature sequences, KMSKS and HIGH, that are common to all class I aaRSs [8]. The editing active site is located in a large connecting polypeptide 1 (CP1) domain spanning about 200 amino acid residues between the third and fourth β-strand of the Rossmann fold [9]. Recently, the conserved T-rich region within the CP1 domains was considered to be crucial for the editing activity [10].

For *Escherichia coli* LeuRS, mutation of the highly conserved threonine (T252) residue in the T-rich region to alanine (T252A) resulted in the efficient hydrolysis of the correct product by the mutant LeuRS [11], in contrast, mutations of T252 to tyrosine, methionine, phenylalanine or leucine led to the mischarging of $tRNA^{Leu}$ [12,13]. Thus, it had been proposed that the size of side chain of 252 residue played major role in the editing. Subsequent crystal structures of LeuRSTT complexed with editing substrates showed the importance of T-rich

☆ *Abbreviations:* aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; LeuRSTT, *Thermus thermophilus* LeuRS; *leuS*, LeuRS gene; WT, wild-type LeuRS; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; CP1, connective polypeptide 1; MS, mineral standard medium; Abu, α-amino-butyrate.

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region for the formation of amino acid binding pocket in CP1 domain [14]. Specially, the extremely conserved T252 in the T-rich region was indicated to interact with the amino acid substrate [11,14]. However, a recent study revealed that removal of the terminal methyl group of threonine resulted in the hydrolysis of Leu-tRNA^{Leu}, while substitution of valine for the conserved threonine appeared similar in aminoacylation and editing activity to the wild-type enzyme [15]. Mainly based on the combined computational data, it was suggested that groups on the side chain of T252 might play important role in maintaining the editing active site geometry within the CP1 domain [15].

In our laboratory, several LeuRSs with mutations in the CP1 domain of *E. coli* LeuRS have been obtained [16]. Within which, LeuRS-T252E (mutation T252 to glutamic acid) is full of aminoacylation activity. Because the hydroxyl moiety on the side chain of T252 has been suggested to be important for the stabilizing position of its own and nearby residue V338 within the editing pocket [15], we are interested in whether the highly polar carboxyl group on the side chain of T252E would interfere with the stabilization. Additionally, we further mutated the T252 to aspartic acid (D) or glycine (G), except E. Steady-state kinetics and editing assays for these mutants were performed. These results, and combined in vivo data from studying the relationship between the editing defect of LeuRS and the growth phenotype, confirmed the importance of group on the side chain of T252 residue for the editing.

Materials and methods

Materials. All chemicals were purchased from Sigma, except otherwise noted. Kinase, ligase, and restriction endonucleases were obtained from Roche (USA). [^{32}P]pyrophosphate was purchased from Dupont (Boston, MA, USA). [^{14}C]leucine, [^{14}C]isoleucine, and [γ-^{32}P]ATP were obtained from Amersham (England). *E. coli* in vivo tRNA^{Leu} was purified in our laboratory. *E. coli* tRNA^{Leu} (GAG) transcript was prepared as mentioned before [16]. The *leuS* temperature-sensitive strain KL231 (*F*$^{-}$, *leuS31, thyA6, rpsL120, deoC1*) was obtained from the *E. coli* Genetic Stock Center, Yale University, USA.

Mutagenesis, expression and purification of LeuRS, and its mutants. *E. coli* LeuRS-T252E had been constructed previously in our laboratory [16]. T252 mutants were constructed by in vitro site-specific mutagenesis as reported [7]. LeuRS and its mutants were purified from *E. coli* TG1 transformants by two-step chromatography [16].

Aminoacylation and misaminoacylation assays. Aminoacylation activity was determined at 37 °C in a reaction mixture consisting of 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 *E. coli* tRNA^{Leu}, and appropriate amounts of ^{14}C-labeled amino acids and enzymes. The kinetic constants of the enzymes were determined using various concentrations of the relevant substrates. To assay the mischarging of tRNA^{Leu}, noncognate amino acid, [^{14}C]isoleucine was used in the aminoacylation assay [7]. The reaction was initiated by adding LeuRS or its mutants.

Measurement of ATP consumption in editing. Assays for overall editing of norvaline by measuring ATP hydrolysis were performed as described by Du et al. [17]. The rate of ATP hydrolysis is the average of triplicate. Reactions with no tRNA were used as control for a small background hydrolysis. Determination of ATP consumed in overall editing of leucine was performed by replacing norvaline with leucine (1 mM).

Preparation and deacylation of radiolabeled aminoacyl-tRNA^{Leu}. Radiolabeled aminoacyl-tRNA^{Leu} was prepared by aminoacylation of tRNA^{Leu}. Reaction mixtures containing 20 μM tRNA^{Leu}, 1 mM C^{14}-labeled amino acid, 4 mM ATP, and 5 μM LeuRS-T252E in the assay buffer were incubated at 37 °C for 15 min and extracted with phenol (saturated with 0.1 M sodium acetate, pH 5.0). Aminoacyl-tRNA was precipitated by 95% ethanol. Residual low molecular weight components were removed by repeated washings with 80% ethanol. The C^{14}-labeled aminoacyl-tRNA was deacylated at 37 °C in the presence or absence of 100 nM LeuRS or its mutants in the standard buffer. The hydrolysis of the C^{14}-labeled aminoacyl-tRNA was monitored by trichloroacetic acid precipitation.

The complement assay of the E. coli LeuRS T252 mutants. Positive and negative clones could easily be selected by plating on LB plates supplemented with 100 μg/ml ampicillin and 200 μg/ml thymine. After transformation, *E. coli* KL231 could grow at 42 °C, showing the presence of a cloned gene encoding active LeuRS. The *leuSs* were transferred into temperature-sensitive strain KL231 (Δ*leuS* at 42 °C). The cells were grown overnight and adjusted to the same OD_{600} value. For each strain, 10 μl of bacterial cells was dropped onto the LB plate with ampicillin and thymine and incubated at 42 °C for 24 h, and another copy of plate was incubated at 30 °C as a positive control.

Analysis of growth phenotypes. Overnight cultures of each strain (wild-type, T252G, T252E, and T252D LeuRS) were grown at 30 °C in LB supplemented with 100 μg/ml ampicillin and 200 μg/ml thymine. Each culture was diluted 1:100 into LB supplemented with 100 μg/ml ampicillin and 200 μg/ml thymine. Diluted cultures were shaken at 30 and 42 °C for each strain. Absorbance readings were measured at 600 nm at time intervals.

Response to high levels of α-amino-butyrate. The assays were performed as described previously with a little modification [18]. Briefly, inhibition "halo experiments" were performed using mineral standard medium (MS) supplemented with ampicillin and 200 μg/ml thymine. KL231 cells harboring the *leuSs* were grown overnight to stationary phase at 30 °C, diluted 1:100 into MS, plated, and allowed to dry. A central hole was created in each plate and 10 μl of 100 mM α-aminobutyrate (Abu) was added in the hole. The plates were incubated for 48 h at 42 °C. The response to the non-cognate Abu could be evaluated based on the cell death in the inhibition zone.

Results and discussion

Purification of LeuRS and its mutants

LeuRS and other mutants were overproduced in *E. coli* at similar levels, except a lower expression of LeuRS-T252G. All LeuRS mutants were purified to homogeneity in SDS–PAGE (data not shown).

Steady-state kinetics of LeuRS and mutants

The amino acid activation activities of LeuRS and three mutants (LeuRS-T252E, -T252D, and -T252G) are almost the same (data not shown). LeuRS-T252G had less than 1% aminoacylation activity in comparison with LeuRS, so its kinetic parameters were not determined. The aminoacylation kinetics of other two mutants were assayed and compared with those of the

Table 1
Kinetic constants of the aminoacylation reaction of LeuRS and its two mutants

Substrate:	ATP			Leucine			$tRNA^{Leu}$		
Constants:	K_m (μM)	k_{cat} (s^1)	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Enzyme									
LeuRS	240	5.0	21	20	5.1	255	2.6	5.1	1923
LeuRS-T252E	220	4.9	22	19	4.9	258	3.3	5.1	1545
LeuRS-T252D	230	4.8	21	19	4.6	242	2.5	4.7	1880

The kinetic constants of enzymes were determined as described in experimental procedures. All the data in this table were the average values with a variation of <5% from three independent determinations.

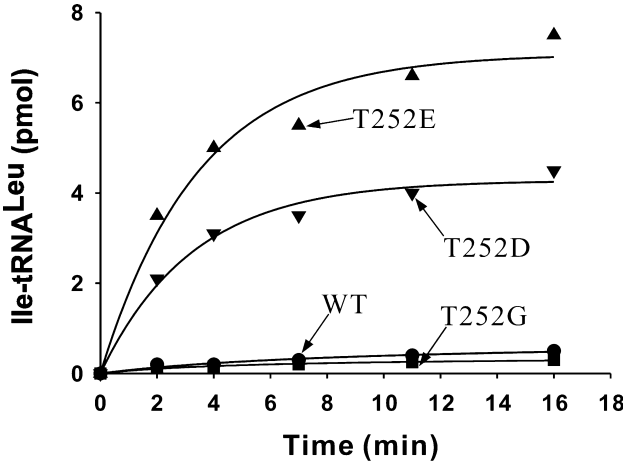

Fig. 1. Isoleucylation of $tRNA^{Leu}$ by *E. coli* LeuRS and its mutants. Isoleucylation of 10 μM $tRNA^{Leu}$ (transcript) in the presence of 1 mM [^{14}C]isoleucine at 37 °C, pH 7.8, by 1 μM *E. coli* LeuRS (WT), LeuRS-T252E, LeuRS-T252D, or LeuRS-T252G.

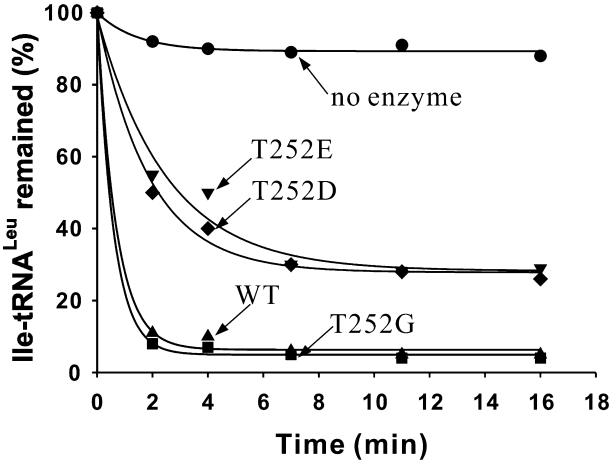

Fig. 2. Deacylation of Ile-$tRNA^{Leu}$ by LeuRS and mutants. Deacylation was performed at 37 °C, pH 7.8, by 100 nM LeuRS or LeuRS-T252E, -T252D, and -T252G. The control was performed in the absence of the enzyme.

native enzyme (Table 1). The K_m values of the two mutants and the native LeuRS for three substrates appeared almost the same. LeuRS-T252E and -T252D were fully active, even though the volume of aspartic acid (111 Å^3) is a little small than that of the conserved threonine (116 Å^3) [19].

Effect of T252 substitutions on the editing activity of LeuRS in vitro

To reveal the function of T252 in *E. coli* LeuRS during editing (the fine sieve), the misaminoacylation of $tRNA^{Leu}$ was assayed in the presence of 1 mM [^{14}C]isoleucine instead of 0.1 mM [^{14}C]leucine. As Fig. 1 shows, LeuRS-T252G, like the native LeuRS, did not mischarge $tRNA^{Leu}$ with isoleucine. In contrast, the substitutions of T by E, or D resulted in the isoleucylation of $tRNA^{Leu}$. The editing active site of LeuRS was considered to be a hydrolytic site for removing the non-cognate aminoacyl-tRNA [12,15]. For mischarged $tRNA^{Leu}$, LeuRS, and LeuRS-T252G hydrolyzed it completely; LeuRS-T252E and -T252D also deacylated Ile-$tRNA^{Leu}$, but incompletely (Fig. 2). Though LeuRS-T252E or -T252D exhibited full aminoacylation activity, the editing active site was impaired by the substitution of T with E or D.

Editing by LeuRS consumes ATP and can be measured by tRNA-dependent breakdown of ATP in the presence of non-cognate amino acid, such as norvaline or α-amino-butyrate [17]. In the presence of norvaline, the rate of ATP hydrolysis by LeuRS-T252E, -T252D was only 18%, 27% in comparison with the native LeuRS. Whereas, ATP consumption in editing norvaline by LeuRS-T252G was almost twofold of that by LeuRS (Table 2).

In accordance with the previous results (T252A), mutation to the glycine (T252G) resulted in the fast hydrolysis of correctly charged aminoacylation products in the presence of leucine, which led to the fast ATP hydrolysis and almost no detection of aminoacylation activity (Table 2 and data not shown). While LeuRS and the mutants with full aminoacylation activity (LeuRS-T252E, -T252D) almost do not hydrolyze ATP (Table 2).

The tertiary structure of LeuRSTT reported by Lincecum [14] showed that the conserved T252 was located at the bottom of amino acid binding pocket and could block leucine binding by crashing with γ-methyl

Table 2
$tRNA^{Leu}$-dependent ATP hydrolysis by *E. coli* LeuRS

LeuRS	ATP hydrolysis rates (pmol/min)[a]	Relative rates[a]	ATP hydrolysis rates (pmol/min)[b]	Relative rates[b]
LeuRS	112	1.0	4.0	1.0
T252E	20	0.18	3.5	0.86
T252D	30	0.27	3.9	0.98
T252G	240	2.1	238	60

[a] Norvaline as the editing substrate.
[b] Leucine as the editing substrate.

group of leucine, while non-cognate amino acids without the γ-methyl group such as norvaline would enter the binding pocket. Recently, based on the T252 mutation assays and the combined modeled structure data, it was suggested that the group of the side chain not only hindered the cognate product into the editing pocket but also played a role in maintaining the overall structure of the editing site [15]. It is also proposed that the stabilization effect by the hydroxyl group of T252 may be dependent on the hydrogen bonding to a water molecule [13,15]. Furthermore, the crystal structures of LeuRSTT complexed with editing substrates showed that several hydrogen bonds were formed in the amino acid binding pocket and might maintain the functional conformation of the editing site to facilitate the editing hydrolysis [14]. Thus, the hydroxyl group of T252 should be essential for the network. Although, the modeled editing sites of T252V mutant LeuRS were slightly distorted and compressed, none mischarged tRNA could be detected in the editing assays [15]. Here for the first time, we revealed that by mutation the T252 to E or an even smaller residue (D) mischarged tRNA could be detected. For T252D or T252E, the additional carboxyl group might interfere with the nearby hydrogen bonds, leading to the impairment of editing activity by greatly disturbing the editing pocket geometry.

The rescue of KL231 by LeuRS and its mutants

Escherichia coli KL231 grows at 30 °C very well, but stops growing at 42 °C. In vivo active LeuRS can rescue *E. coli* KL231 at 42 °C. The growth at 42 °C and 30 °C of KL231 containing LeuRS, LeuRS-T252D, LeuRS-T252E, and T252G was investigated and is shown in Fig. 3A. At 42 °C, all the mutants could rescue KL231, while the negative control using pTrc-99B failed to do so. The aminoacylation activity of LeuRS-T252G was very low in vitro, but it could also rescue KL231 in vivo, though the cells grew slower at 42 °C. The complement assay (Fig. 3A) showed that the cell growth was inhibited by LeuRS-T252G, which hydrolyzes the correct products, even with endogenous expression of wild-type LeuRS at 30 °C. To elucidate the slow-growth phenotype, we next determined growth curves of KL231 containing LeuRS, LeuRS-T252D, LeuRS-T252E or T252G at 30 and 42 °C. A prominent growth defect was observed for KL231 cells bearing the T252G (Fig. 3B). No growth defect (under these conditions) was seen in

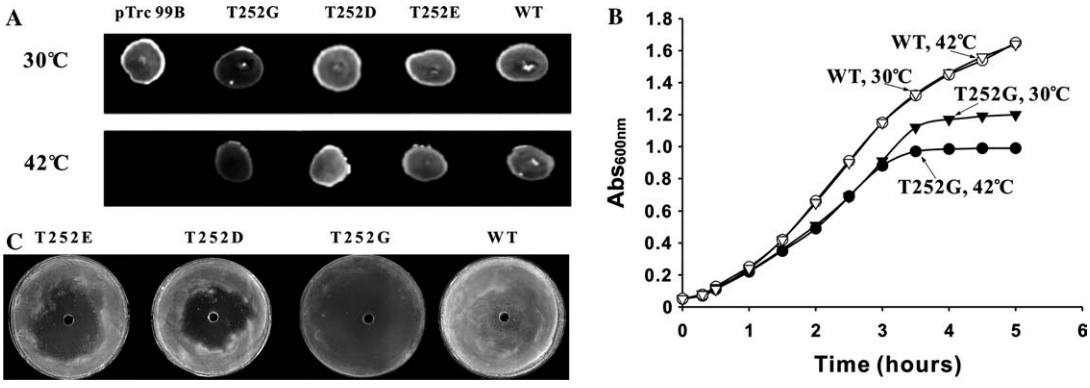

Fig. 3. In vivo assays using temperature-sensitive strain, KL231. (A) Rescue of temperature-sensitive strain by LeuRS and its mutants. Temperature-sensitive strain, KL231 (Δ*leuS* at 42 °C) cells harboring pTrc-99B, LeuRS-T252G, LeuRS-T252D, LeuRS-T252E, or LeuRS were incubated on LB medium at 30 °C or 42 °C. (B) The T252G substitution results in a slow-growth phenotype. Growth curves of KL231 containing wild-type LeuRS versus T252G are shown. (C) Relationship between defective editing of LeuRS mutants and Abu toxicity phenotypes. KL231 cells harboring LeuRS-T252E, LeuRS-T252D, LeuRS-T252G or LeuRS were incubated at 42 °C on MS medium with Abu in the central well.

strains encoding the T252E and T252D protein (data not shown). It was noticed that cells harboring T252G grew better at 30 °C than 42 °C, which was reasonable because of endogenous expression of wild-type LeuRS at 30 °C. LeuRS-T252G fast hydrolyzed correct products and led to an almost no detection of aminoacylation activity, therefore the growth defect was likely due to the deficiency of leu-tRNA^{Leu} in vivo, suggesting the LeuRS-T252G as a toxic protein to the cell. The unstable possibility for LeuRS-T252G was precluded because even under longtime incubation at 40 °C, LeuRS-T252G had almost the same activity to catalyze the editing reaction (data not shown). Moreover, growth curve of ordinary *E. coli* strain containing T252G under 37 °C also confirmed the slow growth phenotype (data not shown).

Response to high levels of α-amino-butyrate

The misaminoacylation of tRNA^{Leu} by mutant enzymes, LeuRS-T252E and -T252D, may cause problems in protein biosynthesis. Although KL231 containing the above genes grew very well at 42 °C (Fig. 3A), in the presence of high concentration of Abu, it may be misincorporated into cellular proteins in vivo and may be toxic to the cells.

To determine the in vivo effect of defect in editing, cells bearing the mutant *leuS* were treated with high concentration of Abu. Cells containing the gene encoding LeuRS-T252E or -T252D were sensitive to high concentrations of Abu (Fig. 3C). This phenotypic sensitivity was seen as a "halo" of cell death around the central well where Abu was added. In contrast, cells harboring native LeuRS were not sensitive to Abu. For cells with LeuRS-T252G, growth inhibition was seen without obvious "halo," which is believed to be caused by its fast hydrolysis of correctly charged aminoacylation products. Therefore, this toxicity was dependent solely on the presence of non-cognate amino acid and editing defective LeuRS. The in vivo results presented here showed that the editing defect in *E. coli* LeuRS could lead to genetic code ambiguity and the editing function is essential for the fidelity of protein biosynthesis. Thus, combined with previous results [11–13,15] and our complement assay (Figs. 3A and B), it is clear why T252 has been selected in evolution for the specific recognition of amino acid.

Recently, some results provided strong evidence that the editing domain was critical for maintaining the genetic code [18,20]. According to the aaRS evolution hypothesis, the appending function domain, such as the editing domain, is recruited later and the crucial residues in the editing domain play an indispensable role in aaRS evolution [20]. However, even in the *Aquifex aeolicus*, which locates in the root of evolution tree, LeuRS harbors the functional CP1 editing domain (unpublished data), suggesting that the editing domain has been incorporated early to establish the genetic code.

Acknowledgments

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