Isoleucyl-tRNA synthetase (IleRS) links tRNA^{ile} with not only its cognate isoleucine but also the nearly cognate valine. The CP1 domain of IleRS deacylates, or edits, the mischarged Val-tRNA^{ile}. We determined the crystal structures of the Thermus thermophilus IleRS CP1 domain alone, and in its complex with valine at 1.8- and 2.0-Å resolutions, respectively. In the complex structure, the Asp^{228} residue, which was shown to be critical for the editing reaction against Val-tRNA^{ile} by a previous mutational analysis, recognizes the valine NH$_3^+$ group. The valine side chain binding pocket is only large enough to accommodate valine, and the placement of an isoleucine model in this location revealed that the additional methylene group of isoleucine would clash with His$^{159}$. The H319A mutant of Escherichia coli IleRS reportedly deacylates the cognate Ile-tRNA$^{ile}$ in addition to Val-tRNA$^{ile}$, indicating that the valine-binding mode found in this study represents that in the Val-tRNA$^{ile}$ editing reaction. Analyses of the Val-tRNA$^{ile}$ editing activities of T. thermophilus IleRS mutants revealed the importance of Thr$^{228}$, Thr$^{229}$, Thr$^{328}$, and Asp$^{328}$, which are coordinated with water molecules in the present structure. The structural model for the Val-adenosine moiety of Val-tRNA$^{ile}$ bound in the IleRS editing site revealed some interesting differences in the substrate binding and recognizing mechanisms between IleRS and T. thermophilus leucyl-tRNA synthetase. For example, the carbonyl oxygens of the amino acids are located opposite to each other, relative to the adenosine ribose ring, and are differently recognized.

Crystal Structures of the CP1 Domain from Thermus thermophilus Isoleucyl-tRNA Synthetase and Its Complex with l-Valine*

Ryuya Fukunaga‡, Shuya Fukai§‡, Ryuichiro Ishitani‡, Osamu Nureki†§, and Shigeyuki Yokoyama‡‡†**

From the ‡Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, §PRESTO, Japan Science and Technology, 1-8-4 Honcho, Kawaguchi, Saitama 332-0012, Japan, the ¶RIKEN Genomic Sciences Center, 1-7-22 Suehiro-cho, Takumis, Yokohama 239-0045, Japan, and the **Cellular Signaling Laboratory, RIKEN Harima Institute, 1-1-1 Kouto, Mihazuki-cho, Sayo-gun, Hyogo 679-5148, Japan

Isoleucyl-tRNA synthetase (IleRS) links tRNA$^{ile}$ with not only its cognate isoleucine but also the nearly cognate valine. The CP1 domain of IleRS deacylates, or edits, the mischarged Val-tRNA$^{ile}$. We determined the crystal structures of the Thermus thermophilus IleRS CP1 domain alone, and in its complex with valine at 1.8- and 2.0-Å resolutions, respectively. In the complex structure, the Asp$^{228}$ residue, which was shown to be critical for the editing reaction against Val-tRNA$^{ile}$ by a previous mutational analysis, recognizes the valine NH$_3^+$ group. The valine side chain binding pocket is only large enough to accommodate valine, and the placement of an isoleucine model in this location revealed that the additional methylene group of isoleucine would clash with His$^{159}$. The H319A mutant of Escherichia coli IleRS reportedly deacylates the cognate Ile-tRNA$^{ile}$ in addition to Val-tRNA$^{ile}$, indicating that the valine-binding mode found in this study represents that in the Val-tRNA$^{ile}$ editing reaction. Analyses of the Val-tRNA$^{ile}$ editing activities of T. thermophilus IleRS mutants revealed the importance of Thr$^{228}$, Thr$^{229}$, Thr$^{328}$, and Asp$^{328}$, which are coordinated with water molecules in the present structure. The structural model for the Val-adenosine moiety of Val-tRNA$^{ile}$ bound in the IleRS editing site revealed some interesting differences in the substrate binding and recognizing mechanisms between IleRS and T. thermophilus leucyl-tRNA synthetase. For example, the carbonyl oxygens of the amino acids are located opposite to each other, relative to the adenosine ribose ring, and are differently recognized.

Aminoacyl-tRNA synthetases (aaRSs)$^3$ catalyze the esterification of an amino acid to its cognate tRNA. This reaction proceeds in two steps: the synthesis of an aminoacyl adenylate, as an activated intermediate, from the amino acid and ATP, and the transfer of the aminoacyl moiety to the 3'-terminal of the cognate tRNA to yield the aminoacyl-tRNA (1). To maintain accurate protein biosynthesis, each aaRS must discriminate between its cognate amino acid and other similar amino acids (2, 3). Some aaRSs, including the isoleucyl-, leucyl-, and valyl-tRNA synthetases (IleRS, LeuRS, and ValRS, respectively), have a specific editing activity that hydrolyzes the misaminoacylated tRNAs ("post-transfer editing") (4–6). For example, IleRS also recognizes valine, which is smaller than the cognate isoleucine by only one methylene group, and mischarges it with tRNA$^{ile}$. Then, the mischarged Val-tRNA$^{ile}$ is hydrolyzed to valine and tRNA$^{ile}$ in the post-transfer editing pathway. As for IleRS, another editing pathway (pre-transfer editing) also exists, in which the misactivated Val-AMP is directly hydrolyzed to valine and AMP in the presence of tRNA$^{ile}$ (7, 8). Biochemical experiments linked the specific location of the editing site to the connective polypeptide 1 (CP1) domain, a large insertion in the aminoacylation catalytic Rossmann-fold domain (9).

Previously, we determined the crystal structures of the Thermus thermophilus full-length IleRS complexed with isoleucine and with valine, and showed that the editing site is in the highly conserved threonine-rich region of the CP1 domain (10). In addition, the crystal structure of Staphylococcus aureus IleRS complexed with tRNA$^{ile}$ and mupirocin (an analogue of isoleucyl-adenylate) revealed that the 3'-terminal of tRNA$^{ile}$ is located in the CP1 domain (although it was not completely resolved) (11). This suggested that when a nearly cognate amino acid is charged to a tRNA, the acceptor stem flips from the aminocacylation site to the editing site, while the rest of the tRNA remains bound. However, the B factors of many atoms in the CP1 domains of these structures were high and some residues were disordered, since the CP1 domain is quite mobile relative to the rest of the protein (10, 11). Furthermore, in our previous study (10), the omit map electron density for valine was not clear enough for us to determine its orientation precisely, and the coordinates were determined by analogy with the related ValRS system (12). The crystal structure of T. thermophilus ValRS complexed with tRNA$^{val}$ accurately revealed the location of the 3'-terminal of tRNA$^{val}$ in the CP1 domain with complete resolution, although that of threonine (which is edited by ValRS) remains to be elucidated (12). Based

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The atomic coordinates and structure factors (code 1UDZ and 1UE0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ Present address: Dept. of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Inst. of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa 229-8501, Japan.

† To whom correspondence should be addressed. Tel.: 81-3-5841-4392; Fax: 81-3-5841-8057; E-mail: yokoyama@biochem.s.u-tokyo.ac.jp.

¶ The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase.

Aminoacyl-tRNA synthetases (aaRSs)$^3$ catalyze the esterification of an amino acid to its cognate tRNA. This reaction proceeds in two steps: the synthesis of an aminoacyl adenylate, as an activated intermediate, from the amino acid and ATP, and the transfer of the aminoacyl moiety to the 3'-terminal of the cognate tRNA to yield the aminoacyl-tRNA (1). To maintain accurate protein biosynthesis, each aaRS must discriminate between its cognate amino acid and other similar amino acids (2, 3). Some aaRSs, including the isoleucyl-, leucyl-, and valyl-tRNA synthetases (IleRS, LeuRS, and ValRS, respectively), have a specific editing activity that hydrolyzes the misaminoacylated tRNAs ("post-transfer editing") (4–6). For example, IleRS also recognizes valine, which is smaller than the cognate isoleucine by only one methylene group, and mischarges it with tRNA$^{ile}$. Then, the mischarged Val-tRNA$^{ile}$ is hydrolyzed to valine and tRNA$^{ile}$ in the post-transfer editing pathway. As for IleRS, another editing pathway (pre-transfer editing) also exists, in which the misactivated Val-AMP is directly hydrolyzed to valine and AMP in the presence of tRNA$^{ile}$ (7, 8). Biochemical experiments linked the specific location of the editing site to the connective polypeptide 1 (CP1) domain, a large insertion in the aminoacylation catalytic Rossmann-fold domain (9).

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Crystal Structure of the Isoleucyl-tRNA Synthetase CP1 Domain

on the location of valve in the IleRS CP1 domain and that of the tRNA 3'-terminal adenosine in the ValRS CP1 domain, we built the structural models for Val-tRNA<sup>iso</sup> in IleRS and Thr-tRNA<sup>iso</sup> in ValRS, assuming that their binding modes are analogous (12). However, differences between their binding modes were subsequently reported (13). In addition, no water molecules could be assigned around the valve bound to the CP1 domain in the full-length T. thermophilus IleRS structure. Therefore, the precise valve-binding mode and the mechanisms by which the IleRS CP1 domain selectively recognizes valine and catalyzes the hydrolysis of Val-tRNA<sup>iso</sup> remained to be elucidated.

Recently, the crystal structure of T. thermophilus IleRS complexed with the post-transfer editing substrate analogue was determined (14). In comparison with IleRS, the CP1 domain is inserted at a different point in the enzyme, and its rotational orientation differs by nearly 180° from IleRS (14, 15). The structure provided the precise substrate-binding mode, in which conserved Thr residues recognize the substrate (14). However, the LeuRS crystal structures revealed that both the pre- and post-transfer editing analogues are bound in one site in the CP1 domain, although a mutational analysis showed that distinct residues are needed between the pre- and post-transfer editing reactions in IleRS (16). Therefore, the substrate-binding modes may differ between IleRS and LeuRS.

In the present study, to study specifically the post-transfer editing mechanism of IleRS, we isolated the T. thermophilus IleRS CP1 domain (201–384 amino acids), which contains the post-transfer editing site, but lacks a part of the pre-transfer editing site, based on the previous model (12, 16). We determined its crystal structure at 1.8-Å resolution. Then, we determined its cocrystal structure with valine at 2.0-Å resolution. In the complex structure, valine is located in the same pocket as in the case of the modeled Val-tRNA<sup>iso</sup> (12, 16); however, its orientation within the pocket is quite different. This new valine-binding mode can explain the mutational data well, unlike the previous model. Furthermore, we examined the post-transfer editing activity of some mutant IleRSs. Based on the high resolution structural data and these and other mutational data, we created a new structural model of the Val-adenosine moiety of Val-tRNA<sup>iso</sup> in the editing state. In this model, the substrate recognition differs from that in the LeuRS system.

EXPERIMENTAL PROCEDURES

Protein Preparation—The gene fragment encoding the T. thermophilus IleRS CP1 domain (201–384 amino acids with the initiating methionine) was subcloned into pET28b (Novagen). The construct was designed to have only the post-transfer editing site, but not the pre-transfer editing site, based on the previous model (12, 16), to study specifically the post-transfer editing mechanism. The plasmid was transformed into E. coli strain JM109(DE3). For protein overexpression, the cells were grown to an OD<sub>600</sub> of 0.8, and the expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. The cells were harvested and sonicated in 50 mM K<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0) containing 15 mM MgCl<sub>2</sub> 10 mM dithiothreitol, and 1 mM butylsulfonyl fluoride. The insoluble cell debris was removed by centrifugation at 15,000 × g for 30 min at 4°C. The supernatant was heat-treated at 70°C for 30 min to denature the E. coli proteins. The heat-treated mixture was centrifuged at 15,000 × g for 1 h at 4°C. Then, 2.4 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the supernatant to a final concentration of 0.3 M. The mixture was applied to a 50 ml column of butyl-Toyopearl (Tosoh) equilibrated with 50 mM K<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0) containing 0.8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol. The CP1 domain was eluted with a linear gradient of 0–1 M NaCl and was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM MgCl<sub>2</sub> and 5 mM β-mercaptoethanol.

The final purity of the protein was monitored by SDS-PAGE.

Crystallization and Data Collection—For crystallization, the hanging drop vapor diffusion method was used, by mixing 1 μl of the protein solution (10 mg/ml) with 1 μl of the reservoir solution (50 mM Hepes-NaOH buffer (pH 7.0) containing 2.0 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 5% 2-propanol) and by equilibrating this mixture against 500 μl of the reservoir solutions at 20°C. Crystals (space group P4<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, unit-cell parameters a = b = 102.7 Å, c = 83.8 Å) were grown for 3 days to dimensions of ~0.3 × 0.3 × 0.3 mm<sup>3</sup>. To obtain cocrystals of the CP1 domain complexed with valine, reservoir solutions containing 100 mM valine were used, and the crystals thus obtained were transferred to a solution containing 55 mM Hepes-NaOH buffer (pH 7.0), 2.2 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.5% 2-propanol, and 100 mM valine, 24 h before the data collection. The diffraction datasets of the bound-free crystal were collected at beamline BL44XU at SPring-8 to 2.0 Å resolution. A single crystal, flash-frozen at a temperature of ~173°C, was used for each experiment. Before flash-cooling, the crystals were transferred into a cryoprotective solution containing 20% (v/v) ethylene glycol. The ligand-free data sets were processed using the HKL2000 program (17), and the complex data sets were processed using MOSFLM/SCALA (18).

Data collection statistics are summarized in Table 1.

Structure Determination and Refinement—We carried out molecular replacement with MOLREP (19), starting with the coordinates of the CP1 domain from the full-length T. thermophilus IleRS, which we previously determined at 2.5-Å resolution (Protein Data Bank ID, ILE) (10). The rotated and translated model was first subjected to rigid-body refinement using 20–2.5 Å data sets collected from the ligand-free crystal. The model refinement was carried out using the program CNS (20). After several rounds of Cartesian coordinate energy minimization, simulated annealing, B factor refinement, automatic water picking, and manual revision of the model, using the program O (21), R and R<sub>free</sub> decreased to 20.3% and 25.0%, respectively. A random sample containing 5% of the total reflections in the data sets was excluded from the refinement, to calculate R<sub>free</sub>. Using the data sets of the complex crystals, molecular replacement was carried out with the coordinates of the ligand-free CP1 domain structure. The structure refinement was performed in the same way. After building the valine ligand, further rounds of refinement were performed. The model refinement of the complex structure finally converged to an R value of 20.3% and an R<sub>free</sub> of 24.8%, with good stereochemistry. Ramachandran plot analysis using the program PROCHECK (22) showed that all of the residues in both

| Table 1 | Data collection and model refinement statistics |
|---------|-----------------------------------------------|
| R<sub>f</sub> = Σ||F<sub>o</sub> - |F<sub>c</sub>||/Σ|F<sub>d</sub>|, where I<sub>k</sub> and I<sub>j</sub> are the intensity of measurement and the mean intensity for the reflection with indices hkl, respectively. R = Σ|F<sub>o</sub> - kF<sub>c</sub>|/ΣF<sub>o</sub>, where k is a scale factor and R<sub>f</sub> is the R factor for the test set of reflections not used during refinement (5% of the data set). |
| Data set | CP1 domain | CP1 domain + valine |
|---------|------------|---------------------|
| Space group | P4<sub>1</sub>2<sub>1</sub>2<sub>1</sub> | P4<sub>1</sub>2<sub>1</sub>2<sub>1</sub> |
| Unit-cell parameters (Å) | a = 102.73, b = 102.73, c = 83.83 | a = 102.73, b = 102.73, c = 84.03 |
| Resolution range (Å) | 50.0–1.8 | 50.0–2.0 |
| Redundancy | 15.8 | 8.9 |
| Completeness (%) (last shell) | 99.1 (98.2) | 98.8 (99.2) |
| I/σ (last shell) | 45.3 (3.7) | 7.1 (2.6) |
| R<sub>f</sub> (%) (last shell) | 8.1 (28.2) | 4.7 (28.5) |
| Structure refinement | | |
| No. of reflections: working set/test set | 39682/20836 | 28641/1492 |
| No. of atoms | 2806 | 2843 |
| No. of water molecules | 251 | 166 |
| R factor (%): working set/test set | 20.3/25.0 | 20.3/24.8 |
| Average B factor (Å<sup>2</sup>) | 40.2 | 48.4 |
| Root mean square bonds | 0.020 | 0.011 |
| Root mean square angles (°) | 1.98 | 1.65 |
the ligand-free and complex structures, except for one residue in each structure, were in the most favored or additionally allowed regions. Omit maps for the valine and surrounding water molecules were calculated at the end of the refinement.

Post-transfer Editing Assay of Mutant IleRSs—The full-length *T. thermophilus* IleRS gene was cloned into pET28a (Novagen). This plasmid encodes IleRS with a His tag on its C-terminal end. The mutations, T229A, T230A, T230A, T233A, T233A, T229A/T230A, T233A, T233A, D328A, were introduced using a QuikChange mutagenesis kit (Stratagene). Plasmids were transformed into *E. coli* strain BL21(DE3). Cells were cultured and harvested by the same procedure used for the CP1 domain. Cells were sonicated in 30 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM MgCl₂, 10 mM imidazole, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (buffer A). The insoluble cell debris and the *E. coli* proteins were removed by the same procedure used for the CP1 domain. The supernatant was applied to a 1-ml HiTrap chelating column (Amersham Biosciences) chelated with Ni²⁺ ions and equilibrated with buffer A. After the column was washed with buffer A, the mutant IleRSs were eluted with 30 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl, 5 mM MgCl₂, 500 mM imidazole, and 5 mM β-mercaptoethanol and were dialyzed against 150 mM Tris-HCl buffer (pH 7.5) containing 150 mM KCl and 10 mM MgCl₂. The final purity of the proteins was monitored by SDS-PAGE.

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^{14}C\text{Val-tRNA}^\text{Val} \text{ was prepared as described previously (23, 24). Decacylation assays were performed at 37 °C in 150 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl₂, 150 mM KCl, [^{14}C\text{Val-tRNA}^\text{Val}] (1.4 μCi, 1000 cpm/μmol), and each mutant IleRS (20 nM). Aliquots were removed at specific time points and quenched on filter papers (Whatman, 3 mm) equilibrated with 10% trichloroacetic acid. The filters were washed 3 times with 5% trichloroacetic acid and once with 100% ethanol. The radioactivities of the precipitates were quantitated by scintillation counting.}
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RESULTS AND DISCUSSION

Overall Structure—We determined the high resolution crystal structure of the isolated *T. thermophilus* IleRS CP1 domain, containing the post-transfer editing site, at 1.8 Å resolution (Protein Data Bank ID, 1UDZ). The root-mean-square deviation between the isolated CP1 domain and the CP1 domain in the full-length IleRS over all Cₐ atoms is 0.91 Å, which is small enough to show that these two structures are quite similar to each other. Indeed, the secondary structures and the locations of the individual residues are almost the same, and therefore, the structure of the CP1 domain in this study reflects that of the full-length IleRS. Next, we determined the crystal structure of the CP1 domain complexed with valine at 2.0-Å resolution (Protein Data Bank ID, 1UE0) (Fig. 1A). Its overall structure is also quite similar to that of the CP1 domain in the full-length IleRS (Fig. 1B), and thus, no large conformational change occurs upon the valine binding.

Valine-binding Mode—The editing active site is formed mainly by one β-strand (β8) and two almost parallel α-helices (α1 and α5). In the Fₜ – Fₜ, omit map of the complex structure, there is a strong and clear electron density that could be attributed to the valine molecule in the editing active site (Fig. 2A), and there are some electron densities that could be attributed to water molecules around the valine. The high resolution complex structure revealed the precise valine recognition mechanism (Fig. 2B and C, and Fig. 4A). The valine NH₃⁺ hydrogen bonds to the O₆ of Asp²²⁸ and the α-CO of His²¹⁹, and the valine COO⁻ group hydrogen bonds to the O₇ atoms of Thr²²⁹ and Thr²³⁰, and the α-CO groups of Trp²²⁷ and Phe²³⁴, through water molecules. The valine side chain is surrounded by the side chains of Thr²³³, His²¹⁹, Ala²³¹, and Phe²³⁴, and the α-CO of Phe²³⁴. All of these residues are highly conserved in the IleRSs from other species (Fig. 6A). We previously proposed a valine-binding mode in the post-transfer editing state, which was modeled on the basis of the electron density corresponding to valine (although it was not clear enough to locate it unambiguously) in the full-length IleRS structure, and the 3′-terminal adenosine of tRNA⁷⁷⁵ in the *T. thermophilus* ValRS-tRNA⁷⁷⁵ cocrystal structure (12). In the present structure, the valine is located in the same pocket as in the case of the modeled Val-tRNA⁷⁷⁵, but its orientation within the pocket is different from that in the model (Fig. 3).

This valine-binding mode can nicely explain the mutational data of the *E. coli* H319A mutant IleRS, which the previous model could not. The H319A mutant hydrolyzes not only the nearly cognate Val-tRNA⁷⁷⁵ but also the cognate Ile-tRNA⁷⁷⁵ (16). In the present structure, valine fits well in the editing active site (the distance between the Ne2 of His²¹⁹ and the Cy of valine is 3.5 Å) (Fig. 4A), but when the isoleucine model is placed in the same site, the additional methylene group clashes with the side chain of His²¹⁹ (the distance between the Ne2 of His²¹⁹ and the Cβ of isoleucine would be 2.1 Å) (Fig. 4B). If this His²¹⁹ residue was mutated to Ala, then the distances between the Ala Cβ and the valine Cy or the isoleucine Cβ would be 5.4
or 4.7 Å, respectively. These data support the idea that the location of the valine in the determined structure represents the post-transfer editing state, while the previous model for the valine-binding mode could not explain this mutational analysis. Therefore, we concluded that the valine-binding mode in the present structure precisely reflects that of the valyl moiety of Val-tRNA\textsuperscript{Ile} in the editing state.

Post-transfer Editing Assay of Mutant IleRSs—Previous biochemical experiments using \( E. \, coli \) IleRS showed that the T242A (corresponding to Thr\textsuperscript{229} in \( T. \, thermophilus \) IleRS) mutant is defective in the total editing activity (whether it affects post- or pre-transfer editing or both is unknown), while the T241A, T243A, and T246A \( E. \, coli \) IleRS mutants (Thr\textsuperscript{228}, Thr\textsuperscript{230}, and Thr\textsuperscript{233} in \( T. \, thermophilus \) IleRS, respectively) can still perform the total editing activity (10, 25). In addition, the D342A \( E. \, coli \) IleRS mutant (Asp\textsuperscript{328} in \( T. \, thermophilus \) IleRS) is defective in the post-transfer editing activity (26). Thus far, all of the mutant analyses have been carried out using \( E. \, coli \) IleRS, with most examining only the aspect of total editing and not distinguishing between pre- and post-transfer editing. For
a more precise understanding of the post-transfer editing reaction, we analyzed the post-transfer editing activity of Ala-replaced mutants of *T. thermophilus* IleRS. As a result, the T228A, T229A, T228A/T230A, T229A/T230A, and D328A mutants had some defects, while T230A, T233A, and T230A/T233A retained the full activity (Fig. 5). These data show that Thr228, Thr 229, Thr 230 (detectable only when Thr 228 is also mutated to Ala), and Asp328 (Thr241, Thr 242, Thr 243, and Asp342, in *E. coli* IleRS, respectively) play some role in the post-transfer editing reaction.

Previous results using *E. coli* IleRS showed that Thr228 and Thr230 were not critical for the total editing (10, 25). The Ala-replacing mutation of Thr230 leads to the defect only when Thr228 is also mutated to Ala, whereas the previous analysis examined only the single mutation of Thr230. The T228A defect is not as severe in the post-transfer editing, and in IleRS, the post-transfer editing was estimated to account for only 25% of the overall editing, while the remaining 75% was attributed to the pre-transfer editing (7). By assuming that the T228A mutant is only defective in the post-transfer editing, but not in the pre-transfer editing, we can account for the previous mutational analysis by not being able to detect the slight defect of the T228A mutant in the total editing. The more severe defect of the T228A/T230A double mutant than that of the T228A single mutant also shows the importance of the Thr228 residue in the post-transfer editing (Fig. 5).

**A Model for the Editing Mechanism**—We tried to build a structural model for the Val-adenosine moiety of Val-tRNA<sup>15</sup>. First, we superposed *T. thermophilus* ValRS complexed with tRNA<sup>val</sup> onto the *T. thermophilus* IleRS CP1 domain. However, the 3’-terminal adenosine of tRNA<sup>val</sup> and valine could not be ester bonded, even with a slight adjustment. The distance between the OH group of the tRNA<sup>val</sup> adenosine ribose and the valine COO<sup>−</sup> group was too far (data not shown). Then, we superposed *T. thermophilus* LeuRS complexed with a post-transfer editing substrate analogue onto the *T. thermophilus* IleRS CP1 domain (Fig. 6B). This time, although there were some differences in the editing site architecture, especially in the adenine base recognition region and the conserved threonine-rich region, the distance between the OH group of the adenosine ribose and the valine COO<sup>−</sup> group was close enough for linkage with a slight adjustment. We translated the adenosine to allow the ribose 2’-OH group to form an ester bond with the valine COO<sup>−</sup> group, and rotated it around the 2’-C–O bond. We also rotated the γ angle of the adenine base to adjust for the differences between the adenine base recognition regions of IleRS and LeuRS (Fig. 6A). Finally, we could build a fine model for the Val-adenosine moiety of Val-tRNA<sup>15</sup>.
a similar way. These Asp residues are completely conserved among IleRSs, LeuRSs, and ValRSs (Fig. 6A) and are critical in the IleRS post-transfer editing reactions (Fig. 5 and Ref. 23) and in the LeuRS total editing reactions (25). Therefore, the interaction between the amino acid NH$_3^+$ group and the Asp residue must be vital for the editing reaction in both IleRSs and LeuRSs and maybe also in ValRSs. However, the orientations of the ester bond in the editing substrates are quite different between IleRS and LeuRS, and the threonine-rich region is closer to the substrate in LeuRS than in IleRS (Fig. 6B). In LeuRS, the amino acid carbonyl group is oriented toward the threonine-rich region and is recognized by Thr$_{247}$ (Thr$_{228}$ in IleRS) through the water molecule (Fig. 2). In contrast, in IleRS, it is located opposite to the threonine-rich region and is recognized by the $\alpha$-CO of His$_{319}$ through the water molecule, not by the conserved threonine residue (Fig. 6C). In IleRS, only Thr$_{229}$ hydrogen bonds to the substrate 5′-OH, unlike the other conserved threonine residues (Fig. 6C). These findings can account for the mutational data that Thr$_{229}$ plays some role in the post-transfer editing, but Thr$_{230}$ does not (Fig. 5). Furthermore, the water molecule, which hydrogen bonds to Asp$_{328}$ and the NH$_3^+$ group of the substrate, is favorably located to act as the catalytic nucleophile for the ester bond hydrolysis (Fig. 6C). Conversely, in the determined valine-bound structure, Thr$_{230}$ hydrogen bonds to the valine COO$^-$ group through the water molecule (Fig. 2, A and B), but the water molecule clashes with the ribose 3′-OH of the modeled substrate, and cannot exist in the same place in the model (data not shown). We will discuss the importance of Thr$_{228}$ and Thr$_{230}$ below.

In the aminoclaylation reaction by IleRS, valine is first mischarged to the 2′-OH of the tRNA 3′-terminal adenosine ribose (13). Under normal conditions, valine is transacylated to the 3′-OH from the 2′-OH, and vice versa, and stays in equilibrium. Until now, we have discussed the 2′-OH valylated tRNA$^{\text{Ile}}$ model. Biochemical analyses using aminoclaylated tRNA$^{\text{Ile}}$s with a deoxygenized 2′-OH or 3′-OH of the 3′-terminal adenosine ribose, which the amino acid cannot transacylate, suggested that IleRS specifically deacylates valine from the 3′-OH but not from the 2′-OH, whereas ValRS can deacylate the mischarged threonine from the 2′-OH (13).

Considering these data, we built the binding mode model of another editing substrate, the 3′-OH valylated adenosine moiety of Val-tRNA$^{\text{Ile}}$ (Fig. 7). Unlike the case of the 2′-OH valylated model, to make the 3′-OH valylated model, we moved the valyl moiety from the observed location in the determined valine-bound structure. In the 3′-OH valylated model, the recognition modes of the NH$_3^+$ group and the adenine base are similar to those in the 2′-OH valylated model (Figs. 6C and 7).
In the 3'-OH valylated model, Thr\textsuperscript{229} hydrogen bonds directly to the adenosine 5'-O, as in the 2'-OH valylated model, and Thr\textsuperscript{228} and Thr\textsuperscript{230} hydrogen bond to the adenosine 3'-O through the water molecule (Fig. 7), as observed in the determined valine-bound structure (Fig. 2, A and B). This water molecule, which hydrogen bonds to Thr\textsuperscript{228} and Thr\textsuperscript{230}, is favorably located to act as a catalytic nucleophile for the ester bond hydrolysis (Fig. 7). In this model, Thr\textsuperscript{228} and Thr\textsuperscript{230} participate in the substrate recognition, in contrast to the 2'-OH valylated model, thus confirming the mutational analysis that revealed the importance of these residues in the editing reaction. Although Thr\textsuperscript{231} is highly conserved in IleRSs, it is replaced by the highly conserved Arg residue in LeuRSs and ValRSs (Fig. 6A). This is consistent with the data that the mischarged amino acid is deacylated from the 3'-OH only in IleRS (13) but is deacylated from the 2'-OH in ValRS (13) and maybe also in LeuRS (14). Moreover, these two binding-mode models are not mutually exclusive. Instead, both binding mechanisms might be used. That is, first the 2'-OH valylated tRNA\textsuperscript{3}

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**Fig. 7. Structural model of the 3'-OH valylated adenosine moiety of Val-tRNA\textsuperscript{3} (stereo view).** The two water molecules are located in the same places as in the determined structure (light blue). Ionics bonds and hydrogen bonds are shown by dashed blue lines. The water molecule hydrogen bonding to Thr\textsuperscript{228} and Thr\textsuperscript{230} is favorably located to act as a nucleophile in the ester bond hydrolysis reaction.

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**Concluding Remarks**—The high resolution crystal structure of the *T. thermophilus* IleRS CP1 domain complexed with the nearly cognate valine provides a precise understanding of the selective valine recognition mechanism. In addition, we were able to build the binding mode models of the two substrates, the 2'-OH valylated and the 3'-OH valylated adenosine moieties of Val-tRNA\textsuperscript{3}. These structure models illustrate the mutational data well and suggest a specific editing mechanism for IleRS, which is distinct from those of LeuRS and ValRS. Actually, only in IleRS, the pre-transfer editing is predominant (75\% of the total editing) (7). However, we still need to determine whether the active site accommodates 2'-OH or 3'-OH valylated tRNA\textsuperscript{3} or both and to elucidate the precise binding mode. Moreover, the binding mode of the pre-transfer editing substrate is still unknown in IleRS, although in LeuRS, both substrates are recognized in the same binding site in similar manners (14). This structural study has provided significant clues toward elucidating the editing mechanism of IleRS. For a more precise understanding of both pre- and post-transfer editing, the determination of the IleRS structures complexed with nonhydrolyzable analogs of pre- and post-transfer editing substrates is required.

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