Articles

Two Conserved Threonines Collaborate in the *Escherichia coli* Leucyl-tRNA Synthetase Amino Acid Editing Mechanism†

Yuxin Zhai and Susan A. Martinis*

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

Received July 22, 2005; Revised Manuscript Received September 29, 2005

ABSTRACT: The aminoacyl-tRNA synthetases covalently link transfer RNAs to their cognate amino acids. Some of the tRNA synthetases have employed an editing mechanism to ensure fidelity in this first step of protein synthesis. The amino acid editing active site for *Escherichia coli* leucyl-tRNA synthetase resides within the CP1 domain that folds discretely from the main body of the enzyme. A portion of the editing active site is lined with conserved threonines. Previously, we identified one of these threonine residues (Thr^{252}) as a critical amino acid specificity factor. On the basis of X-ray crystal structure information, two other nearby threonine residues (Thr^{247} and Thr^{248}) were hypothesized to interact with the editing substrate near its cleavage site. Single mutations of either of these conserved threonine residues had minimal effects on amino acid editing. However, double mutations that deleted the hydroxyl group from the neighboring threonine residues abolished amino acid editing activity. We propose that these threonine residues, which are also conserved in the homologous isoleucyl-tRNA synthetase and valyl-tRNA synthetase editing active sites, play a central role in amino acid editing. It is possible that they collaborate in stabilizing the transition state.

The fidelity of protein synthesis relies on accurate recognition of amino acid and transfer RNA (tRNA)[1] substrates by the aminoacyl-tRNA synthetases (aaRSs) (*1*). This family of enzymes is responsible for linking the correct amino acid to its cognate tRNA isoacceptors. Each aaRS activates a single amino acid for aminoacylation in a two-step reaction mechanism:

$$\text{amino acid (AA)} + \text{ATP} \rightleftharpoons \text{AA}-\text{AMP} + \text{pyrophosphate } (PP_i) \quad (1)$$

$$\text{AA}-\text{AMP} + \text{tRNA} \rightarrow \text{AA}-\text{tRNA} + \text{AMP} \quad (2)$$

The charged tRNA then shuttles the amino acid to the ribosome where it is incorporated during protein synthesis.

Some tRNA synthetases misactivate closely related noncognate amino acids (*2*). Many of these aaRSs have developed hydrolytic amino acid editing mechanisms to decrease the extent of incorporation of incorrect amino acids into proteins:

$$\text{AA}-\text{AMP} \rightarrow \text{AA} + \text{AMP (pre-transfer editing)} \quad (3)$$

$$\text{AA}-\text{tRNA} \rightarrow \text{AA} + \text{tRNA (post-transfer editing)} \quad (4)$$

Leucyl-tRNA (LeuRS), isoleucyl-tRNA (IleRS), and valyl-tRNA synthetases (ValRS) utilize a "double-sieve" mechanism (*3*−*5*) that introduces two separate amino acid binding sieves or pockets with different strategies for recognition to enhance fidelity. The amino acid binds first to a "coarse sieve", which corresponds to the aminoacylation active site. The cognate amino acid, its analogues, and smaller amino acids can "pass through" this sieve or bind productively in the amino acid binding pocket of the aminoacylation active site. The activated aminoacyl adenylates or charged tRNAs are then transferred to a "fine sieve" within a separate editing active site. Importantly, the fine sieve blocks the correct or cognate amino acid, but binds to misactivated amino acids and/or mischarged tRNAs for hydrolysis.

The two amino acid recognition sieves in LeuRS have been identified by mutational and structural analysis (*6*−*10*). The amino acid editing active site is located in a discretely folded domain called connective peptide 1 (CP1; *11*) that is distinct from the main aminoacylation body of the enzyme (*6*−*8*, *10*, *12*). The amino acid binding pocket of the LeuRS editing active site has been precisely located within a threonine-rich region (*7*, *8*). Within this amino acid binding pocket, a conserved threonine residue (Thr^{252} in *Escherichia coli*

† This work was supported by The Robert A. Welch Foundation (Grant E-1404) and the National Institutes of Health (Grant GM63789).

* To whom correspondence should be addressed. Present address: Department of Biochemistry, University of Illinois, 419 RAL, Box B4, 600 S. Mathews Ave., Urbana, IL 61801-3732. Phone: (217) 244-2405. Fax: (217) 244-5858. E-mail: martinis@ life.uiuc.edu.

[1] Abbreviations: tRNA, transfer RNA; aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; CP1, connective peptide 1; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; HEPES, hydroxyethylpiperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

10.1021/bi0514461 CCC: $30.25
Published on Web 11/05/2005

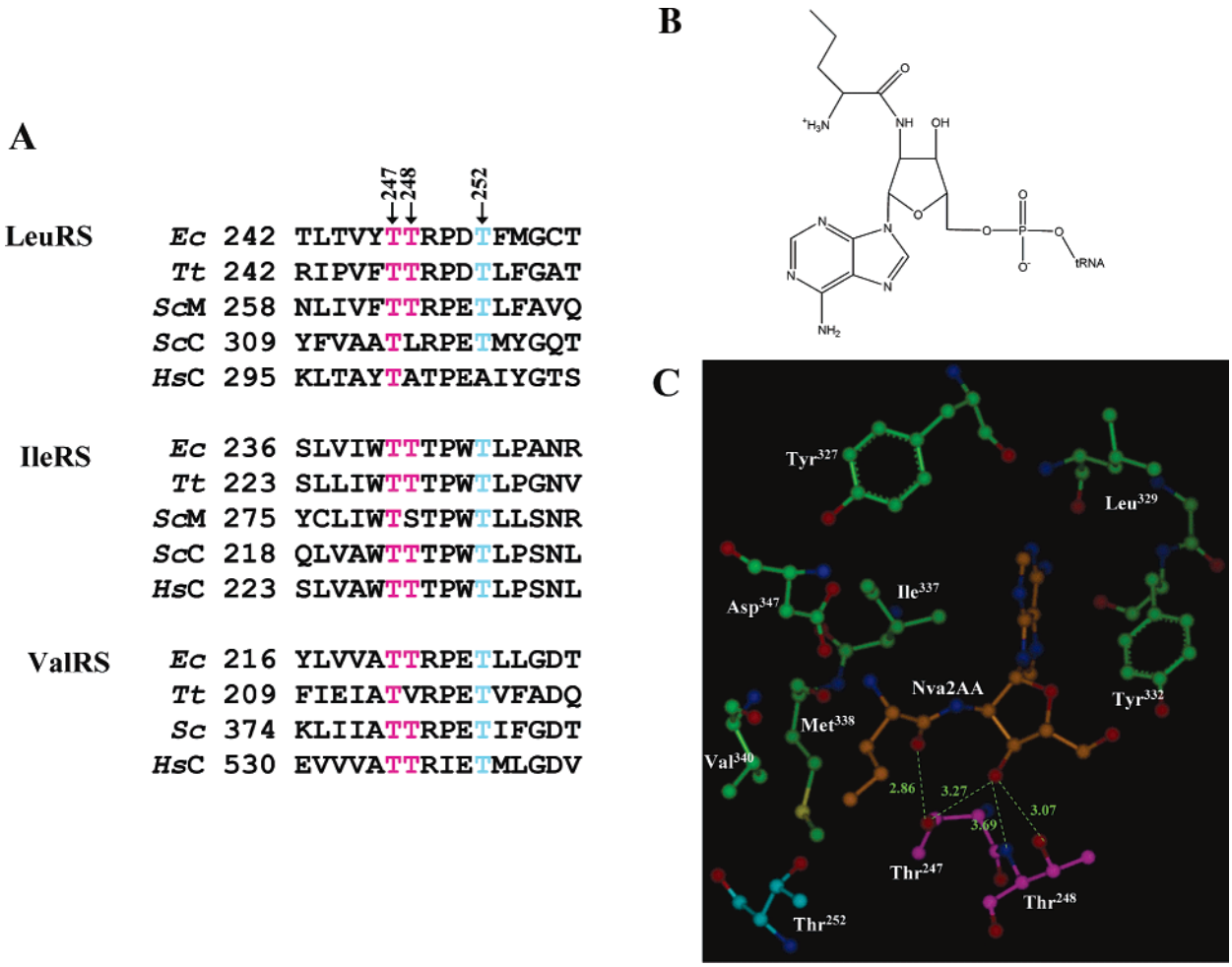

FIGURE 1: Primary and tertiary structure analysis of conserved threonine residues in the LeuRS CP1 domain editing active site. (A) Primary sequence alignment of the threonine-rich editing region within the CP1 domains of LeuRS, IleRS, and ValRS. Thr^{247} and Thr^{248} are indicated by arrows and colored pink. The Thr^{252} that is critical to amino acid specificity in the threonine-rich region is colored light blue. Residue numbers for the first amino acid shown in the primary sequence are indicated at the beginning of each line. Abbreviations are as follows: *Ec*, *E. coli*; *Tt*, *T. thermophilus*; *Sc*, *Saccharomyces cerevisiae*; *Hs*, *Homo sapiens*; C, cytoplasmic; M, mitochondrial. Yeast cytoplasmic and mitochondrial ValRSs share the same protein sequence. (B) Chemical structure of 2′-(L-norvalyl)amino-2′-deoxyadenosine (Nva2AA), the post-transfer editing substrate analogue that is bound in the X-ray crystal structure of *T. thermophilus* (*7*). (C) *T. thermophilus* LeuRS editing active site (*7*). The hydrogen bonds between the post-transfer editing analogue (Nva2AA) and Thr^{247} and Thr^{248} in the *T. thermophilus* LeuRS cocrystal structure are highlighted with green dashed lines (*7*). The hydrogen bond lengths are given in angstroms. The hydroxyl group of Thr^{247} forms a hydrogen bond with both the carbonyl oxygen and the 3′-OH of ribose. The main chain amide and the hydroxyl group of Thr^{248} each participate in a hydrogen bond with the 3′-OH of ribose. Atoms are represented by the following colors: blue for nitrogen, red for oxygen, yellow for sulfur, pink for Thr^{247} and Thr^{248} carbons, light blue for Thr^{252} carbons, orange for substrate analogue carbons, and green for all other carbons.

LeuRS) acts as a fine discriminator to block leucine from editing (*8*). The role of two other threonines, Thr^{247} and Thr^{248} in *E. coli* LeuRS, which are also conserved within the homologous CP1 domains of IleRS and ValRS (Figure 1A), has been less clear.

The cocrystal structure of *Thermus thermophilus* LeuRS bound to a post-transfer editing substrate analogue containing norvaline (Nva2AA; Figure 1B) shows that the hydroxyl group of Thr^{247} forms a hydrogen bond with the carbonyl oxygen of the aminoacyl link (*7*). This Thr^{247} hydroxyl group also interacts with the 3′-hydroxyl group of the ribose ring, which additionally forms two hydrogen bonds with the backbone amide and hydroxyl group of Thr^{248} (*7*). We hypothesized that the hydroxyl group of each of these threonine residues is important for orienting the substrate in the editing active site for catalysis by LeuRS. Herein, we introduced mutations at each of these sites to disrupt hydrogen bonding. Our results show that Thr^{247} and Thr^{248} are two key residues in the *E. coli* LeuRS editing active site and appear to collaborate in the hydrolytic cleavage mechanism.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC). Tritium-labeled amino acids were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). *Dpn*I restriction enzyme was obtained from Promega (Madison, WI). Cloned *Pfu* DNA polymerase and dNTP mix were acquired from Stratagene (La Jolla, CA). Crude *E. coli* $tRNA^{Leu}$ was purchased from Hoffmann-La Roche Ltd. (Basel, Switzerland). The restriction enzyme *Bst*NI was obtained from New England BioLabs Inc. (Beverly, MA).

RNA Preparation. A total of 450 μg of plasmid ptDNAleu (*13*) containing the gene for *E. coli* $tRNA^{Leu}_{UAA}$ was digested

overnight with 25 units of *Bst*NI in a 1 mL reaction mixture at 60 °C and then used as a template for *in vitro* transcription (*14*). Each transcription reaction mixture contained 40 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), 30 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.01% Triton X-100, 50 μg/mL bovine serum albumin (BSA), NTPs (4 mM each), 80 mg/mL PEG^{8000}, 0.02 unit/μL RNase inhibitor (Eppendorf, Hamburg, Germany), 2 mM spermidine, 0.4 mg/mL template, 0.01 mg/mL pyrophosphatase (Sigma, St. Louis, MO), and 800 nM T7 RNA polymerase. The reaction mixture was incubated overnight at 37 °C and the RNA product purified on a 10% polyacrylamide gel. The $tRNA^{Leu}_{UAA}$ band was detected by UV shadowing, excised from the gel, crushed, and then shaken overnight at 37 °C in 0.5 M NH_4OAC and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The supernatant was collected and the gel extracted two more times. Butanol extractions were used to concentrate the RNA. A 10 μL aliquot of 25 mg/mL glycogen was added to 500 μL of concentrated tRNA followed by ethanol precipitation. The pellet was washed twice with 70% ethanol and dried followed by resuspension in nuclease-free water (Ambion, Austin, TX). The concentration was determined on the basis of the absorbance at 260 nm at 80 °C using the conversion factor of $A_{260} = 40$ μg/mL tRNA.

Mutagenesis and Purification of E. coli LeuRS. Wild-type LeuRS was mutated to T247V,[2] T248V, T247/8V, T247/8A, and T247/8S using polymerase chain reaction (PCR)-based mutagenesis. Each 50 μL PCR mixture contained 25 ng of plasmid p15ec3-1 (*15*), 125 ng of each forward and reverse primer, 10 mM dNTPs, and 2.5 units of *Pfu* DNA polymerase (Stratagene) in commercially prepared buffer. The reaction mixtures were heated at 95 °C for 1 min, and then DNA was amplified by PCR for 25 cycles under the following conditions: 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min. Each PCR mixture was restriction digested with 20 units of *Dpn*I and then used for transformation of *E. coli* DH5α (Stratagene). The DNA sequences of the mutated LeuRS genes were confirmed by Lone Star Labs (Houston, TX).

Mutant and wild-type plasmids were used to transform *E. coli* strain BL21 (Stratagene). A single transformant was transferred to 3 mL of LB with 100 μg/mL ampicillin and incubated at 37 °C overnight. A 1 mL aliquot of the overnight culture was transferred to 500 mL of LB with 100 μg/mL ampicillin and grown at 37 °C. When the OD_{600} was increased to 0.6, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce LeuRS expression at 37 °C for 2 h. The cells were harvested by centrifugation at 8000 rpm for 15 min in a Beckman J2-HS centrifuge and stored at −80 °C.

The pellet was resuspended in 10 mL of solution I [20 mM sodium phosphate ($Na{\cdot}P_i$), 10 mM Tris (pH 8.0), 300 mM NaCl, and 5% glycerol] and then sonicated at 50% power for 1.5 min using a Vibra Cell sonicator. The sonication was repeated, and the cells were centrifuged at 8000 rpm in the Beckman J2-HS centrifuge for 15 min at 4 °C. The lysate was combined with HIS-Select HF Nickel Affinity Gel (Sigma) that had been pre-equilibrated with solution I. The gel and lysate were mixed at 4 °C for 30 min, followed by clarification in a low-speed clinical centrifuge. The gel was washed with 10 mL of solution II [20 mM $Na{\cdot}P_i$, 10 mM Tris (pH 7.0), 500 mM NaCl, and 5% glycerol] for a total of five times for 30 min each at 4 °C. The LeuRS with the N-terminal six-histidine tag was eluted by mixing the gel with 1 mL of elution buffer [20 mM $Na{\cdot}P_i$, 10 mM Tris (pH 7.0), 100 mM NaCl, 10% glycerol, and 100 mM imidazole]. The protein product was concentrated using a centricon-50 apparatus (Amicon, Bedford, MA). The final concentration was determined by a Bio-Rad protein assay as described in the commercial protocol.

Aminoacylation Assays. Each aminoacylation reaction mixture contained 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 4 mg/mL crude *E. coli* $tRNA^{Leu}$. The enzyme and amino acid concentrations are given in the appropriate figure legend. The reactions were initiated by adding 4 mM ATP. Aliquots were quenched at specific time points and transferred to filter pads that were presoaked with 5% trichloroacetic acid (TCA). The pads were washed for 10 min each with cold 5% TCA for a total of three times and twice with 70% ethanol. The pads were incubated in anhydrous ether, dried, and quantified in a Beckman LS 6000IC scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Amino Acid Editing Assays. Crude *E. coli* $tRNA^{Leu}$ was aminoacylated with [^{3}H]isoleucine (650 μCi/mL) or [^{3}H]-valine (650 μCi/mL) by Y330A/D342A/D345A mutant LeuRS (T. L. Lincecum, R. S. Mursinna, and S. A. Martinis, unpublished data) as described above for 60 min at 37 °C. The reaction was quenched with 0.17% acetic acid (*16*). Protein was removed by extraction with a phenol/chloroform/isoamyl alcohol mixture (125:24:1; Fisher Biotech, Fair Lawn, NJ) that had been pre-equilibrated to pH 4.3. The RNA was ethanol precipitated and the dried pellet resuspended in 10 mM potassium phosphate buffer (pH 5.0). Hydrolytic editing assays were carried out in 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 0.25 mg/mL misaminoacylated $tRNA^{Leu}$. Each reaction was initiated by adding 10 nM enzyme. The reactions were quenched at specific time points and processed as described above.

Mischarged Ile-$tRNA^{Leu}_{UAA}$ *in vitro* transcripts were generated for kinetic analysis as described above except that 60 mM hydroxyethylpiperazineethanesulfonic acid (HEPES) (pH 7.5) was used as the buffer. At the end of the reaction, a total of six 3 μL duplicate aliquots were transferred onto TCA-soaked pads. Half of the pads were washed extensively as described above to quantify the Ile-$tRNA^{Leu}_{UAA}$ product. The other half were air-dried to determine the total amount of free and aminoacylated isoleucine. The yield of mischarged Ile-$tRNA^{Leu}_{UAA}$ varied from 15 to 30% of the total free and charged $tRNA^{Leu}_{UAA}$ for different preparations. This compared to a yield of nearly 100% for the more efficiently aminoacylated Leu-$tRNA^{Leu}_{UAA}$. Each amino acid editing reaction included 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, and Ile-$tRNA^{Leu}_{UAA}$ (0.1−1.0 μM). The concentrations of wild-type and mutant enzymes used to initiate the reactions were as follows: 5 nM wild-type or T247/8S LeuRS, 30 nM T247V LeuRS, 25 nM T248V LeuRS, and 1.0 μM T247/

[2] The mutants are named X###Ys, in which X is the wild-type amino acid, ### is the residue number, and Y is the mutant amino acid. For example, the threonine at position 247 is replaced with a valine in the T247V mutant enzyme. In addition, 247/8 represents mutations at both positions 247 and 248.

8V LeuRS. Because of the difficulty in obtaining sufficient levels of mischarged tRNA for post-transfer editing assays, kinetic analysis was based on apparent values. Temperature-dependent reactions that were used to construct an Arrhenius plot were carried out at 21, 24, 27, 30, 33, and 36 °C.

Free Energy of Activation Calculations. The change in the free energy required to form the transition state complex (LeuRS−S‡) from the free enzyme and substrate (LeuRS + S) for post-transfer editing is represented by $\Delta G^{\ddagger}$. Mutation-dependent changes in the free energy of activation ($\Delta\Delta G^{\ddagger}$) for post-transfer editing of Ile-tRNA$^{Leu}_{UAA}$ by LeuRS can be defined by

$$\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{mt} - \Delta G^{\ddagger}_{wt} \quad (5)$$

where the subscripts mt and wt correspond to the mutant and wild-type enzyme, respectively. The $\Delta\Delta G^{\ddagger}$ was calculated using the measured apparent Michaelis constant for hydrolysis of Ile-tRNA$^{Leu}_{UAA}$ (K_M) and the corresponding apparent turnover number (k_{cat}) as follows (*17*−*19*):

$$\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_M)_{mt}/(k_{cat}/K_M)_{wt}] \quad (6)$$

The free energy changes of the two single mutants, T247V and T248V, should be related to that of the double mutant (T247/8V) by eq 7:

$$\Delta\Delta G^{\ddagger}_{T247/8V} = \Delta\Delta G^{\ddagger}_{T247V} + \Delta\Delta G^{\ddagger}_{T248V} + \Delta\Delta G^{\ddagger}_{I} \quad (7)$$

where $\Delta\Delta G^{\ddagger}_{I}$ represents the coupling free energy between the two mutation sites (*17*, *18*).

RESULTS

A homologous conserved threonine-rich region in the CP1 domain is crucial to amino acid editing by LeuRS, IleRS, and ValRS (*8*, *20*, *21*). The conserved Thr^{252} residue in LeuRS acts as an important specificity factor and efficiently blocks the cognate leucine amino acid from hydrolysis in the editing reaction (*8*). The Thr^{247} and Thr^{248} sites are highly conserved not only in LeuRS but also in the homologous IleRS and ValRS editing enzymes (Figure 1A). The *T. thermophilus* LeuRS cocrystal structure has been determined with a norvalyl-adenosine post-transfer editing substrate analogue (*7*). The hydroxyl group of Thr^{247} forms a hydrogen bond with both the carbonyl oxygen and the 3′-hydroxyl group of the ribose ring. The main chain amide and the hydroxyl group of Thr^{248} also contribute two hydrogen bonds to the 3′-hydroxyl group of the ribose ring (Figure 1C). Since these two highly conserved threonine sites appeared to anchor the central region of the editing substrate that is targeted for hydrolysis, we hypothesized that they might play a role in amino acid editing.

We mutationally analyzed each of the threonine residues by introducing an isosteric valine that lacks hydrogen bonding potential. We also substituted these threonines with serines that retain the capacity to form hydrogen bonds. Five single and double mutant LeuRSs, including T247V, T248V, T247/8V, T247/8A, and T247/8S, were constructed and purified to homogeneity via affinity chromatography. Each of the mutant enzymes exhibited leucylation activities similar to that of wild-type LeuRS (Figure 2).

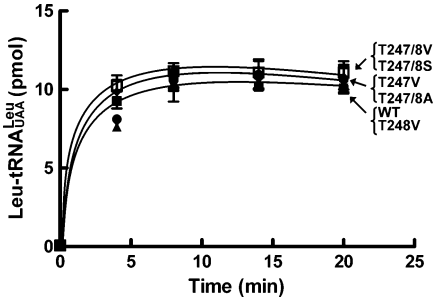

FIGURE 2: Aminoacylation activity of wild-type and mutant LeuRSs. Each aminoacylation reaction was carried out in the presence of 4 μM tRNA$^{Leu}_{UAA}$, 25 μM [^{3}H]leucine (150 μCi/mL), and 20 nM enzyme: wild type (■), T247V (▲), T248V (▼), T247/8V (◆), T247/8A (●), and T247/8S (□). Error bars are based on the reaction being repeated at least in triplicate.

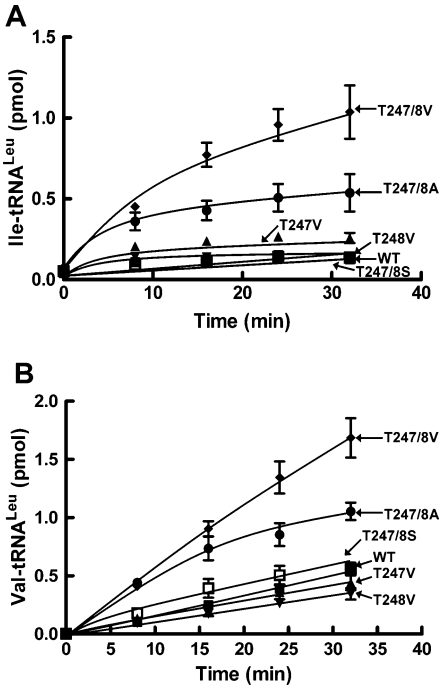

FIGURE 3: Misaminoacylation activity of wild-type and mutant LeuRSs. Panels A and B refer to isoleucylation and valylation mischarging activity, respectively. Each misaminoacylation reaction was carried out in the presence of 4 mg/mL crude *E. coli* tRNA^{Leu}, 25 μM [^{3}H]isoleucine (150 μCi/mL) or [^{3}H]valine (150 μCi/mL), and 3.7 μM enzyme: wild type (■), T247V (▲), T248V (▼), T247/8V (◆), T247/8A (●), and T247/8S (□). Error bars are based on the reaction being repeated at least in triplicate.

Mischarging activity for isoleucine and valine was also tested to identify mutation-dependent editing defects. As found for the single-alanine substitutions at these sites (R. S. Mursinna and S. A. Martinis, unpublished data), each of the single-valine mutations had low, if any, mischarging activity compared to that of wild-type *E. coli* LeuRS (Figure 3). However, the combined T247/8V LeuRS double mutant exhibited significantly higher levels of isoleucylation (Figure 3A) and valylation (Figure 3B) mischarging activity than the corresponding single-mutant proteins. We also constructed double mutants that changed both of the conserved threonines to alanines or serines. The T247/8A double mutant LeuRS exhibited mischarging activity, albeit not as high as that of the T247/8V mutant LeuRS (Figure 3). In contrast, the T247/8S mutant exhibited only low levels of mischarging activity for both isoleucine and valine that were comparable

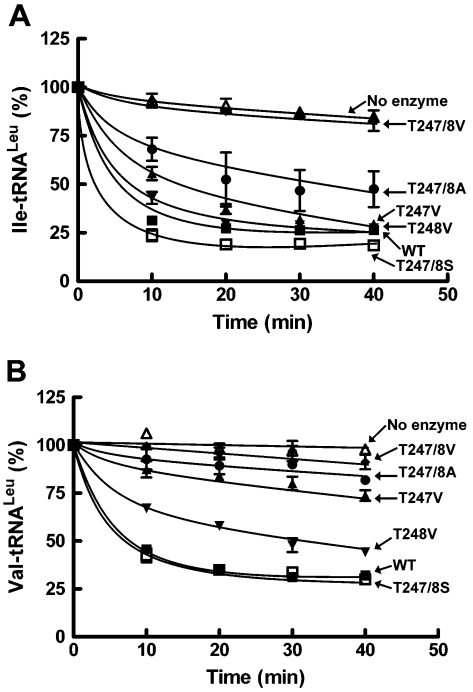

FIGURE 4: Hydrolytic editing activity of wild-type and mutant LeuRSs. Panels A and B refer to the isoleucine and valine editing activity of the mischarged tRNA, respectively. Each hydrolytic editing reaction was carried out in the presence of 10 nM enzyme and 0.2 μM Ile-tRNA^{Leu} or 0.1 μM Val-tRNA^{Leu}. Mischarged tRNA was prepared from crude *E. coli* tRNA purchased from Hoffmann-La Roche Ltd.: wild type (■), T247V (▲), T248V (▼), T247/8V (◆), T247/8A (●), T247/8S (□), and no enzyme (Δ). Error bars are based on the reaction being repeated at least in triplicate.

Table 1: Apparent Kinetic Constants and Thermodynamic Parameters of Wild-Type and Mutant LeuRSs for Post-Transfer Editing Activity of Ile-tRNA$^{Leu}_{UAA}$

parameter	wild type	T247V	T248V	T247/8V	T247/8A	T247/8S
K_M (μM)[a]	1.3	2.6	2.2	4.4	3.6	2.4
k_{cat} (s^{-1})[a]	3.2	0.8	0.9	2×10^{-2}	0.1	5.2
k_{cat}/K_M (μM^{-1} s^{-1})[a]	2.4	0.3	0.4	4.5×10^{-3}	3.1×10^{-2}	2.2
$\Delta\Delta G^{\ddagger}$ (kcal/mol)	–	1.2	1.1	3.7	2.5	0.05
E_a (kcal/mol)	3.5	5.5	4.4	15.5	13.2	3.7

[a] The reported K_M, k_{cat}, and k_{cat}/K_M measurements are apparent values.

to the wild-type activity (Figure 3). This suggests that the side chain hydroxyl moiety at both of these conserved positions is important to fidelity.

Mischarged tRNAs, including Ile-tRNA^{Leu} and Val-tRNA^{Leu}, were isolated to directly test the wild-type and mutant enzymes' post-transfer editing activities. Although the single-valine mutants showed some decreases in editing activity in this direct measurement of post-transfer editing activity (Figure 4), it was not high enough to sustain significant production of mischarged product (Figure 3). This has been found for other mutants that have small decreases in editing activity (*10*). As would be expected from the misaminoacylation activities, the T247/8V mutant LeuRS's hydrolytic editing activity of misisoleucylated tRNA$^{Leu}_{UAA}$ (Figure 4A) was greatly decreased by more than 500-fold (Table 1) with activities similar to that of a control that lacked enzyme. The apparent k_{cat}/K_M was determined to be 4.5×10^{-3} μM^{-1} s^{-1}, which corresponded to a $\Delta\Delta G^{\ddagger}$ of 3.7 kcal/mol. Likewise, the T247/8A double LeuRS mutant post-transfer editing activity was also significantly altered with an apparent k_{cat}/K_M of 3.3×10^{-2} M^{-1} s^{-1}. In both cases, this decrease was primarily due to a significant decrease in the apparent k_{cat}. In contrast, the apparent kinetic parameters for the T247/8S double mutant and single-valine mutant LeuRSs showed little change. Analysis using double mutant cycles (Figure 5) demonstrates that the sum of the $\Delta\Delta G^{\ddagger}$ of each of the single mutants was significantly smaller than the $\Delta\Delta G^{\ddagger}$ of the double T247/8V mutant LeuRS and introduces a coupling energy of 1.4 kcal/mol. Thus, it is possible that the conserved Thr^{247} and Thr^{248} might function cooperatively or their activities are at least weakly coupled (*19*).

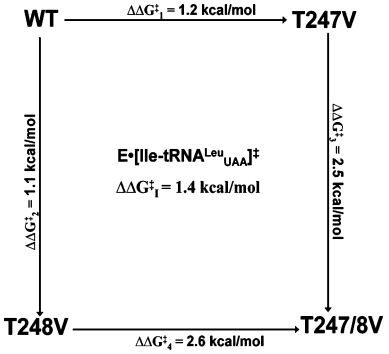

FIGURE 5: Double mutant cycle of Thr^{247} and Thr^{248}. $\Delta\Delta G^{\ddagger}_1 = \Delta\Delta G^{\ddagger}_{T247V}$. $\Delta\Delta G^{\ddagger}_2 = \Delta\Delta G^{\ddagger}_{T248V}$. $\Delta\Delta G^{\ddagger}_3 = \Delta\Delta G^{\ddagger}_{T247/8V} - \Delta\Delta G^{\ddagger}_{T247V}$. $\Delta\Delta G^{\ddagger}_4 = \Delta\Delta G^{\ddagger}_{T247/8V} - \Delta\Delta G^{\ddagger}_{T248V}$. The coupling energy between Thr^{247} and Thr^{248} is calculated on the basis of the relation $\Delta\Delta G^{\ddagger}_I = \Delta\Delta G^{\ddagger}_4 - \Delta\Delta G^{\ddagger}_1$ as described in more detail in Experimental Procedures.

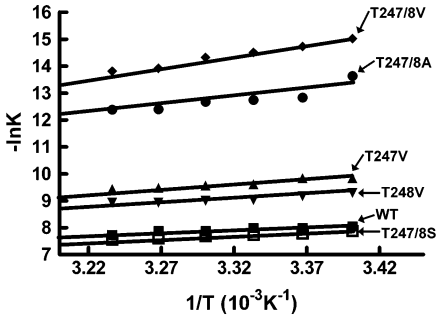

FIGURE 6: Arrhenius plots of wild-type and mutant LeuRS post-transfer editing activity. The amino acid editing reactions were carried out at 21, 24, 27, 30, 33, and 36 °C. The concentrations of enzymes were as follows: 5 nM wild-type or T247/8S LeuRS, 30 nM T247V LeuRS, 25 nM T248V LeuRS, and 1.0 μM T247/8V LeuRS. The concentration of Ile-tRNA$^{Leu}_{UAA}$ was 0.2 μM: wild type (■), T247V (▲), T248V (▼), T247/8V (◆), T247/8A (●), and T247/8S (□).

An Arrhenius plot in which the post-transfer editing activities were measured as a function of temperature determined that the apparent mutant LeuRS's energy of activation (E_a) was increased from 3.5 to 15.5 kcal/mol when both Thr^{247} and Thr^{248} were replaced with valines (Figure 6 and Table 1). The E_a for the double T247/8A mutant LeuRS was also significantly increased. In comparison, the single mutants and retention of the hydroxyl group in the T247/8S double mutant LeuRS resulted in only very small changes in their respective E_a measurements that were consistent with their wild-type-like editing activities. Thus, we hypothesize that these two collaborating threonine residues might aid in

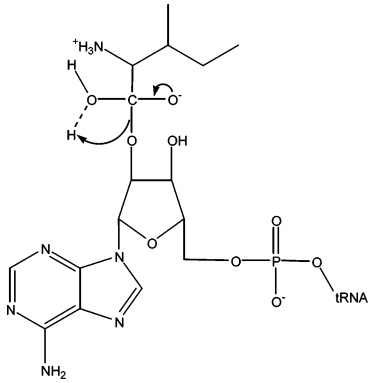

FIGURE 7: Tetrahedral structure of the putative oxyanion transition state intermediate during post-transfer editing activity. The representative mischarged amino acid is isoleucine. Arrows indicate the proposed movement of electrons to hydrolyze mischarged tRNA.

transition state stabilization by donating hydrogen bonds to the activated editing complex.

DISCUSSION

Although parts of the aaRS CP1-based editing active sites have been localized and some key amino acids that are critical to editing activity have been identified (*6*−*10*, *12*, *20*, *22*−*25*), the hydrolytic mechanism for amino acid editing remains poorly understood. Herein, we characterized two nearby threonines that might collaborate during catalysis and also in stabilizing the transition state of the hydrolytic reaction of *E. coli* LeuRS. The cocrystal structure of LeuRS shows that the post-transfer editing substrate analogue is bound in the active site via a consortium of redundant interactions (Figure 1C; *7*). Thr^{247} and Thr^{248} contribute four of these hydrogen bonds. Single mutations of these two threonines did not yield a significant difference in amino acid editing activity compared to wild-type LeuRS. Similarly, mutation of the equivalent conserved threonine residues in *E. coli* IleRS (Thr^{241} and Thr^{242}) showed little difference in activity compared to the wild-type IleRS editing activity (*20*, *26*). This network of hydrogen bonding interactions may be universal to the homologous LeuRS, IleRS, and ValRS CP1 domains.

The 3′-hydroxyl group of the ribose ring is involved in multiple hydrogen bonds that include the main chain amide and the side chain hydroxyl group of Thr^{248} and also the side chain hydroxyl group of Thr^{247}. A single mutation of Thr^{248} only eliminates one of these triplicated hydrogen bonds and results in little, if any, effect on amino acid editing. Similarly, this would explain in part why the Thr^{247} single mutant did not show much difference compared to wild-type LeuRS. Although no obvious effect is observed, these hydrogen bonds and also the universally conserved Asp^{345} (*7*) likely function collectively to orient the cleavage site during amino acid editing.

Thr^{247} of wild-type LeuRS also forms a hydrogen bond at the cleavage site to the bound amino acid's carbonyl oxygen (Figure 1C; *7*). It has been proposed that this carbonyl would form an oxyanion intermediate during catalysis (Figure 6; *7*). If this were the case, then Thr^{247} would be poised to stabilize the transition state by donating a hydrogen bond. Since a single mutation of this conserved threonine had little effect on editing, it is possible that small structural perturbations allow the neighboring threonine residue in the class Ia synthetases to form a hydrogen bond with the carbonyl oxygen and can significantly compensate for the missing hydroxyl group in the mutant protein.

Because of their close positions, it is also possible that the neighboring threonines both form hydrogen bonds with the putative oxyanion intermediate during catalysis. In the ground state, the distance between the oxygen of the Thr^{248} side chain hydroxyl group and the carbonyl oxygen is 5.96 Å. Thus, minimal conformational changes within the enzyme could facilitate hydrogen bond interactions in either the ground or the transition state. Conformational changes are likely required since the current consortium of X-ray crystal structures for class IA editing tRNA synthetases in the presence and absence of editing substrate analogues (*6*, *7*, *20*, *21*, *23*, *27*−*31*) fail to identify an obvious oxyanion hole (*32*) that would be expected to stabilize the transition state intermediate. Access of both threonines to the carbonyl oxygen could also be aided by shifting the carbonyl carbon from a trigonal geometry in the ground state to a tetrahedral geometry in the transition state. Although we performed modeling studies using a bound tetrahedral substrate analogue, we still were unable to detect an oxyanion hole (Y. Zhai and S. A. Martinis, unpublished results). However, the distance between the threonine side chains and the oxyanion remained less than 6 Å. If these two threonine side chains contribute multiple hydrogen bonds to the transition state, the energy of activation would be lowered and facilitate catalysis. As shown for other examples of biological catalysts, such as the hairpin ribozyme, multiple mutations of sites that donate redundant hydrogen bonds to the substrate transition state could be required before catalytic activity is appreciably affected (*33*).

When Thr^{247} and Thr^{248} are simultaneously mutated to valine or alanine, two important hydrogen bonds in the substrate-bound ground state are eliminated. The significantly reduced post-transfer editing activity is largely due to a k_{cat} effect (Table 1). If redundant hydrogen bonds exist between the putative oxyanion intermediate and hydroxyl side chains of Thr^{247} and Thr^{248}, their absence would be expected to increase the activation energy and lower editing activity (Table 1). The T247/8V double mutant LeuRS exhibited a dramatic increase in mischarging activity due to poor post-transfer amino acid editing that corresponded to an increase in the activation energy of ∼12 kcal/mol. In contrast, maintenance of a hydroxyl group by introduction of serine at both positions maintained a wild-type-like activity that correlated with similar activation energies. Notably, the T247/8A mutant LeuRS exhibited some editing activity. In the absence of a bulky side chain, we hypothesize that a water molecule with hydrogen bonding potential could fit in the void similar to that found for serine protease mutants (*34*) and at least partially fulfill a role that requires a hydrogen bond.

Whether interactions with the ground state, transition state, or both are affected, thermodynamic measurements support the idea that the neighboring threonines are collaborating for catalysis. Double mutant cycle analysis shows that the sum of the $\Delta\Delta G^{\ddagger}$ of the single-mutant T247V and T248V LeuRSs is significantly smaller than the $\Delta\Delta G^{\ddagger}$ for the double-mutant T247/8V LeuRS (Table 1) and introduces a coupling energy of ∼1.4 kcal/mol. This suggests that the

neighboring threonines at least weakly interact or collaborate to aid in either transition state stabilization or the reaction mechanism of hydrolytic editing (*17*).

These neighboring threonine residues are highly conserved in the threonine-rich region of LeuRS, ValRS, and IleRS. In addition, the cocrystal structure of LeuRS shows that the both threonines interact with moieties of the substrate that are in the proximity of the cleavage site and that are common to the editing substrates for ValRS and IleRS. Thus, we propose that these two threonines play similar roles in the catalytic mechanism for IleRS and ValRS. This contrasts with a nearby conserved threonine (Thr^{252}) that is also conserved within the threonine-rich area, but was shown to serve as a critical specificity factor for LeuRS and interacts with the side chain of the bound mischarged amino acid (*8*).

ACKNOWLEDGMENT

We thank Drs. Richard S. Mursinna and Tommie L. Lincecum for technical advice. We are grateful to Drs. James M. Briggs and Wei Fu for providing their SGI and expertise.

REFERENCES

1. Martinis, S. A., Plateau, P., Cavarelli, J., and Florentz, C. (1999) Aminoacyl-tRNA synthetases: A family of expanding functions, *EMBO J. 18*, 4591−4596.
2. Hendrickson, T. L., and Schimmel, P. (2003) Transfer RNA-dependent amino acid discrimination by aminoacyl-tRNA synthetases, in *Translation Mechanisms* (LaPointe, J., and Brakier-Gingras, L., Eds.) pp 34−64, Klumer Academic/Plenum Publishers, New York.
3. Fersht, A. R. (1998) Sieves in sequence, *Science 280*, 541.
4. Fersht, A. R. (1977) Editing mechanisms in protein synthesis. Rejection of valine by the isoleucyl-tRNA synthetase, *Biochemistry 16*, 1025−1030.
5. Fersht, A. R., and Dingwall, C. (1979) Evidence for the double-sieve editing mechanism in protein synthesis. Steric exclusion of isoleucine by valyl-tRNA synthetases, *Biochemistry 18*, 2627−2631.
6. Cusack, S., Yaremchuk, A., and Tukalo, M. (2000) The 2 Å crystal structure of leucyl-tRNA synthetase and its complex with a leucyl-adenylate analogue, *EMBO J. 19*, 2351−2361.
7. Lincecum, T. L., Jr., Tukalo, M., Yaremchuk, A., Mursinna, R. S., Williams, A. M., Sproat, B. S., Van Den Eynde, W., Link, A., Van Calenbergh, S., Grøtli, M., Martinis, S. A., and Cusack, S. (2003) Structural and mechanistic basis of pre- and posttransfer editing by leucyl-tRNA synthetase, *Mol. Cell 11*, 951−963.
8. Mursinna, R. S., Lincecum, T. L., Jr., and Martinis, S. A. (2001) A conserved threonine within *Escherichia coli* leucyl-tRNA synthetase prevents hydrolytic editing of leucyl-$tRNA^{Leu}$, *Biochemistry 40*, 5376−5381.
9. Mursinna, R. S., Lee, K. W., Briggs, J. M., and Martinis, S. A. (2004) Molecular dissection of a critical specificity determinant within the amino acid editing domain of leucyl-tRNA synthetase, *Biochemistry 43*, 155−165.
10. Mursinna, R. S., and Martinis, S. A. (2002) Rational design to block amino acid editing of a tRNA synthetase, *J. Am. Chem. Soc. 124*, 7286−7287.
11. Starzyk, R. M., Webster, T. A., and Schimmel, P. (1987) Evidence for dispensable sequences inserted into a nucleotide fold, *Science 237*, 1614−1618.
12. Chen, J. F., Guo, N. N., Li, T., Wang, E. D., and Wang, Y. L. (2000) CP1 domain in *Escherichia coli* leucyl-tRNA synthetase is crucial for its editing function, *Biochemistry 39*, 6726−6731.
13. Tocchini-Valentini, G., Saks, M. E., and Abelson, J. (2000) tRNA leucine identity and recognition sets, *J. Mol. Biol. 298*, 779−793.
14. Sampson, J. R., and Uhlenbeck, O. C. (1988) Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed *in vitro*, *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033−1037.
15. Martinis, S. A., and Fox, G. E. (1997) Non-standard amino acid recognition by *Escherichia coli* leucyl-tRNA synthetase, *Nucleic Acids Symp. Ser. 36*, 125−128.
16. Schreier, A. A., and Schimmel, P. R. (1972) Transfer ribonucleic acid synthetase catalyzed deacylation of aminoacyl transfer ribonucleic acid in the absence of adenosine monophosphate and pyrophosphate, *Biochemistry 11*, 1582−1589.
17. Wells, J. A. (1990) Additivity of mutational effects in proteins, *Biochemistry 29*, 8509−8517.
18. Xin, X., Xi, L., and Tu, S. C. (1994) Probing the *Vibrio harveyi* luciferase β subunit functionality and the intersubunit domain by site-directed mutagenesis, *Biochemistry 33*, 12194−12201.
19. Wilkinson, A. J., Fersht, A. R., Blow, D. M., and Winter, G. (1983) Site-directed mutagenesis as a probe of enzyme structure and catalysis: Tyrosyl-tRNA synthetase cysteine-35 to glycine-35 mutation, *Biochemistry 22*, 3581−3586.
20. Nureki, O., Vassylyev, D. G., Tateno, M., Shimada, A., Nakama, T., Fukai, S., Konno, M., Hendrickson, T. L., Schimmel, P., and Yokoyama, S. (1998) Enzyme structure with two catalytic sites for double-sieve selection of substrate, *Science 280*, 578−582.
21. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylyev, D. G., and Yokoyama, S. (2000) Structural basis for double-sieve discrimination of L-valine from L-isoleucine and L-threonine by the complex of $tRNA^{Val}$ and valyl-tRNA synthetase, *Cell 103*, 793−803.
22. Tang, Y., and Tirrell, D. A. (2002) Attenuation of the editing activity of the *Escherichia coli* leucyl-tRNA synthetase allows incorporation of novel amino acids into proteins *in vivo*, *Biochemistry 41*, 10635−10645.
23. Fukunaga, R., Fukai, S., Ishitani, R., Nureki, O., and Yokoyama, S. (2004) Crystal structures of the CP1 domain from *Thermus thermophilus* isoleucyl-tRNA synthetase and its complex with L-valine, *J. Biol. Chem. 279*, 8396−8402.
24. Hountondji, C., Lazennec, C., Beauvallet, C., Dessen, P., Pernollet, J. C., Plateau, P., and Blanquet, S. (2002) Crucial role of conserved lysine 277 in the fidelity of tRNA aminoacylation by *Escherichia coli* valyl-tRNA synthetase, *Biochemistry 41*, 14856−14865.
25. Hountondji, C., Beauvallet, C., Dessen, P., Hoang-Naudin, C., Schmitter, J. M., Pernollet, J. C., and Blanquet, S. (2000) Valyl-tRNA synthetase from *Escherichia coli* MALDI-MS identification of the binding sites for L-valine or for noncognate amino acids upon qualitative comparative labeling with reactive amino-acid analogs, *Eur. J. Biochem. 267*, 4789−4798.
26. Hendrickson, T. L., Nomanbhoy, T. K., and Schimmel, P. (2000) Errors from selective disruption of the editing center in a tRNA synthetase, *Biochemistry 39*, 8180−8186.
27. Fukunaga, R., and Yokoyama, S. (2005) Crystal structure of leucyl-tRNA synthetase from the archaeon *Pyrococcus horikoshii* reveals a novel editing domain orientation, *J. Mol. Biol. 346*, 57−71.
28. Silvian, L. F., Wang, J., and Steitz, T. A. (1999) Insights into editing from an Ile-tRNA synthetase structure with $tRNA^{Ile}$ and mupirocin, *Science 285*, 1074−1077.
29. Nakama, T., Nureki, O., and Yokoyama, S. (2001) Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase, *J. Biol. Chem. 276*, 47387−47393.
30. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Vassylyev, D. G., and Yokoyama, S. (2003) Mechanism of molecular interactions for $tRNA^{Val}$ recognition by valyl-tRNA synthetase, *RNA 9*, 100−111.
31. Fukunaga, R., and Yokoyama, S. (2005) Structural basis for non-cognate amino acid discrimination by the valyl-tRNA synthetase editing domain, *J. Biol. Chem. 280*, 29937−29945.
32. Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972) Subtilisin: A stereochemical mechanism involving transition-state stabilization, *Biochemistry 11*, 4293−4303.
33. Rupert, P. B., Massey, A. P., Sigurdsson, S. T., and Ferre-D'Amare, A. R. (2002) Transition state stabilization by a catalytic RNA, *Science 298*, 1421−1424.
34. Carter, P., and Wells, J. A. (1990) Functional interaction among catalytic residues in subtilisin BPN′, *Proteins 7*, 335−342.

BI0514461