Structural Basis for Discrimination of L-Phenylalanine from L-Tyrosine by Phenylalanyl-tRNA Synthetase

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Summary

Aminoacyl-tRNA synthetases (aaRSs) exert control over the faithful transfer of amino acids onto cognate tRNAs. Since chemical structures of various amino acids closely resemble each other, it is difficult to discriminate between them. Editing activity has been evolved by certain aaRSs to resolve the problem. In this study, we determined the crystal structures of complexes of T. thermophilus phenylalanyl-tRNA synthetase (PheRS) with L-tyrosine, p-chlorophenylalanine, and a nonhydrolyzable tyrosyl-adenylate analog. The structures demonstrate plasticity of the synthetic site capable of binding substrates larger than phenylalanine and provide a structural basis for the proofreading mechanism. The editing site is localized at the B3/B4 interface, 35 Å from the synthetic site. Glu334 plays a crucial role in the specific recognition of the Tyr moiety in the editing site. The tyrosyl-adenylate analog binds exclusively in the synthetic site. Both structural data and tyrosine-dependent ATP hydrolysis enhanced by tRNA^Phe provide evidence for a preferential posttransfer editing pathway in the phenylalanine-specific system.

Introduction

The accuracy of genetic code translation is vitally important for the function of living cells and is intimately associated with the proper operation of correctly assembled macromolecules. The aaRSs play a crucial role in the maintenance of faithful translation, promoting close control over the two-step aminoacylation reaction and thus establishing a linkage between the cognate amino acid and three nucleotides of the tRNA anticodon(s). In the first step, aaRS activates the amino acid and forms an aminoacyl-adenylate intermediate. In the second step, the amino acid moiety is transferred to the 3'-terminal ribose of tRNA. The rate of misincorporation of noncognate amino acids in vivo is approximately 1 out of 10^4–10^5 reactions (reviewed in Jakubowski and Goldman, 1992). This brings up the question of how such a level of fidelity can be achieved for those aaRSs that have to distinguish between amino acids with very similar chemical structures. The problem has been resolved with the discovery of proofreading activity associated with a distinct active site at which misactivated aminoacyl-adenylate (pretransfer editing) (Fersht, 1977) or misaminoacylated tRNA (posttransfer editing) (Eldred and Schimmel, 1972) are hydrolyzed.

The “double-sieve” model for aminoacylation and editing proposed by Fersht (1977) has been visualized in the crystal structure of class I isoleucyl-tRNA synthetase (IleRS) complexed with Ile or Val (Nureki et al., 1998). The synthetic catalytic site, which is suggested to act as “coarse sieve,” binds isosteric or smaller amino acids and discards those that are larger than or dissimilar to the cognate one. The smaller substrates form incorrect aminoacyl-adenylates or aminoacyl-tRNAs hydrolyzed at the editing site, which acts as “fine sieve.” The availability of the “fine sieve” for IleRS derives from the detection of a second binding site for Val (Nureki et al., 1998). The synthetic and editing sites are widely spaced from each other (for a review, see Jakubowski, 2004). Even though the double-sieve model allows us to explain the proofreading in IleRS, ValRS, and LeuRS, it could not give insight into the editing activities of MetRS, AlaRS, and PheRS, shown to misactivate amino acids larger than the correct ones (ethionine, Ser, and Tyr, respectively) (Tsui and Fersht, 1981; Lin et al., 1983; Jakubowski and Goldman, 1992).

Until recently, the editing activity of class II aaRSs was not characterized sufficiently. Structural and biochemical studies of ThrRS have revealed that the zinc ion directly involved in amino acid recognition is responsible for rejection of isosteric Val at the activation step (Sankaranarayanan et al., 2000). The system-specific N2 domain employed to correct misacylation of tRNA^Thr with Ser is 39 Å away from the synthetic site (Dock-Bregereen et al., 2000). A seryl-adenylate analog (Ser-AMS) has been shown to bind in the active site of the isolated N2 domain, although the existence of pretransfer hydrolytic activity is not evidenced by these data (Dock-Bregereen et al., 2000, 2004). Based on comparative modeling, a similar architecture of the editing domain was predicted for AlaRS (Beebe et al., 2003), shown earlier to hydrolyze misacylated Gly and Ser (Tsui and Fersht, 1981). In E. coli ProRS, editing activity directed toward misactivated Ala and misacylated Ala-tRNA^Pro is associated with an INS domain located between signature motifs 2 and 3 and not maintained in the sequences of eukaryote/archaeon-like ProRSs (Wong et al., 2003). The editing domains of class II aaRSs are more diverse in amino acid sequences and distinguishing features of their folds in comparison with those of the class I enzymes.

A double-rejection mechanism of noncognate Tyr, using pretransfer and posttransfer hydrolysis, has been suggested for yeast PheRS (Lin et al., 1983, 1984), the most complex representative of class II aaRSs. During evolution, the (αβ)2 subunit organization of cytoplasmic PheRS is markedly conserved from prokaryotes to eukaryotes. The 3D structure of T. thermophilus PheRS and its complexes with functional substrates (Mosyak...
et al., 1995; Goldgur et al., 1997; Reshetnikova et al., 1999; Fishman et al., 2001) reveals that PheRS is both structurally and functionally an \((\alpha\beta)_2\) heterodimer; the \(\beta\) subunit is not directly involved in the catalytic reaction, but it exerts control over the recognition and binding of cognate tRNA\(^{Phe}\). Three domains of the \(\beta\) subunit (B2, B4, and B5) do not contact tRNA\(^{Phe}\), and, until recently, there was no clear notion of their functional role (Safro et al., 2004). As proposed in early studies of yeast PheRS, the pretransfer hydrolysis triggered by native tRNA\(^{Phe}\) makes a major contribution to the Tyr rejection (Lin et al., 1984). It was recently shown that \(E.\ coli\) PheRS also misactivates Tyr and further corrects such errors (Roy et al., 2004). Based on structural modeling and an in vivo genetic screen, a few residues of the \(E.\ coli\) PheRS \(\beta\) subunit (from the B3/B4 domains) presumably associated with the editing activity of the enzyme were identified. The editing activity of \(E.\ coli\) PheRS against Tyr occurs predominantly at the posttransfer stage. For yeast PheRS, however, a major role was assigned to the pretransfer editing stage.

This work presents the structural basis of editing activity of \(T.\ thermophilus\) PheRS directed toward misactivated Tyr. The crystal structures of the bacterial enzyme complexes with noncognate Tyr, an unnatural para-substituted Phe analog, \(p\text{-Cl-Phe}\), and a nonhydrolyzable analog of tyrosyl-adenylate (Tyr-AMS) have been determined. The key feature of the complexes is binding of Tyr in both the synthetic active site and an editing site localized on the \(\beta\) subunit. Protein residues involved in the sculpting of the editing site were identified. Discrimination of the editing site against cognate Phe is ensured by specific recognition of the \(p\)-hydroxyl group of Tyr. A second key feature is the binding of Tyr-AMS exclusively in the synthetic site, in close proximity to the position of phenylalanyl-adenylate. The structural data combined with a kinetic study of the substrate properties of Tyr provide evidence for a preferential posttransfer editing mechanism in PheRS, i.e., deacylation of the incorrectly charged Tyr-tRNA\(^{Phe}\).

### Results

**Tyr as Substrate of PheRS**

The ability of L-Tyr to act as a substrate of \(T.\ thermophilus\) PheRS was initially investigated by steady-state aminoacylation kinetics. No charging of tRNA\(^{Phe}\) with the noncognate Tyr, an unnatural para-substituted Phe analog, \(p\text{-Cl-Phe}\), and a nonhydrolyzable analog of tyrosyl-adenylate (Tyr-AMS) have been determined. The key feature of the complexes is binding of Tyr in both the synthetic active site and an editing site localized on the \(\beta\) subunit. Protein residues involved in the sculpting of the editing site were identified. Discrimination of the editing site against cognate Phe is ensured by specific recognition of the \(p\)-hydroxyl group of Tyr. A second key feature is the binding of Tyr-AMS exclusively in the synthetic site, in close proximity to the position of phenylalanyl-adenylate. The structural data combined with a kinetic study of the substrate properties of Tyr provide evidence for a preferential posttransfer editing mechanism in PheRS, i.e., deacylation of the incorrectly charged Tyr-tRNA\(^{Phe}\).

### Table 1. Inhibition Properties of L-Tyr and Synthetic Ligands

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(K_i) ((\mu)M)</th>
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</thead>
<tbody>
<tr>
<td>L-Tyr (Phe)</td>
<td>3100</td>
</tr>
<tr>
<td>L-Tyr-AMS (Phe)</td>
<td>0.045</td>
</tr>
<tr>
<td>L-Tyr-AMS (ATP)</td>
<td>0.043</td>
</tr>
<tr>
<td>L-PheOH-AMP (Phe)</td>
<td>0.089</td>
</tr>
<tr>
<td>L-PheOH-AMP (ATP)</td>
<td>0.085</td>
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\(K_i\) values of competitive inhibitors were determined in the aminoacylation reaction catalyzed by \(T.\ thermophilus\) PheRS with respect to the substrate indicated in parentheses. The \(K_a\) value for L-Phe is 1.8 \(\mu\)M, and the value for ATP is 130 \(\mu\)M.

### Figure 1. ATP Hydrolysis by \(T.\ thermophilus\) PheRS

(A and B) (A) ATP hydrolysis stimulated by Phe. (B) ATP hydrolysis stimulated by noncognate Tyr. The reaction mixture contained 100 \(\mu\)M ATP, 0.2 \(\mu\)M PheRS, 300 \(\mu\)M amino acid, and other constituents as described in Experimental Procedures. The reaction was performed without addition (1) or with the addition of 1 \(\mu\)M \(T.\ thermophilus\) tRNA\(^{Phe}\) (2), 4 U/ml inorganic pyrophosphatase (3), and pyrophosphatase (4).
smaller effect compared to the cognate reaction and is independent of tRNA_Phe concentration (in the range of 0.5–10 μM). The activating effect of pyrophosphatase on Tyr-stimulated ATP hydrolysis was significantly suppressed in the presence of tRNA_Phe, indicating that tRNA-dependent ATP consumption is the prevailing reaction pathway. tRNA_Phe shortened by one nucleotide from the 3′ end and hence deprived of charging activity failed to activate ATP hydrolysis (data not shown). Thus, the tRNA-dependent editing activity against Tyr revealed for yeast (Lin et al., 1984) and E. coli PheRSs (Roy et al., 2004) is also characteristic of the T. thermophilus enzyme.

Three noncognate L-amino acids (Ile, His, and Trp) were tested additionally for their ability to inhibit aminoacylation of tRNA_Phe and act as substrates in activation and aminoacylation reactions. The K_i value for Trp (4.6 mM) was found to be closely similar to that of Tyr, while the other two amino acids showed no inhibition effect even at 20 mM concentration. None of the three amino acids stimulated ATP hydrolysis (in the absence or presence of tRNA_Phe) or can be attached to tRNA_Phe. These results suggest that the editing (or hydrolytic) site of PheRS specifically rejects only Tyr, while the other naturally occurring amino acids are efficiently discriminated in the synthetic active site.

The Structure of PheRS Complexed with Tyr
We have determined the crystal structure of PheRS with Tyr at 2.7 Å resolution (see Table 2, PDB ID code 2AMC). A clear electron density (Figure 2A) on the (F_{obs} - F_{calc}) map may be unambiguously attributed to the Tyr molecule located at the synthetic site. The anchoring of the a-NH_3+ group of Tyr is achieved by its interactions with the O_g atom of Ser_a180 and with a well-ordered water molecule, S6 (see Table S1 in the Supplemental Data available with this article online), which in turn is located at an H bonding distance from O_g of Thr_a179, N_e2 of Gln_a218, and O_e1 of Glu_a220. The network is completed by the interaction of Ser_a180 with Glu_a220. The a-carboxylate of the substrate makes H bonds with the side chains of Trp_a149, His_a178, and the class II invariant Arg_a204.

Figure 2. The Synthetic Active Site of the T. thermophilus PheRS with Bound Ligands
(A–C) The T. thermophilus PheRS is orange, and the ligands are slate. The electron density maps calculated as described in Experimental Procedures are contoured at 2.5σ, 3σ, and 3σ for (A) Tyr, (B) Tyr-AMS, and (C) p-Cl-Phe, respectively. Dashed lines show direct and water-mediated H bonds. The water molecules are depicted by red/magenta spheres.
Stereospecific discrimination between the cognate substrate and Tyr is not easy to achieve, as the latter has the aromatic side chain of Phe with only one extra OH group, which makes the phenyl ring less hydrophobic than that of Phe. However, being a neutral polar amino acid, Tyr may participate in various hydrophobic interactions. More specifically, Tyr participates in aromatic-aromatic interactions that characterize the amino acid binding site of T. thermophilus PheRS. The binding mode of Tyr, much like that of Phe (Reshetnikova et al., 1999; Fishman et al., 2001), is also characterized by “edge-to-face” interactions with Phe\textsubscript{258} and Phe\textsubscript{260}. The displacement of Tyr for about 0.8–1.0 Å toward the entrance of the active site cleft as compared to the position of the Phe moiety in the complex with Phe-AMP or to that of Phe itself does not cause steric clashes of the Tyr OH group with water S\textsubscript{98} and the carbonyl oxygen of Ala\textsubscript{314}. The Tyr is additionally stabilized by interactions of the OH group with the main chain oxygen of Phe\textsubscript{260} and O\textsuperscript{11} of Gln\textsubscript{183}.

A second Tyr was identified at the interface between the B3 and B4 domains (Figure 3A). The B3 domain resembles an \( \alpha\)-\( \beta\) sandwich with a layer of two \( \alpha\) helices packed against a four-stranded antiparallel \( \beta\) sheet. There are three insertions in this domain; the largest insertion (residues 265–328) constitutes domain B4. In complexes with Phe-AMP (Fishman et al., 2001) and Phe alone (unpublished data), no electron density was detected in the cavity between domains B3 and B4. The distinct pocket visualized on the \( \beta\) subunit is specific for Tyr and is most likely related to the editing activity of PheRS. By analogy with the synthetic site, the editing site is designed to bind the aromatic amino acid: the phenyl ring of Phe\textsubscript{360} and the side chain of Pro\textsubscript{259} provide “edge-to-face” interactions with Tyr. Phe\textsubscript{360} is strictly conserved in eubacterial PheRSs, while, in several cases, Pro\textsubscript{259} is replaced with Ile or Leu capable of participating in hydrophobic interactions. The anchoring of the Tyr OH group is achieved by its interactions with the O\textsuperscript{1} of Glu\textsubscript{334} and the main chain amide of Gly\textsubscript{315} (Figure 3B). A remarkable structural peculiarity of the editing site is the appearance of the invariant hydrophilic Glu\textsubscript{334} in a fully hydrophilic environment formed by Phe\textsubscript{213}, Leu\textsubscript{215}, Phe\textsubscript{263}, Ile\textsubscript{300}, Ala\textsubscript{314}, Gly\textsubscript{315}, Ala\textsubscript{336}, and Phe\textsubscript{338}. Consequently, the Tyr aromatic ring is placed into the hydrophilic environment. Thus, Glu\textsubscript{334} plays a critical role in specific recognition of the Tyr moiety.

The main chain of Tyr forms both direct and water-mediated contacts within the editing site (see Figure 3B). Whereas water molecule S\textsubscript{99} bridges the amino group of Tyr to the hydroxyl of Thr\textsubscript{354} and the main chain amide of Ala\textsubscript{356}, the COO\textsuperscript{−} group is locked in a position by interaction with His\textsubscript{261} and extensive H bonding mediated by three water molecules (see Table S1). The carboxyl group of Tyr interacts through the S14 molecule with O\textsuperscript{1} of Thr\textsubscript{249}, through S\textsubscript{80} with O\textsuperscript{2} of Glu\textsubscript{323}, and uses S112 to make contact with O\textsuperscript{11} of Asn\textsubscript{300}.

The distance between the C\textsuperscript{α} atoms of two Tyr molecules located in the synthetic and the editing sites is about 35 Å. It is similar to the distances that occur in class I IleRS (34 Å), ValRS (35 Å), LeuRS (38 Å), and class II ThrRS (39 Å). No electron density was observed in the area in which the Tyr moiety may be anchored by the main chain carbonyl of Leu\textsubscript{311} and the side chain hydroxyl of Ser/Thr\textsubscript{322}, as was expected by comparative structural modeling (Roy et al., 2004).

The Structure of PheRS Complexed with 5'-O-[N-(L-tyrosyl)sulphamoyl]adenosine
The crystal structure of the PheRS complex with 5'-O-[N-(L-tyrosyl)sulphamoyl]adenosine (Tyr-AMS), a nonhydrolyzable Tyr-AMP analog, determined at 2.6 Å resolution (PDB ID code 2ALY) reveals clear density for the ligand in the synthetic site only (see Table 2 and Figure 2B). In Tyr-AMS, the sulphamoyl group replaces...
the phosphate group. The conformation of Tyr-AMS and its principal interactions within the active site are closely similar to those observed in the complex of PheRS with Phe-AMP. The exception is provided with a lack of the H bond between the carbonyl oxygen of Tyr-AMS and the N1 of Trp2149 belonging to a “helical loop” (residues 138–152) of the catalytic α subunit; the two atoms are separated by ~4 Å. In the complex with Phe-AMP, the respective atoms are at H bonding distance. The changes are triggered by a concerted shift of the “helical loop” as a whole. This effect was also observed in the ternary complex of PheRS with tRNA\textsuperscript{Phe} and L-phenylalaninyl-5′-adenylate (PheOH-AMP), a stable analog of Phe-AMP with a methylene group substituted for the carbonyl one (unpublished data), and in the binary complex with PheOH-AMP (Reshetnikova et al., 1999), where Trp2149 appears to be disordered (lacking electron density). Universally conserved Phe260 and Ala314, together with Val261 (replaced in most sequences with isosteric Thr), provide an optimal size for the amino acid binding pocket. However, the distances between the OH group of free Tyr or Tyr-AMS, or between the chlorine atom of p-Cl-Phe (see below) and a pair of atoms, C1 of Val261 and C0 of Ala314 (see Table S1), provide evidence for actual broadening of the Phe discrimination criteria.

Multiple interactions of Phe-AMP and PheOH-AMP (Reshetnikova et al., 1999; Fishman et al., 2001) are retained in the complex with the noncognate adenylate analog. This is consistent with in vitro aminoacylation kinetic data, which show Tyr-AMS to efficiently compete with both Phe and ATP for binding (see Table 1). A 2-fold higher affinity of Tyr-AMS as compared to that of PheOH-AMP presumably results from the presence of a carbonyl group (replaced in PheOH-AMP) shown to make extra contacts with the enzyme.

The Structure of the PheRS Complex with p-Cl-Phe Recognition of unnatural amino acids by appropriate aaRSs and subsequent misacylation of the cognate tRNAs is a feasible way of in vivo incorporation of the noncanonical amino acids into protein. Ibabu et al. (1994) have demonstrated attachment of the p-Cl-substituted analog of Phe to tRNA and in vivo incorporation into cellular protein by the E. coli Alaa234Gly mutant PheRS (Alaa234 in T. thermophilus PheRS) that exhibits relaxed substrate specificity. In contrast to the wild-type E. coli PheRS shown to activate only p-F-Phe, the engineered enzyme can activate all four p-halogenated (F, Cl, Br, and I) Phe derivatives. To elucidate the mechanism of activation of unnatural amino acids and plasticity of the PheRS amino acid binding pocket, the structure of wild-type PheRS complexed with p-Cl-Phe has been determined at 2.8 Å resolution (see Table 2, PDB ID code 2AKW).

The electron density distinct in shape from those attributed to Phe and Tyr is clearly visible at the synthetic site (Figure 2C). The characteristic extension of the density is in agreement with the C-Cl bond distance (in para-position) of ~1.75 Å observed in various p-Cl-substituted Phe derivatives (Shürmann et al., 1999). The p-Cl-Phe fits well into the electron density, and its location at the active site is similar to those observed for Phe, Tyr, and the aromatic moieties of Tyr-AMS, PheOH-AMP, and Phe-AMP. The appearance of p-Cl-Phe in the Phe binding pocket suggests the absence of steric clashes between the Cl atom, and the C0 (3.5 Å) and C1 (3.3 Å) atoms of Ala234 and Val261, respectively. Unlike the Tyr binding mode (see above), the α-NH\textsubscript{2} group of p-Cl-Phe makes direct interactions with the O\textsubscript{5} of Glu220 and the O' of Ser180 (see Table S1). The change from water-mediated (S6) to direct H bonding is dictated by a small rearrangement in the position of the analog and by a conformational switch of the carboxylic group of Glu220. From least squares superpositions of all of the above complexes in pairs (the referenced complex is PheRS/PheAMP), it follows that O' of Ser180 occupies a rather stable position and makes direct H bonds with the α-NH\textsubscript{2} group in all of the complexes, while the conformation of the Glu220 side chain varies substantially.

Localization of Phe, Tyr, and Tyr-AMS Binding Regions in PheRS Using a Docking Approach To further investigate the regions of PheRS most appropriate for pretransfer and posttransfer editing activities, we employed the AutoDock protocol, which has been shown to localize ligand binding sites efficiently (Morris et al., 1998). The docking algorithm treats ligands as flexible molecules, while keeping protein conformation restrained. A properly docked ligand structure should meet the following requirements: the center-of-mass of a “good” candidate have to be found in a well-populated cluster; the rmsd between the docked molecule and the referenced X-ray structure is small (<2 Å); the energy of the docked ligand is low. The results of searching revealed two well-defined clusters of docked T. thermophilus molecules placed at the synthetic site area and the interface between the B3 and B4 domains (Figure S1B). The denser population of the Tyr binding sites is observed in the editing site area.

The docking protocol has also been applied for searching Phe and Tyr-AMS binding regions (Figures S1A and S1C). Calculations revealed the synthetic active site as the most preferable for accommodation of both ligands. There is only one potential energy funnel for Phe. The vast majority of Tyr-AMS docking patterns are also related to the synthetic site. Two attractive energy funnels exist for the Tyr molecule. In contrast to the synthetic site, the editing site prefers the Tyr molecules and “filters out” the Phe molecules. This is further proof of the limited ability of PheRS’s synthetic active site to effectively discriminate between the two amino acid substrates.

Discussion Specificity of the PheRS Editing Site Complexes with Tyr, p-Cl-Phe, and Phe provide evidence that the noncognate substrates bind in the amino acid binding pocket in a manner closely similar to that of Phe. Moreover, activation of Tyr by the wild-type T. thermophilus PheRS immediately follows from the ATP consumption experiments, as does the close similarity of the Phe-AMP and Tyr-AMS binding modes in the synthetic site. The direct observation of the presence of unnatural p-Cl-Phe in the amino acid binding pocket of T. thermophilus PheRS and the previously revealed
ability of *E. coli* to incorporate p-F-Phe into repetitive polypeptides (Yoshikawa et al., 1994) suggest that not only the *E. coli* mutant Gly294PheRS (Ibba et al., 1994), but also wild-type *T. thermophilus* PheRS, can activate p-Cl-Phe. In contrast to the PheRS/Tyr complex, no electron density was observed in the editing pocket of PheRS complexed with p-Cl-Phe. The space composed of the invariant Gly315, Glu334, Phe360, and Leu286 together with Pro(Ile/Leu)/259, His(Asn)/261, and Ala(Se)/356 is just large enough to accommodate the Cl extremity in the para position of Phe. The only result that would impair the appearance of p-Cl-Phe in the hydrolytic pocket is an unfavorable contact with the side chain of Glu334. The exclusive binding of p-Cl-Phe in the synthetic site is consistent with previous biochemical experiments and is indicative of PheRS editing activity against Tyr only. Stable attachment of para-substituted Phe analogs to tRNA*Phe* by PheRS (Ibba et al., 1994; Kirshenbaum et al., 2002) implies that they are not substrates for editing.

The all-important steric factor of the editing site interior is Gly315 localized at the end of the β hairpin 312–322. The appearance of any residue with a bulky side chain at this position may interlock the correct positioning of the Tyr moiety. The signature sequence of the β hairpin “**A** G/AG **G** G**x**S/T” (where **x** stands for small G, A, or S residues, **G** stands for hydrophobic residues, and **S** stands for any residue) displays a high level of conservation, strongly suggesting the stability of its conformation in eubacterial PheRSs. Thus, invariance of Glu334 and Gly315, and conformation of the β hairpin, adds up to the conservation of the editing site interior in the prokaryotic PheRSs.

**Posttransfer Editing by PheRS**

The lack of Tyr-AMS from the editing site suggests that the B3/B4 interface excludes binding and pretransfer editing of the translocated Tyr-AMP. On the other hand, close structural similarity in the positioning of Phe-AMP, PheOH-AMP, and Tyr-AMS within the synthetic active site bears witness to the absence of certain steric hindrance for the transfer of activated Tyr onto tRNA*Phe* and the subsequent formation of the Tyr-tRNA*Phe* intermediate. Furthermore, the K<sub>i</sub> value determined for Tyr-AMS from kinetic measurements (see Table 1) implies that it binds tightly to the synthetic site of PheRS in complex with tRNA*Phe*. The data obtained by an ATP consumption assay clearly show that Tyr-stimulated ATP hydrolysis is enhanced by tRNA*Phe*, and that the tRNA-dependent reaction dominates alternative pathways controlled, in particular, by pyrophosphate dissociation. The activating effect of tRNA*Phe* is related to its charging capability. This is further confirmed by detailed analysis of the triggering role of tRNA*Phe* in the editing activity of *E. coli* PheRS (Roy et al., 2004). Both structural and biochemical data are indicative of the posttransfer editing activity of prokaryotic PheRSs against misacylated Tyr. Nevertheless, it is pertinent to note that 2 out of 27 docked Tyr-AMS positions with the lowest binding energies are visible in the editing site (Figure S1C). It is likely that calculations based on PheRS structure determined at higher resolution provide a more precise energy spectrum, which, in turn, will bring the docking calculations into better agreement with experiment.

**Interpretation of Site-Directed Mutagenesis Experiments in Terms of Structural Data**

A few replacements of residues within the proposed editing site of *E. coli* PheRS, impairing tRNA-dependent proofreading activity, have been reported (Roy et al., 2004). His/261Ala or His/261Leu mutations break down the entire net of H bonds within the editing site. First, they destabilize the positioning of Tyr, breaking the bond between its COO<sup>−</sup> group and N<sup>4</sup> of His/261. Second, His/261 is located within the core of the extensive network of H bonds formed by water molecules S12, S14, S20, S80, and S112 (via COO<sup>−</sup>) and side chains of Thr/249, Asn/250, Ser/322, and Glu/323 (see Figure 3B). The rupture of water-mediated contacts in the vicinity of Tyr prevents a cleavage of the ester bond between the tRNA and the amino acid. Significant losses in hydrolytic activity caused by the Glu/334Ala mutation are consistent with the critical role of Glu/334 and the fact that the methyl group of Ala renders anchoring of the Tyr OH group impossible. A Trp replacement of Ala/356, whose side chain is exposed to the B3/B4 interface area, may result in substantial conformational changes preventing penetration of Tyr into the editing site cavity. It is notable that residues with the shortest side chains (Ala or Ser) appear in the equivalent position of the β subunit in almost all sequences of eubacterial PheRSs. Thr/354 located at the entrance into the editing site is the only residue whose side chain makes H bonding contact (via S99) with the x amino group of Tyr. Its replacement by Trp would impair the correct positioning of the substrate in the editing site and, moreover, can make the pocket beyond the reach of the substrate.

**Translocation of Tyr-tRNA*Phe* toward the Editing Site**

The two Tyr molecules bound within the editing and synthetic sites are separated by ~35 Å. This implies that Tyr-AMP or Tyr-tRNA*Phe* has to be transferred from the synthetic to the editing site in order for hydrolysis to proceed. A clamp binding mode of tRNA*Phe*, where the anticodon arm is sandwiched between the N-terminal coiled-coil of the x subunit and domain B8 of the β subunit (x stands for the symmetry-related heterodimer), suggests that tRNA and PheRS are specifically fitted together. From an in-depth analysis of the binary complex, it is obvious that there is no alternative “editing tRNA/PheRS” binding mode, in which misacylated tRNA dissociated from PheRS will realign to the new position that is favorable for hydrolysis. This observation is in line with the commonly accepted model of posttransfer editing that suggests the translocation of the tRNA acceptor end loaded with the incorrect amino acid (Dock-Bregenon et al., 2000). The N-terminal module comprising the B3/B4 domains is covalently connected by the extended segment Leu/474-Ala/482 with the “catalytic-like” module (B6-B7). These two are separated by a large cavity wherein the catalytic module (A1-A2) is inserted (Mosyak et al., 1995). The translocation process that may substantially displace the B3/B4 domains will disrupt the network of interactions centered on a Mg<sup>2+</sup> ion, which is located at the β/β subunit interface (near the active site) and exerts control over the catalytic reaction. Thus, shuttling from the synthetic to the editing site is the only course of translocation for the CCA end esterified with the noncognate substrate. Based on the
arrangement of Tyr (in both the synthetic and editing sites) and tRNA\textsuperscript{Phe} (as it was observed in the ternary complex of PheRS with PheOH-AMP and tRNA\textsuperscript{Phe}), we have modeled the CCA end to bring A76 to the position occupied by Tyr in the editing site (Figure 4A). This model shows how A76 could be placed and stabilized to facilitate the hydrolysis of misacylated Tyr-tRNA\textsuperscript{Phe} (Figure 4B). The anchoring of the adenosine moiety is established by hydrophobic interactions with Ile\textsubscript{242} and Leu\textsubscript{286}. Ile\textsubscript{242} belongs to a conserved motif, “I/LN/DxV/LVD/N” (positions 242–247), of domain B3. Leu\textsubscript{286} localized on the extremity of the B4 domain \( \beta \) hairpin 282–292 is an invariant residue in prokaryotic PheRSs and obviously bears functional loads. It is of interest that neighboring Asp\textsubscript{287} is also highly conserved. Together with Thr\textsubscript{285} and Arg\textsubscript{291}, it is implicated in stabilization of the \( \beta \) hairpin conformation. The plane of the A76 base occupies the position midway between the above-mentioned structural elements. The appearance of hydrophobic residues on both sides of the adenine ring reflects the largely hydrophobic nature of this region and is compatible with the idea of generally high propensities of hydrophobic residues to interact with adenine (Moodie et al., 1996).

**Structural Aspects of Hydrolytic Activity in PheRS**

So far, all known aaRSs catalyze ester bond formation between the activated \( \alpha\)-COOH group of the amino acid and the 2’- or 3’-OH group of the tRNA 3’-terminal adenosine. Thus, the posttransfer editing, thought of as a correction of the erroneously acylated tRNA\textsuperscript{Phe} as a prevalent mode in the Phe system, should be associated with cleavage of the ester bond between the carbonyl carbon of the aminoacyl moiety and the oxygen atom of the terminal ribose. The presence of His\textsubscript{261}, Glu\textsubscript{334}, Asn\textsubscript{250}, Thr\textsubscript{249}, and Glu\textsubscript{323} in the vicinity of the ester bond subjected to hydrolysis is reminiscent of the active site of peptidyl-tRNA hydrolase (PTH) dealing with hydrolysis of the ester bond between tRNA and the peptide (Schmitt et al., 1997). Residues Asn\textsubscript{10}, His\textsubscript{20}, and Asp\textsubscript{93} considered as being crucial for PTH activity (Schmitt et al., 1997; Goodall et al., 2004) are similar to the PheRS triad, Asn\textsubscript{250}, His\textsubscript{261}, and Glu\textsubscript{323}, on which they can be superimposed with an rmsd of 1.4 \( \text{Å} \) for the \( \text{C}^\alpha \) atoms. However, the general topology of the B3/B4 domains (\( \alpha\)-\( \beta\) sandwich and slightly distorted \( \beta\) barrel) differs from that of PTH (single \( \alpha\)/\( \beta\) globular domain), and the respective triads belong to different structural elements. Obviously, the two-esterase activities are spanned on the distinct folds. Close inspection of the editing site with the built-in Tyr-A76 shows that the nearest position to the cleaved bond is occupied by water molecule S80. It is conceivable that substrate hydrolysis may be initiated via nucleophilic attack of water S80 oriented by an activating Glu\textsubscript{323}. Interestingly, the “catalytic” water molecule coordinated by His\textsubscript{261} and Glu\textsubscript{323} is present in the editing site. The position of S80 is additionally stabilized by H bonds with Thr\textsubscript{249} and the amide group of Ala\textsubscript{262} via water molecule S14. The activating role of Glu\textsubscript{323}, which is not invariant, may be performed by Thr\textsubscript{249} (via highly the coordinated water S14 observed in all available PheRS structures) or His\textsubscript{261}, which are both well conserved in eubacterial PheRSs.

Manual fitting of the CCA-Tyr moiety into the cavity of the B3/B4 interface gives rise to more than one plausible conformation of C74 and C75 nucleotides of the mischarged tRNA. This is due to the hydrophilic character of the interface and the high level of conservation of residues covering the inner surface of the cavity (Asp/ Glu\textsubscript{33}, Glu/Asp\textsubscript{127}, Asp/Glu\textsubscript{157}, Arg/Lys\textsubscript{240}, Glu/ Asp\textsubscript{355}, Arg/Lys\textsubscript{362}, etc.). Further determination of PheRS structures with nonhydrolyzable analogs of the posttransfer substrates is necessary to detail the translocation and editing events.

**Experimental Procedures**

**Materials and General Methods**

PheRS and native tRNA\textsuperscript{Phe} (isocopyator I, 1700 pmol of Phe/A205 unit) from *T. thermophilus* HB8 were isolated and purified as described (Ankilova et al., 1988; Stepanov et al., 1998). *E. coli* tRNA\textsuperscript{Phe} was synthesized by using runoff transcription of a synthetic gene. 

![Figure 4. Model for tRNA\textsuperscript{Phe} Translocation](image-url)
with T7 RNA polymerase, followed by electrophoretic isolation of the correct length transcript as described (Vasil’eva et al., 2002). Plasmid DNA containing a gene of E. coli tRNA^Phe^ was under control of the phage T7 promoter was a gift from O.C. Uhlenbeck. The tRNA^Phe^ transcript lacking the 3’-terminal nucleotide was prepared as described (Vasil’eva et al., 2002). Radiochemicals were purchased from Amersham Biosciences. L-Phe was purchased from Sigma, and other unlabeled L-amino acids (Ile, His, Trp, and p-CI-Phe) were purchased from Fluka. L-Tyr from Merck and Fluka was assayed by AccQTag amino acid analysis for Phe contamination and purified by freeze-dried recrystallization in water as described (Lin et al., 1983). Tyr-AMS was a gift from M. Tukalo and S. Cusack. PheOH-purified by 4-fold recrystallization in water as described (Vasil’eva et al., 2002). Radiochemicals were purchased from Fluka. L-Tyr from Merck and Fluka was ascribed (Vasil’eva et al., 2002).}

Aminoacylation of tRNA^Phe^ (native T. thermophilus or E. coli tRNA^Phe^ transcript) with T. thermophilus PheRS was performed in conditions described previously (Vasil’eva et al., 2002). Direct attachment of noncognate amino acids to tRNA^Phe^ was measured in similar conditions by the addition of 100 μM L-[14C]Tyr, L-[14C]Ile, or L-[14C]His or 20 μM L-[3H]Tyr to the reaction mixture instead of 14C/3H-labeled Phe. Inhibition of Phe-tRNA^Phe^ synthesis with noncognate amino acids was measured in the presence of a nonsaturating concentration of L-[14C]Phe (0.5 mM); concentrations of Ile and His varied in the range of 0.1–20 mM, and those of Trp and Tyr were in the range of 0.1–4 mM. In all inhibition analyses, conditions were selected to ensure linear reaction rates. The Phe concentration used as variable substrate was 0.4–4 mM, and the concentration of ATP varied in the range of 0.05–0.6 mM. Concentrations of inhibitors were in the range of their inhibition constants. Inhibition types were analyzed from double-reciprocal plots (Cornish-Bowden and Wharton, 1976). The kinetic parameters (Kcat, KM, and Kapp = KM/[1+I/Ki]) for competitive inhibitors, where I is concentration of the inhibitor) were calculated by using the Microcal Origin 4.10 program. The reported KM and K values represent the average of at least two determinations with standard deviations less than 15%.

ATP Hydrolysis Assay
ATP hydrolysis catalyzed by PheRS was measured at 37°C. A 20 μl reaction mixture contained 50 mM Tris-HCl (pH 8.5), 9 mM MgCl2, 2–200 μM [14]HATP, and 2–300 μM Phe or 2–1200 μM Tyr (or other noncognate amino acids added at 300 μM). A total of 0.5–10 μM tRNA^Phe^ (native T. thermophilus or E. coli tRNA^Phe^ transcript, correct length or lacking the 3’-terminal nucleotide) and 4 μM yeast inorganic pyrophosphatase (Sigma) were added when indicated. The reactions were initiated by the addition of 0.2 μM PheRS. At variable incubation times (2–40 min), samples of 1 μl were spotted onto silicagel sheets (DC-Plastikfolien Kieselgel 60 F254, Merck). Unlabeled ATP and AMP were applied to the plate at the origin prior to use. The nucleotides were separated by TLC developed in a mixture of dioxane, concentrated ammonia, and water (6:1:4, v/v). The spots were marked in UV light and cut out, and the radioactivity was measured in a liquid scintillation counter.

Crystal Preparation and Data Collection
The crystals of native T. thermophilus PheRS were grown as described previously (Chernaya et al., 1987). The protein concentration in the drop was 3 mg/ml. Crystals grew within 3 months to maximum dimensions of 0.3 × 0.3 × 0.2 mm3.

To produce complexes, the crystals were soaked for 48 hr in a mixture containing the crystallization buffer and 1 mM ligand (Tyr, Tyr-AMS, or p-CI-Phe). Data were collected at the synchrotron radiation source at ESRF (beamline ID14), France and on the in-house Rigaku R-axix IV+ image plate detector. Before flash cooling, soaked crystals were transferred into a cryoprotectant containing 30% (v/v) glycerol. Diffraction intensities were evaluated and integrated by using the HKL package (Otwinowski and Minor, 1997) and were scaled further by using programs from the CCP4 package. A summary of the data collection statistics is given in Table 2.

Structure Determination and Refinement
The 2.9 Å refined structure of T. thermophilus PheRS (Mosyak et al., 1995, 1pys) was used as a starting model. After rigid-body refinement and cycles of simulated annealing and conjugate gradient

Docking PheRS with Phe, Tyr, and Tyr-AMS
To generate the input files, AutoDockTools (ADT) (http://www.scripps.edu/pub/olson-web/doc/autodock/tools.html) was used. Gasteiger-Hückel charges were assigned to ligand atoms. Water molecules were eliminated from PDB files to suit ADT requirements. To include binding regions of both the active and editing sites, the simulation box 50 Å × 50 Å × 50 Å has been chosen. Grids for potential evaluation had a resolution of 0.45 Å. The parameters of the Lamarckian genetic algorithm correspond to the AUTODOCK 3.05 default values. All α bond torsion angles were free to rotate; 3 bonds for Phe and p-CI-Phe, 4 bonds for Tyr, and 14 bonds for Tyr-AMS.

Supplemental Data
Supplemental Data including a graphical representation of the ligand-binding sites deduced from the AutoDock protocol and a list of the interactions in the described complexes are available at http://www.structure.org/cgi/content/full/13/12/1799/DC1/.

References


Determined by amino acid binding pocket size in Escherichia coli.


Accession Numbers

Coordinates have been deposited in the PDB with accession codes 2AMC, 2AKW, and 2ALY.