Molecular Mechanism of Amino Acid Discrimination for the Editing Reaction of *E.coli* Leucyl-tRNA Synthetase

Keun Woo Lee, Minky Son, Chanin Park, and Ayoung Baek

Abstract—Certain tRNA synthetases have developed highly accurate molecular machinery to discriminate their cognate amino acids. Those aaRSs achieve their goal via editing reaction in the Connective Polypeptide 1 (CP1). Recently mutagenesis studies have revealed the critical importance of residues in the CP1 domain for editing activity and X-ray structures have shown binding mode of noncognate amino acids in the editing domain. To pursue molecular mechanism for amino acid discrimination, molecular modeling studies were performed. Our results suggest that aaRS bind the noncognate amino acid more tightly than the cognate one. Finally, by comparing binding conformations of the amino acids in three systems, the amino acid binding mode was elucidated and a discrimination mechanism proposed. The results strongly reveal that the conserved threonines are responsible for amino acid discrimination. This is achieved through side chain interactions between T252 and T247/T248 as well as between those threonines and the incoming amino acids.

Keywords—Amino acid discrimination, Binding free energyLeucyl-tRNAsynthetase, Molecular dynamics.

I. INTRODUCTION

ACCUARCY in protein synthesis is highly dependent on the correct aminoacylation of an amino acid to its cognate tRNA [1]-[3]. This very important reaction is controlled by a family of enzymes called aminoacyl-tRNAsynthetases (aaRSs). The overall reaction is achieved by an aaRS through two steps. First, an aminoacyl-adenylate is synthesized from the amino acid and ATP, and the aminoacyl moiety is then transferred from the adenylate to the 3′-terminal adenosine (3′-A) of tRNA [4]. Some aaRSs have developed very accurate molecular recognition mechanisms to discriminate their cognate amino acids against amino acids containing even just a one methyl difference. Previously, using the theory of thermodynamics Pauling estimated the error rate that the isoleucyl-tRNAsynthetase (IleRS) distinguishes between valine and isoleucine to be 1/5 [5], however, the measured rate

in the real experiments is less than 1/3000 [6]. This high accuracy is achieved through a proofreading or editing reaction that hydrolyzes incorrectly activated or charged amino acids in a tRNA-dependent manner [7]-[11]. The specificity of the amino acid activation and that of the subsequent editing activity of the aaRS has been referred to as adouble sieve mechanism since the specificity is conferred through the action of separate amino acid activation and editing catalytic sites [11]-[14]. The activation/aminoacylation site is in the ATP-binding Rossman fold domain, which is common to all class I aaRSs, and the editing active site lies in a large inserted domain called Connective Polypeptide 1 (CP1) [15]-[18]. In general, there are two different editing substrates, pre- and post-transfer [8]-[9], [11]. In the pre-transfer editing pathway, aaRS complexed with the cognate tRNA directly hydrolyzes the amino acid (AA)-AMP to AA + AMP. While, in post-transfer editing, the amino acid moiety is already transferred to the 3′-end of tRNA, and the AA-tRNA is then hydrolyzed by aaRS. Therefore, in the editing reaction, AA-AMP binds to the editing domain for the pre-transfer editing pathway, while the AA attached to tRNA will bind to the domain in the post-transfer editing reaction.The homology of the CP1 domain is extensively shared among the class IatRNAsynthetases: leucyl-, isoleucyl- and valyl-tRNAsynthetases (LeuRS, IleRS and ValRS). Several X-ray structures for the class IaaaRSs are available [15]-[19]. Among them, two structures revealed an amino acid or analog in the editing domain: a free valine in *T. thermophilus* IleRS15 and an amino acid analog in *T. thermophilus*LeuRS [19]. It was also found that the pre-transfer editing site is overlapping with the post-transfer editing one [19]. In addition to the structural studies, many experimental mutagenesis studies of the editing domains have reported that a universally conserved aspartic acid is critical for the editing activity [19]-[20].Also, certain threonines in the conserved threonine-rich region are involved in the editing activity [21]-[24]. However, the core mechanism for the amino acid discrimination is not yet fully understood. Therefore, we have investigated this classic puzzle of the highly accurate amino acid discrimination mechanism of the editing reaction using state-of the art molecular modeling methods, including MD simulations. One especially pertinent experimental result is that the T252A mutant *E. coli*LeuRS edits its cognate leucine from the aminoacylatedtRNA [22].To investigate this phenomenon, four models were constructed and subjected to MD simulations which were then analyzed to address the puzzle.

Keun Woo Leeis with the Division of Applied Life Science (BK21 program), Gyeongsang National University (GNU), 501 Jinju-daero, Gazwa-dong, Jinju 660-701, Republic of Korea (phone: +82-55-772-1360; fax: +82-55-772-1359; e-mail: kwlee@bio.gnu.ac.kr).

Minky Son is with the Division of Applied Life Science (BK21 program), Gyeongsang National University (GNU), 501 Jinju-daero, Gazwa-dong, Jinju 660-701, Republic of Korea (e-mail: minky@bio.gnu.ac.kr).

Chanin Park is with the Division of Applied Life Science (BK21 program), Gyeongsang National University (GNU), 501 Jinju-daero, Gazwa-dong, Jinju 660-701, Republic of Korea (chip@bio.gnu.ac.kr).

Ayoung Baek is with the Division of Applied Life Science (BK21 program), Gyeongsang National University (GNU), 501 Jinju-daero, Gazwa-dong, Jinju 660-701, Republic of Korea (abaek@bio.gnu.ac.kr).

World Academy of Science, Engineering and Technology
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II. METHODS

A. Protein and Ligand Preparation

Four different models were built to study the amino acid binding mode in the editing domain and then subjected to 2 ns MD simulations. The models included the CP1 domain of wild-type LeuRS (designated as LeuRS_CP1WT( )), bound by leucine (LeuRS_CP1WT(Leu)), or isoleucine (LeuRS_CP1WT(Ile)), and finally the CP1 domain of the T252A mutant LeuRS bound by leucine (LeuRS_CP1T252A(Leu)). The binding conformations for isoleucine and leucine in the T252A model were very similar to that of leucine in the WT model [25]. The amino and carboxyl termini of the docked isoleucine and leucine ligands were charged as in the docking calculations [25]. Unlike the automated docking study where the entire LeuRS structure was considered, only the CP1 (editing) domain containing 194 residues from I224 to L417 were used in the MD simulations in order to reduce the computational cost. This was accomplished by cutting the CP1 domain out of the full-length homology modeled E. coli structure prepared and refined via molecular dynamics in a prior study [25].

B. Computational Details

Four different models of the LeuRS CP1 domain were fully hydrated with water box that contained about 7600 water molecules. Counter ions were then introduced to neutralize the charge of entire system. After initial setup using CHARMM [26], the remaining calculations including minimization, equilibration, and production MD runs were completed using NAMD [27]. CHARMM27 parameters, periodic boundary conditions, and a dielectric constant of 1 were used throughout the simulations. A spherical cutoff of 10.5 Å was used to truncate the pairwise interactions and a switching function was used between 9.5 Å and 10.5 Å so that the potential function would go smoothly to zero by 10.5 Å. Energy minimizations were performed for the entire systems using 1000 steps using the conjugate gradient algorithm. The temperature for each system was increased from 0 K to 298 K, then equilibrated for 100 ps of MD. Initially, 2 ns production runs were performed for all four models, and later for verification purposes of binding stability, the simulation time was extended to 5 ns for two models: wild-type bound to leucine (LeuRS_CP1WT(Leu)) and wild-type bound to isoleucine (LeuRS_CP1WT(Ile)). The temperature and pressure were controlled at 298 K and 1 bar by Berendsen's coupling method [28]. A 2 fs time step was used in the MD simulations along with the SHAKE algorithm to constrain all bonds involving hydrogen atoms. A snapshot of the molecular system was saved every 250 steps (0.5 ps) for later analysis.

C. Essential Dynamics Analysis

Essential dynamics (ED) is a method that can filter out the locally confined fluctuations or vibrational motions in a macromolecular system and focus on the few large, global structural motions [29]. ED analyses were performed for the 4000 conformations from the first 2 ns MD simulation trajectories. Each frame of the trajectory was superimposed onto the starting geometry. ED analysis was focused on the movement of the 194 C atoms of the protein in order to trace the backbone atom movements. The Cartesian coordinates for the atoms result in 582 dimensional displacement vectors. The covariance matrix was constructed based on the coordinates from the 4000 conformations and then diagonalized to obtain its eigenvalues and eigenvectors. Movements in the essential subspace are projected along the eigenvectors from the analyses. By projection of an eigenvector on the matrix, two final protein structures representing the two extreme states in the motion range result. The essential motion for an eigenstate can be obtained from comparison or visualization of the two structures. The ED analyses were performed using the WHATIF program [30].

D. Binding Free Energy Calculations

Among the currently available methods for calculating binding free energies, free energy perturbation (FEP) and thermodynamic integration (TI) methods are the most rigorous ones. However, those methods are not ideally suited for macro molecular systems such as protein-ligand interactions because of the substantial computational cost. Two MD based approaches are linear interaction energy (LIE) [31]-[33] and molecular mechanics-Poisson Boltzmann surface area (MM-PBSA) [34]-[35]. Both methods have reasonable accuracies and require a similar amount of computational effort [35]. In the present study, the LIE method was employed. In this calculation, instead of using the entire 2 ns trajectories, the first 1.5 ns trajectories were used for a fair comparison because there was a significant binding conformation change after ca. 1.8 ns for the LeuRS_CP1WT(Leu) model.

III. RESULTS

2 ns MD simulations were performed for the four different models of the CP1 domain of E. coliLeuRS to investigate the binding modes of cognate and noncognate amino acids in the editing domain. From the models that include a bound amino acid, the LeuRS_CP1WT(Ile) and LeuRS_CP1T252A(Leu) models are expected to be editing models because the Ile-tRNALeu is naturally destined to be edited by the LeuRS and the leucine of Leu-tRNALeu was shown to be hydrolyzed by the T252A mutant LeuRS [22]. Only the LeuRS_CP1WT(Leu) model should not edit because Leu is the cognate amino acid. The initial structure of the protein was obtained from a homology model of E. coli LeuRS built from the X-ray structure of *T. thermophilus* LeuRS [18], [25]. The overall structure of the E. coli LeuRS editing (CP1) domain and the bound leucine is shown in Fig. 1.

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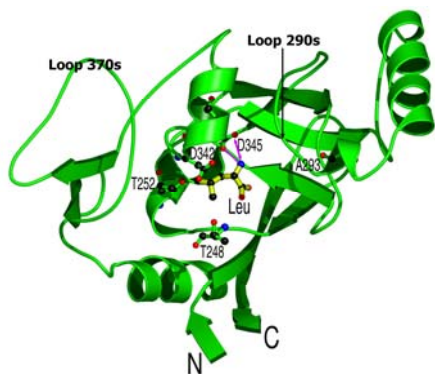International Journal of Bioengineering and Life Sciences
Vol:7, No:1, 2013

Fig. 1 The initial structure of the leucine ligand (yellow) and editing domain of E. coli LeuRS used in the MD simulations

The initial ligand complexes with the CP1 domain for leucine and isoleucine were obtained through systematic docking studies. The results showed that the binding conformations of the ligands were very consistent with valine in the co-crystal structure of the editing pocket of *T. thermophilus*IleRS [15], [25]. The conserved interactionbetween the amino terminus of the bound amino acid and the Oδ (OD) atom of D345 is highlighted in Fig. 1.

A. Basic MD Analyses

The root mean square deviations (RMSDs) for the backbone atoms between the first frame and the subsequent frames were measured (Fig. 2). Overall, the RMSDs stabilized to between 3-4 Å. The RMSD of the WT with leucine (LeuRS_CP1WT(Leu)) was slightly higher than that of the WT without leucine (LeuRS_CP1WT( )), suggesting that the introduction of the leucine ligand resulted in a perturbation in the CP1 domain. However, the introduction of isoleucine (LeuRS_CP1WT(Ile)) resulted in reduction of the RMSD, which seems to indicate that the isoleucine is stably bound to the editing pocket in the CP1 domain. This is the most fundamental evidence suggesting that isoleucine is better accommodated than leucine in the editing domain of LeuRS. The RMSD of the mutant LeuRS with leucine (LeuRS_CP1T252A(Leu)) was very similar to that of the WT LeuRS without leucine (LeuRS_CP1WT( )), suggesting that the mutation of threonine 252 to alanine does not introduce instability in the ligand binding site.
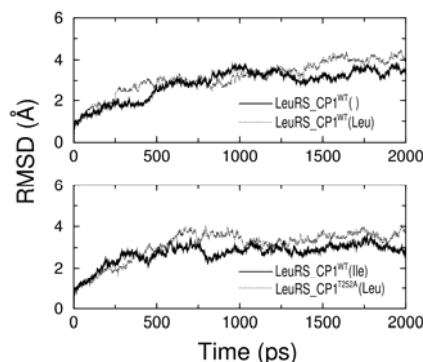

Fig. 2 RMSD plot over the 2 ns MD simulations for the four models

B. Essential Dynamics Analyses

In the ED study, our main concern is to elucidate the largest essential/correlated motion of the protein, which can be obtained by manipulating the first couple of eigenvectors since they are likely to be the most significant motions of the protein.To quantify the essential motion, two protein structures in the two extreme motion ranges of the given eigenvector are overlaid, however, four MD trajectories were analyzed herein. In this study, the Cα-Cα distance measurements were performed for all four models. For the measurements, the maximum and minimum protein structures projected via the first eigenvector were superimposed and the Cα-Cα distances between the two structures were measured to provide quantitative information about the amount as well as the type of movement. The Cα-Cα distance plots for the four models show the range of motion for each residue (Fig. 3). The results reveal two significant motions: loop 290s and loop 370s. The atomic fluctuation calculations focus on the flexibility rather than the range of motion in ED analysis. Results show that the range of motion of the loop 370s was restricted by ligand binding. An interesting result comes from loop 290s. The range of motion for this loop in the ligand free model was about 13-15 Å and was not affected by ligand binding for the editing models. Interestingly, however, when leucine is in the editing site, the movement of the loop 290s was significantly reduced. This phenomenon provides some clues regarding the mode of interaction between the editing site and the bound amino acid ligand. That is, the loop 290s interacts with the amino acid only in the editing-free case (LeuRS_CP1WT(Leu)). This trend will be reinforced in the essential dynamics analyses.
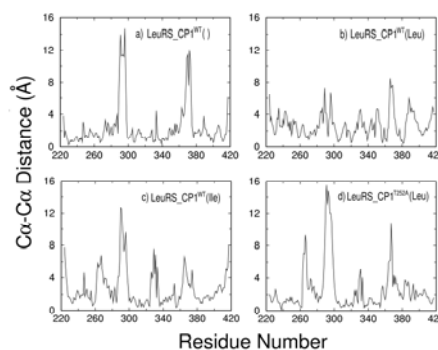

Fig. 3 Cα-Cα distances between two extreme protein structures projected from the 1st eigenvector obtained from essential dynamics analyses were performed for four models

C. Dynamical Behavior of Leucine/Isoleucine

To study the dynamical behavior and stability of bound amino acid ligands in the editing site, the distances between ligand and protein was measured along the simulation trajectory. The O1 atom of D345 in the CP1 domain and the N atom of the ligand were selected for the measurements because all available CP1 X-ray structures containing a noncognate amino acid exhibited a hydrogen-bonding interaction between the amino acid and the universally conserved residue, suggesting its potential importance [15], [19], [25]. Up to 1.7 ns,

World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:7, No:1, 2013

the distances in all models were largely stable at around 2.7 Å (Fig. 4). However, the hydrogenbond in the editing-free model LeuRS_CP1WT(Leu) dissociated at ca. 1.8 ns (Fig. 4a). This suggests that the binding of leucineis weaker than that of isoleucine in the editing site of LeuRS since the noncognate isoleucine showed good stability during the entire 2 ns trajectory (Fig. 4b). This strongly suggests that the editing aaRS binds more tightly with the noncognate amino acid than the cognate one in the editing site. At this point, it is very interesting to check the result for the LeuRS_CP1T252A(Leu) model because the modified aaRS, mutant T252A, recognized the cognate leucine as it would a noncognate alien [22]. The simulation result showed that the binding is very stable, similar to what was seen in LeuRS_CP1WT(Ile) model, since the plot was almost same as Fig. 4b. Based on the above observations, we hypothesize that stable binding of amino acids within the editing site is required for an efficient editing reaction.

Is the 2 ns simulation time enough to evaluate the binding stability of those ligands? In order to address this criticism and to refine the tight binding model, two additional studies were pursued: the MD simulation time was extended to 5 ns and binding free energies were calculated.To answer these questions, two additional 3 ns of MD simulation were performed for the two models of LeuRS_CP1WT(Leu) and LeuRS_CP1WT(Ile). The results of the longer time simulations showed that not only were the isoleucine very stable during the entire 5 ns (Fig. 4d), they also suggest a possible release mode of the ligand (Fig. 4c). After staying near the pocket for about 1.5 ns, the leucine ligand completely dissociated after 3.3 ns. Binding affinities of amino acid ligands in the editing pocket have not yet been reported. In an attempt to account for the thermodynamic aspects of the amino acid binding, we first employed free energy calculations for those amino acids in the editing pocket.
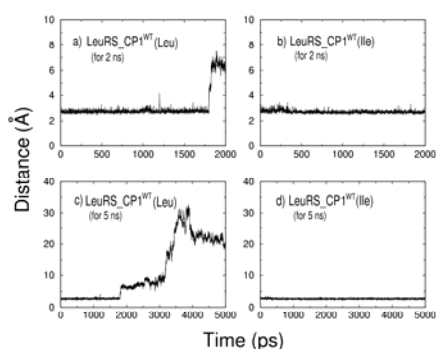

Fig. 4 Interatomic distances between the Oδ1 atom of D345 and the nitrogen terminus of the bound amino acids, leucine or isoleucine

D. Binding Free Energy Calculations

In addition to the assessment of the dynamical behavior of the bound amino acids in the editing domain, the binding affinities of the ligands were also investigated. Linear interaction energy (LIE) [33]-[35] is a relatively rapid and accurate MD-based method for computing binding free energies in macromolecular systems, taking into account

protein dynamics, solvent, and ligand dynamics. 3000 snapshot structures from the first 1.5 ns were used in the computation of ensemble averages of electrostatic and van der Waals interaction energies.Binding in the editing-free model was unfavorable (+1.8 kcal/mol) while the two editing models exhibited favorable and stronger binding affinities (-3.4 and -1.8, respectively). That is, the aaRS binds more tightly to the ligands which will be edited than the one will not be edited.

E. Amino Acid Binding Mode and Position of Nucleophilic Water Molecule

Investigation of the amino acid binding mode in the editing site is of crucial importance to understanding the editing mechanism. The structure that was most similar to the ensemble average structure during the first 1.5 ns of the MD trajectories was selected as the representative structure for each model for comparison: the three snapshot structures were taken at 842 ps, 942 ps, and 587 ps from the trajectories for the models LeuRS_CP1WT(Ile), LeuRS_CP1WT(Leu), and LeuRS_CP1T252A(Leu), respectively (Fig. 5). Unlike for *T. thermophilus*, no editing aaRS structure is available for *E. coli*. Therefore, these MD structures are a unique resource in investigating the numerous experimental data available for the *E. coli* LeuRS editing domain [19]-[24]. Fig. 5a shows the noncognate isoleucine binding conformation in the editing domain of the LeuRS_CP1WT(Ile) model. The amino groups of ligands interact with the universally conserved D345 while the side chains of the ligands lie between the conserved threonine residues.Another hydrogen-bond was found between the amino group and the backbone O atom of M336. This binding format is very consistent with the two available X-ray structures [15], [19]. From comparison with the AA-AMP X-ray structure [19], it turns out that the leftmost carboxyl O atom is supposed to be linked to the tRNA. We argue that our simulation results are reasonable because the tRNA should reside at the outside of the pocket. The X-ray structure also revealed a water molecule between D342 and the carboxyl group of the bound amino acid. It has been proposed that this water may be the nucleophile for the hydrolysis reaction [19]. Our MD structures also identified water molecules with very long residence times. This can be very valuable information as it provides exact positions for potential nucleophiles in the bond cleavage editing reactions or potential locations for inhibitor atoms. The second panel in Fig. 5b shows a leucine bound in the editing pocket of WT LeuRS.

F. Comparison of the BindingModes of Cognate and Non Cognate Amino Acids

To investigate the differences in binding mode between the cognate and noncognate ligands, the three structures of the bound amino acids were superimposed on each other (Fig. 5d-5f). The two editing models, e.g., leucine in the T252A mutant and isoleucine in WT, were surprisingly well aligned (Fig. 5f). The amino and carboxyl terminal atoms of both molecules overlapped perfectly and the two C (CD) side chain atoms of leucine overlap with the terminal C and the C2 atoms

World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:7, No:1, 2013

of isoleucine. However, in the non editing model with the cognate leucine in the WT editing site, the ligand was not well aligned with both of the editing ligands (Fig. 5d-5e). It was in a significantly shifted position and a slightly different shape as compared to the two editing models. The main difference lies in the position of the carboxyl group. The superimposed structure clearly shows an upward shift of the carboxyl group in the leucine ligand as compared to that of the other two ligands (Fig. 5d-5e).This is a key result of this study as it provides critical information concerning the editing conformations of amino acids as well as a demonstration of the accuracy of our computer simulations. We suggest this conformation as the editing mode for the hydrolysis reaction. In the biological setting, the amino acid ligand is connected to tRNA through the COO- moiety in the post-transfer editing reaction. Therefore, the location of the carboxyl group is highly important as this is where the editingreaction occurs. Fig. 5 clearly shows that the leucine ligand in the WT LeuRS is definitely translated upward as compared to the other ligands, potentially in order to avoid fitting. When the cognate leucine is bound, or docking, the side chain of T252 is shifted a bit toward the side chain of T248. It appears that both threonines tightly bind together, thereby preventing the side chain of leucine from being inserted between them.For the non-editing LeuRS_CP1WT(Leu) modele, the closest distance between the heavy atoms of residues 252 and 248 is only 3.8 Å. This is significantly shorter as compared to 6.8 Å for the LeuRS_CP1WT(Ile) and 8.3 Å for LeuRS_CP1T252A(Leu) models. It seems that there is no space between them to accommodate the incoming leucine and the 3 Å distance difference controls the inclusion or exclusion of the terminal methyl group of the bound amino acids.
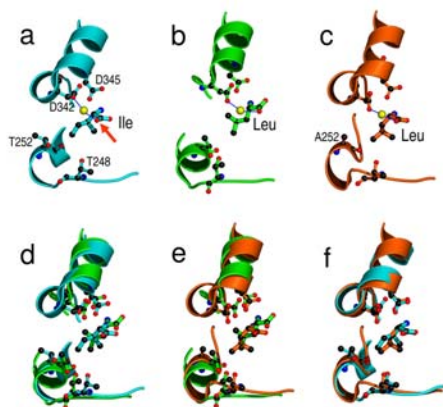

Fig. 5MD snapshot structures showing amino acid binding modes

G. Role of T252in Amino Acid Discrimination

Based on our observations of the MD structures, we identified two key interactions that are required for the editing reaction or to control the editing form/fit. One is the interaction between the amino group of the ligand and the conserved D345 while the other is the interaction between the side chain of the ligand and the two threonines, T252 and T248. It is most likely that the former is responsible for the major anchoring of the ligand while the latter controls fine tuning of the overall ligand

conformation for the editing reaction. That is, interactions between the threonines and the side chain of the ligand control the positioning of the carboxyl group so that it is in the correct position for the editing fit, which then determines the placement of the incoming amino acid for the hydrolysis reaction. The terminal methyl group of isoleucine is inserted between T252 and T248 resulting in an editing competent fit. Both threonines interact with the terminal methyl group of the isoleucine ligand using the hydroxyl group instead of the methyl group. The carboxyl group is well placed for the hydrolysis reaction. In order to identify residues which may be involved in breaking the covalent bond between the amino acid ligand and tRNA, the atoms closest to the carboxyl terminus in the ligand were identified. One side chain carbon atom of V335 was found at a distance of 4.4 Å from one O atom of the carboxyl group in the ligand amino acid. Unlike the binding of isoleucine, the two conserved threonines interact closely with one another when leucine is bound to the editing domain, causing a shift of the two methyl groups of leucine. Due to the partial insertion of leucine, and subsequent distortion of the entire substrate molecule, leucine cannot achieve a proper editing fit and eventually diffused out of the binding pocket. The two methyl groups appear to be too large to fit between the two threonines. Based on these observations, we propose that the two threonines of T252 and T248 are responsible for the amino acid discrimination. The mutation of T252 to alanine in the editing domain provides extra space between the two residues so that the incoming leucine can be aligned for editing. For LeuRS_CP1T252A(Leu), interestingly, the T247 is also involved in interactions with the leucine ligand. Due to the extra space resulting from the mutation and the new interaction with T247, the cognate leucine ligand was able to orient into an editing competent conformation. Finally, we offer a prediction relating to the valine and methionine editing activities based on our binding model. Although the two terminal methyl groups of valine cannot fit between the two threonines, as seen with leucine, the overall length of valine is shorter than leucine. Therefore, valine should fit in the editing pocket, although we expect that the binding energy will be less than that of isoleucine. It should be noted here that there is a possibility for T247 to be involved in the recognition as seen in the T252A mutant LeuRS. For methionine, the terminal methyl group can insert between the two threonines, as with isoleucine. However, methionine has an overall length disadvantage over isoleucine since it is longer by one CH2 unit. We expect that the methionine can compensate for this through changes in side chain dihedrals since all of them are freely rotatable single bonds. Our results also suggest that T252 requires T248 in order to exert its role as discriminator by helping to orient and stabilize the conformation of T252. The two residues are able to distinguish between leucine and isoleucine through side chain interactions among the three residues. If the end of the side chain of the incoming residue is a single methyl group, it can be stabilized between both threonines. However, if the side chain has two methyl groups or something larger at the end, the two threonines bind together, thereby excluding the incoming

World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:7, No:1, 2013

groups. The interaction of these two pairs of hydroxyl and methyl groups is the core of the discrimination between single and multiple terminal methyl groups on incoming amino acid ligands. Definitely, this can be one step or two step improved model of amino acid discrimination mechanism of aaRSs, compared to the pre-existing simple editing models where only the side chain size of amino acid matters [22]-[23]. The mutagenesis results of T248A suggest that the T248 is less crucial than T252 [24].

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