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Rational Design to Block Amino Acid Editing of a tRNA Synthetase

Richard S. Mursinna and Susan A. Martinis*

Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204-5001

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Aminoacyl-tRNA synthetases can be evolved to facilitate incorporation of nonstandard amino acids into proteins.[1−3] In vivo synthesis has recently focused on developing orthogonal tRNA synthetase/tRNA pairs,[3−7] while in vitro translation methods have primarily relied on chemical synthesis of misacylated tRNAs.[8−12] We report an in vitro enzymatic approach that could be used to generate tRNAs linked to nonstandard amino acids. We used an aminoacyl-tRNA synthetase that already possesses misaminoacylation capabilities, but is rationally engineered to block its editing activity.

We previously identified a single threonine (T252) that plays a critical specificity role in amino acid editing by *Escherichia coli* leucyl-tRNA synthetase (LeuRS).[13] When alanine is substituted for T252 (T252A), LeuRS loses its ability to discriminate against the cognate charged leucine amino acid within its post-transfer editing active site. As a result, the T252A mutant LeuRS hydrolyzes the correctly aminoacylated Leu-tRNA^{Leu} product.

The T252 residue resides within a highly conserved threonine-rich region of LeuRS. The X-ray crystal structure of *Thermus thermophilus* LeuRS[15] and the homology model of *E. coli* LeuRS[14] suggest that T252 is near the bottom of a surface depression that we proposed to be the amino acid binding pocket of the editing active site (Figure 1). We hypothesized that T252 acts as a fine discriminator to hinder binding of the leucine amino acid, but allows other misaminoacylated noncognate amino acids (i.e. isoleucine, methionine, norvaline, etc.) to interact effectively. In particular, we proposed that the T252 side chain may sterically clash with the γ-branched methyl moiety of the incoming leucine side chain (Figure 2). Replacement by alanine removes this impediment and increases the volume of the amino acid binding pocket, allowing correctly charged leucine to bind and be hydrolytically cleaved.

Herein, we propose to abolish LeuRS editing activity by rationally obstructing the amino acid binding pocket. We introduced bulky residues (Figure 2C) by substituting T252 with methionine (T252M), phenylalanine (T252F), and tyrosine (T252Y) in *E. coli* LeuRS via polymerase chain reaction-based mutagenesis.[13,16] Each mutant LeuRS was expressed stably and in high yields with a fused six-histidine tag and purified by affinity chromatography.[13,16] *E. coli* tRNA$^{Leu}_{UAA}$ transcripts were generated in vitro by T7 RNA polymerase runoff transcription.[13,17,18]

Leucylation activities of *E. coli* T252M, T252F, and T252Y mutant LeuRSs were similar to that measured for the wild-type enzyme (Figure 3). We also analyzed isoleucine-mischarging activities (Figure 4) using a standard method that relies on trichloroacetic acid precipitation to recover tRNA charged with a radiolabeled amino acid.[13,16] Although the T252M mutant LeuRS did not yield detectable levels of misaminoacylated tRNA, changing the conserved T252 to either the bulky phenylalanine or tyrosine facilitates misaminoacylation of isoleucine to tRNA^{Leu} (Ile-tRNA^{Leu}). These mutant proteins tend to charge tRNA^{Leu} with isoleucine less efficiently than the cognate leucine, as would be expected, since the noncognate amino acid is poorly activated in the first step of the aminoacylation reaction.

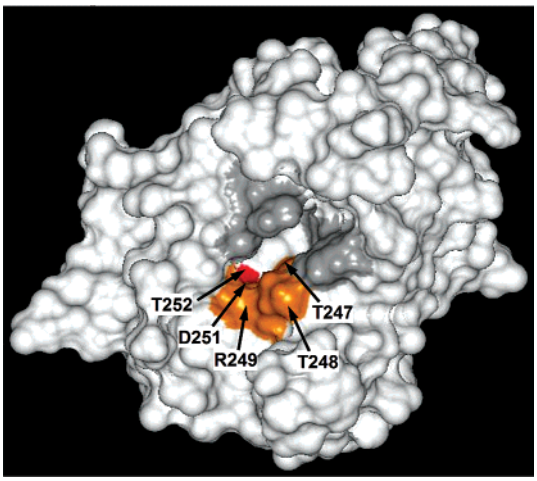

Figure 1. Surface representation of the proposed amino acid binding pocket of the *E. coli* LeuRS editing active site. The homology model[14] was rendered with InsightII software (Accelrys). Only the editing domain is displayed. Orange residues represent the threonine-rich region analyzed previously by alanine-scanning mutagenesis.[13] Residue T252 is colored in red and resides near the bottom of the cavity. Gray highlights indicate other residues on the cavity's rim.

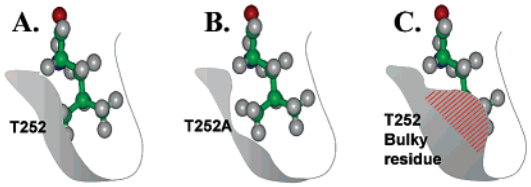

Figure 2. Cartoon of the amino acid binding pocket of the *E. coli* LeuRS editing active site. (A) The conserved T252 may block leucine binding by sterically interfering with one of its γ-branched methyl moieties. (B) When substituted by an alanine,[13] the pocket volume increases allowing leucine to bind. (C) Substitution by a bulky residue such as phenylalanine or tyrosine would significantly reduce the volume of the pocket and may block amino acid binding in general.

Both cognate and noncognate aminoacylation reactions, catalyzed by the wild-type and T252 mutant LeuRSs, were also separated electrophoretically on acidic polyacrylamide gels[19,20] in order to differentiate charged tRNA from protein that was potentially self-labeled.[20,21] The dried gel containing tRNA products charged with either [^{14}C]-leucine or [^{14}C]-isoleucine were phosphorimaged for 3 and 30 days, respectively. As Figure 5A shows, both wild-type and mutant T252 LeuRSs yield correctly aminoacylated Leu-tRNA^{Leu} in comparable amounts. However, only the T252F and

* Corresponding author. E-mail: smartinis@uh.edu.

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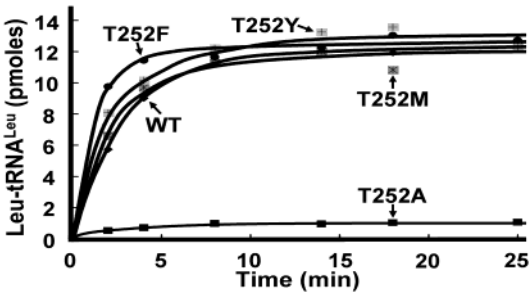

Figure 3. Leucylation of wild-type (WT) and T252 mutant LeuRSs. Assays are described previously[13] and in the Supporting Information.

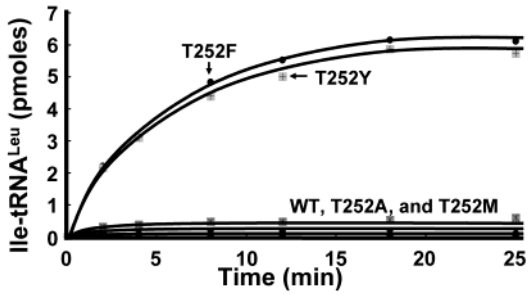

Figure 4. Isoleucylation activities of wild-type (WT) and T252 mutant LeuRSs. Assays are described in the Supporting Information.

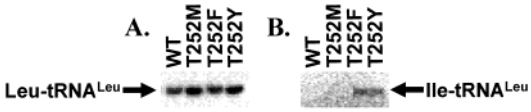

Figure 5. Acid gel analysis of leucylation (A) and isoleucylation (B) activities of wild-type (WT) and T252 mutant LeuRSs. Assays are described previously[19,20] and in the Supporting Information.

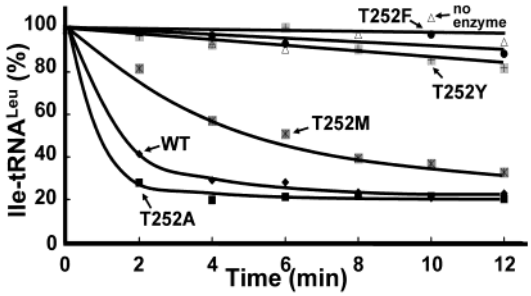

Figure 6. Editing by wild-type (WT) LeuRS and T252 mutants. Assays are described previously[13,22] and in the Supporting Information.

T252Y mutant LeuRSs produce detectable levels of mischarged Ile-tRNA^{Leu} (Figure 5B).

We used T252F and T252Y mutant LeuRSs to generate stable mischarged tRNA^{Leu} with isoleucine as a substrate for hydrolytic editing assays. Wild-type and T252A mutant LeuRSs edited mischarged Ile-tRNA^{Leu} (Figure 6). Although the hydrolysis rate for editing Ile-tRNA^{Leu} by T252M LeuRS was decreased, it was still sufficient to bar stable production of the mischarged tRNA (Figures 4 and 5B). In contrast, the T252F and T252Y mutations essentially eliminated LeuRS editing activity. Alterations in hydrolytic editing activity are largely due to changes in k_{cat} (Table 1). Comparison of k_{cat}/K_M values (listed in Table 1) for editing by T252M versus T252F and T252Y mutant LeuRSs suggests a threshold level of activity that is required to maintain LeuRS fidelity. These results support that the introduction of bulky phenylalanine or tyrosine residues into the amino acid binding pocket of the editing active site effectively abolished hydrolytic cleavage.

Table 1. Kinetic Parameters for Amino Acid Editing

	WT	T252A	T252M	T252F	T252Y
K_M (μM)	0.67	0.78	0.39	0.65	0.82
k_{cat} (s^{-1})	83.3	120.2	27.5	4.1	5.4
k_{cat}/K_M ($\mu M^{-1} s^{-1}$)	124.1	153.1	69.7	6.2	6.5

Inactivation of LeuRS editing has the potential to provide an efficient and facile enzymatic synthesis route to generate tRNAs that are aminoacylated with unusual amino acids. Indeed, a genetically isolated valyl-tRNA synthetase editing mutant yielded proteins in vivo that incorporated threonine, cysteine, and aminobutyrate in place of valine.[2] The amino acid binding pocket volume within the aminoacylation active site of LeuRS allows activation of a wider range of chemically diverse amino acids[16,23−25] because it is relatively large. In addition, since *E. coli* LeuRS does not rely on the anticodon for tRNA identity,[26] limited further engineering may also provide a convenient in vivo route for large-scale production of custom-designed proteins that contain novel amino acids at specific sites.

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Supporting Information Available: Details on all materials and methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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