Substrate Specificity Is Determined by Amino Acid Binding Pocket Size in *Escherichia coli* Phenylalanyl-tRNA Synthetase†

Michael Ibba, Peter Kast,‡ and Hauke Hennecke*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH Zentrum, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

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ABSTRACT: Alanine at position 294 (Ala294) within the motif 3 consensus of *Escherichia coli* phenylalanyl-tRNA synthetase α subunit has previously been implicated as a determinant of amino acid specificity. To characterize the role of Ala294, the catalytic effects of amino acid replacements at this position were tested with purified wild-type and mutant phenylalanyl-tRNA synthetases. We show that Ala294 is involved in amino acid binding and that it influences specificity as a determinant of binding pocket size. Replacement of Ala294 by either glycine or serine, thereby increasing or decreasing the size of the binding pocket, respectively, reduces affinity for phenylalanine. The Gly294 mutant shows a relaxed specificity toward synthetic para-halogenated phenylalanine analogues, the apparent dissociation constant K_m increasing in direct relation to an increase of the van der Waals radius of the para group, thus confirming the role of position 294 in determining amino acid binding pocket size. For the substrate analogue *p*-chlorophenylalanine, attachment to tRNA and *in vivo* incorporation into cellular protein by the Gly294 mutant were demonstrated. Tyrosine activation was also improved with this mutant, but the resulting enzyme–Tyr–adenylate complex was rapidly hydrolyzed, indicating the presence of a proofreading mechanism in *E. coli* phenylalanyl-tRNA synthetase.

Aminoacyl-tRNA synthetases catalyze the charging of specific acceptor tRNAs with their cognate amino acids (Schimmel, 1987; Carter, 1993). They can be divided into two distinct classes on the basis of the presence of mutually exclusive sets of short amino acid sequence motifs (Eriani et al., 1990). Class I synthetases contain a Rossmann dinucleotide-binding fold which features the signature peptides HIGH and (K)MSK(S) in homologous regions (Blow et al., 1983; Rould et al., 1989); class II synthetases contain a central antiparallel β-sheet and can be defined by three sequence motifs (Cusack et al., 1990). One of these, motif 3, is found in all class II synthetases. This division of the aminoacyl-tRNA synthetases has been supported by the solution of several high-resolution crystal structures of enzymes and enzyme–tRNA complexes from both class I (Blow et al., 1983; Rould et al., 1989; Brunie et al., 1990) and class II (Ruff et al., 1991; Leberman et al., 1991). Much work has also focused on the elucidation and functional analysis of identity elements in different tRNAs (Saks et al., 1994) and the specificity of their interaction with cognate synthetases (Cavarelli & Moras, 1993; Weygand-Durasevic et al., 1993). Together, these two approaches have provided a detailed understanding of the processes by which aminoacyl-tRNA synthetases and their cognate tRNAs interact. Less well characterized are the mechanisms by which aminoacyl-tRNA synthetases recognize their cognate amino acids. The most notable exception is the class I tyrosyl-tRNA synthetase of *Bacillus stearothermophilus*, in which the processes of amino acid recognition, activation, and specificity determination have been studied in great detail (Fersht, 1987; de Prat Gay et al., 1993). Based on the crystal structures of the free and Tyr-AMP-complexed enzyme, Fersht and co-workers have constructed a series of nondisruptive mutations in the network of active site residues. Detailed kinetic analysis has led to the elucidation of the mechanism of activation and the roles of specific amino acid side chains of tyrosyl-tRNA synthetase in this process. Such studies have also been undertaken for the methionyl-, glutaminyl-, and aspartyl-tRNA synthetases whose crystal structures have been solved at sufficiently high resolution to allow the characterization of the amino acid activation mechanism (Ghosh et al., 1991; Perona et al., 1993; Cavarelli et al., 1994).

While examples of all of the aminoacyl-tRNA synthetase genes have been cloned and sequenced (Carter, 1993), for the majority no high-resolution structure has yet been solved, and consequently little is known for most synthetases about the role of specific amino acid residues in recognition and catalysis. This is also the case for the class II phenylalanyl-tRNA synthetase (PheRS;[1] EC 6.1.1.20), where analysis at the molecular level has been limited as the three-dimensional structure at 2.9-Å resolution was only recently solved (Mosyak & Safro, 1993). This enzyme is of particular interest as it has the unusual $\alpha_2\beta_2$ quaternary structure which is only possessed by one other aminoacyl-tRNA synthetase, glycyl-tRNA synthetase, which is also a member of class II. PheRS catalyzes amino acid attachment to tRNA in a two-step reaction which can be summarized as follows:

$$E + Phe + ATP \rightleftharpoons [E{\cdot}Phe{\sim}AMP] + PP_i$$

$$[E{\cdot}Phe{\sim}AMP] + tRNA^{Phe} \rightleftharpoons Phe\text{-}tRNA^{Phe} + AMP + E$$

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* Corresponding author. Tel: 41-1-632 3318; Fax: 41-1-262 0647.

‡ Present address: Departments of Chemistry and Molecular Biology, The Scripps Research Institute, MB-15, 10666 N. Torey Pines Rd., La Jolla, CA 92037.

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[1] Abbreviations: PheRS, phenylalanyl-tRNA synthetase; Gly294PheRS, Ala294 → Gly replacement in phenylalanyl-tRNA synthetase; Ser-294PheRS, Ala294 → Ser replacement in phenylalanyl-tRNA synthetase; PP_i, inorganic pyrophosphate; *p*-F-Phe, *p*-Cl-Phe, *p*-Br-Phe, and *p*-I-Phe, *p*-fluoro-, *p*-chloro-, *p*-bromo-, and *p*-iodo-phenylalanines.

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PheRS is the only class II enzyme to catalyze amino acid attachment primarily at the 2′-OH of the tRNA's 3′-terminal ribose, a characteristic of all class I enzymes (Carter, 1993). Despite the lack of sufficiently detailed structural data, the use of molecular biology to analyze mutant enzymes generated by genetic techniques has revealed residues within PheRS that may contribute to the phenylalanine (Phe) binding site. Of particular interest was the analysis of a mutant previously shown to have an altered substrate specificity (Kast & Hennecke, 1991). Earlier studies in *Escherichia coli* showed PheRS to be the target of *p*-fluorophenylalanine (*p*-F-Phe) toxicity (Hennecke & Böck, 1975), whose attachment to tRNA results in incorporation of *p*-F-Phe into cellular protein. Sequence analysis of the *pheS* genes from wild-type and *p*-F-Phe-resistant strains identified position 294 of the PheRS α subunit as the determinant of *p*-F-Phe resistance, specifically an alanine-to-serine exchange. This residue lies in the A5 region of motif 3, which has recently been implicated in amino acid specificity determination (Cavarelli et al., 1994; Belrhali et al., 1994). Site-directed mutagenesis showed that replacement by a glycine at this position resulted in *E. coli* strains acquiring sensitivity to a broader range of para-substituted phenylalanine analogues (Kast & Hennecke, 1991). In the work reported here, these three enzymes with apparently diverse substrate specificities (wild-type and Ser294- and Gly294PheRS) were produced and purified from suitably constructed homologous *E. coli* backgrounds and used to investigate the role of position 294 in determining amino acid specificity. Other replacements at this position have previously been shown to greatly reduce both activity and protein stability and were not used in this work (Kast & Hennecke, 1991).

The potentially broadened substrate range of Gly294PheRS is also of interest as a means of incorporating novel amino acid analogues into proteins. While this process has long been established for a limited range of analogues (Richmond, 1962), it would be of much interest to both extend this range and increase the efficiency of incorporation. Recently, means of expanding the genetic code *in vitro* have been established (Bain et al., 1992; Chung et al., 1993), but to date no comparably specific *in vivo* system exists. One requirement for such an *in vivo* system is an aminoacyl-tRNA synthetase with increased affinity for an amino acid not normally found in proteins (Bain et al., 1992); thus it was of interest to additionally investigate the potential of Gly294PheRS for use in such a system.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strain RR28 (Hennecke et al., 1982) was used directly as the source of Ser294PheRS (Kast & Hennecke, 1991). For the isolation of wild-type (Ala294) and Gly294PheRS, homologous overexpression systems were constructed. In both cases the host strains were derived from *E. coli* NP37 (Comer & Böck, 1976; Kast et al., 1992) as described below. For wild-type PheRS overproduction, strain KA4 was transformed with the multicopy plasmid pKSC-Ala294 which contains the complete wild-type PheRS operon including flanking regions (pKSC-Ala294 is identical to pKSC-W; Kast & Hennecke, 1991). For Gly294PheRS overproduction, strain KA3 was transformed with pKSC-Gly294. This plasmid is identical to pKSC-Ala294 except for a G-to-C exchange at nucleotide position 881 of the *pheS* gene, which encodes the α subunits of PheRS, as present in pKSB1-M4G (Kast & Hennecke, 1991). pKSC-Gly294 was constructed over several cloning steps (not shown) by replacing the 174-bp *Aat*II–*Bst*BI *pheS* fragment of pKSC-Ala294 with the corresponding fragment from pKSB1-M4G to introduce the Gly294 codon into the *pheS* gene.

Strain Construction. Host strains for the overproduction of wild-type PheRS and Gly294PheRS were constructed as follows. The RecA$^+$ *E. coli* strain NP37, which synthesizes a thermosensitive PheRS as a result of the *pheS5* mutation (Kast et al., 1992), was transformed with either pKSC-Ala294 or pKSC-Gly294 as appropriate. Single colonies of transformants were inoculated in LB medium and incubated overnight at 40 °C with vigorous shaking to allow for DNA recombination events within the approximately 10.25 kb of homologous DNA between plasmid and chromosome. Samples were then plated on minimal medium (Kast & Hennecke, 1991) and incubated at a restrictive temperature for NP37 (40 °C), under which conditions pKSC-type plasmids are unstable (P. Kast, unpublished observation). This also selects for replacement of the chromosomal *pheS5* gene against the plasmidial *pheS* gene by double homologous recombination. Resulting isolates were tested for the absence of any plasmid DNA and loss of low-dose (20 μg/mL) ampicillin resistance, indicating that neither extrachromosomal nor cointegrated plasmids were present. Furthermore, appropriate modification of growth phenotypes on media containing para-substituted Phe analogues was verified (Kast & Hennecke, 1991). Replacement of the *pheS5* allele with the appropriate *pheS* gene was confirmed by direct sequencing after asymmetric amplification of the chromosomal *pheS* gene by polymerase chain reaction as described previously (Kast et al., 1992), except that additionally the regions around *pheS* codon 294 were analyzed by sequencing with primer PEKA10 (Kast & Hennecke, 1991). The strain encoding wild-type PheRS was named KA4, whereas the strain encoding Gly294PheRS was named KA3. Both strains are isogenic except for the *pheS* allele. Transformation of KA4 with pKSC-Ala294 thus provided a strain which exclusively overproduced wild-type PheRS, whereas transformation of KA3 with pKSC-Gly294 led to the overproduction of PheRS containing mutant α and wild-type β subunits.

Substrates. *p*-Fluoro, *p*-chloro, *p*-bromo, and *p*-iodo derivatives of L-phenylalanine were from Fluka Chemie AG (Buchs, Switzerland) as was L-tyrosine; *p*-chloro-D,L-phenylalanine was from Aldrich (Steinheim, Germany). L-[U-^{14}C]Phenylalanine and tetrasodium [^{32}P]pyrophosphate were from Amersham International (Amersham, U.K.) and L-[1-^{14}C]tyrosine (Phe free) and *p*-chloro-D,L-[1-^{14}C]phenylalanine from NEN-DuPont (Boston, MA). *E. coli* MRE600 tRNA was from Boehringer (Mannheim, Germany) and *E. coli* tRNA^{Phe} from Sigma (St. Louis, MO).

Culturing Techniques. Media composition and methods for selection and maintenance of strains were as described previously (Kast & Hennecke, 1991). For protein purification the appropriate strain was inoculated into 10 mL of LB medium containing 0.5 mg/mL ampicillin and grown with vigorous shaking for 8 h at 30 °C. This culture was then used to inoculate 1 L of the complex medium MIM (Mott et al., 1985) containing 0.5 mg/mL ampicillin, which was then incubated under identical conditions for 16 h. The synthetic minimal medium M9 was used for analogue incorporation experiments (Atlas, 1993).

Protein Purification. Unless otherwise noted, all manipulations were performed at 4 °C, and PheRS activity was detected using the standard aminoacylation assay (Hennecke & Böck, 1975). One liter of culture broth, prepared as described above, was centrifuged at 12000*g* for 15 min. The

cell pellet was washed in PRS buffer (20 mM Tris-HCl, pH 7.5; 0.2 mM EDTA; 6 mM 2-mercaptoethanol; 30 mM NH_4Cl; 10 mM $MgCl_2$) and centrifuged as before. One liter of culture broth typically yielded 15 g wet weight of cells. The cells were resuspended in PRS buffer, and RNase-free DNase (Promega, Madison, WI) was added to a final concentration of 3 units/g wet weight of cells. The cell suspension was then passed twice through a French press cell at 18000 psi prior to centrifugation at 27500*g* for 20 min. The resulting supernatant was removed and ultracentrifuged at 120000*g* for 90 min. The supernatant was again removed and adjusted to 50% $(NH_4)_2SO_4$ followed by gentle stirring for 1 h and then centrifugation for 30 min at 27500*g*. The pellet obtained was then dissolved in the minimum volume necessary of PRS buffer and dialyzed overnight against 150 times its own volume. The dialyzed sample was applied to a Sephacryl S300 high-resolution (Pharmacia, Uppsala, Sweden) gel filtration column which was eluted with PRS buffer. The PheRS-containing fractions were pooled and concentrated by ultrafiltration, and the gel filtration step was repeated. PheRS-containing fractions were pooled and concentrated, followed by adjustment to 1.5 M $(NH_4)_2SO_4$. This sample was then applied to a Bio-Rad HIC hydrophobic interaction chromatography column (Bio-Rad, Hercules, CA), which was developed using a 1.5–0 M $(NH_4)_2SO_4$ gradient in PRS buffer. PheRS-containing fractions were pooled and dialyzed as described above. This sample was applied to a Mono-Q anion-exchange chromatography column (Pharmacia, Uppsala, Sweden), which was developed using a 0–250 mM KCl gradient in PRS buffer. PheRS-containing fractions were then checked for electrophoretic purity (Laemmli, 1970) with silver staining (Saase & Gallagher, 1992), pooled, and concentrated by ultrafiltration, and the enzyme concentration was determined by active site titration (see below). The enzyme was then adjusted to 20% glycerol and stored at −20 °C.

Steady-State Kinetic Analysis. All kinetic analyses were performed at 37 °C.

(a) Active Site Titration. Enzyme concentration was determined by active site titration (Fersht et al., 1975). The procedure was as previously described (Wilkinson et al., 1983) except that [^{14}C]tyrosine was replaced with [^{14}C]phenylalanine, and the reaction was performed for 5 min. Calculations were based on half-of-the-sites reactivity for *E. coli* PheRS with phenylalanine as previously reported (Baltzinger & Holler, 1982).

(b) PP_i Exchange. The PP_i exchange reaction was carried out as described previously (Wells et al., 1991) except that tyrosine was replaced as indicated for particular experiments. Data were analyzed by least-squares regression using Eadie–Hofstee plots (Fersht, 1985). All values given are means from at least 3 experiments with a variation of less than 5%.

(c) Aminoacylation. The aminoacylation reaction was performed as described previously (Kast & Hennecke, 1991), except that bulk tRNA was replaced with tRNAs of specific acceptor activity in certain cases as detailed in the results. Data analysis was as under (b) above.

In Vivo Incorporation of Phenylalanine Analogues. A 10-mL culture was grown overnight in M9 medium to an absorbance at 600 nm in the range of 1.5–2. The culture was centrifuged, and the cells were resuspended and diluted to give a final absorbance of 0.5. Either *p*-chloro-D,L-[^{14}C]-phenylalanine or [^{14}C]phenylalanine was added to a final concentration of 80 μM (8.7 μCi/μmol) of the L-enantiomer and the culture incubated with shaking at 37 °C. One-milliliter samples were periodically removed, and the cells were washed in 50 mM Tris-HCl, pH 7.5, and lysed as detailed elsewhere (Cull & McHenry, 1990). The lysate was then briefly centrifuged and the supernatant retained, mixed with 900 μL of 10% trichloracetic acid, and kept on ice for 10 min. The resulting suspension was then processed as for the aminoacylation reaction to measure incorporation of radioactivity into cellular protein.

Table 1: Purification and Activities of PheRS Enzymes

PheRS	purification factor	sp act. [nmol of Phe attached (mg of protein)$^{-1}$ min^{-1}]
Ala294 (wild-type)	24	861
Gly294	33	221
Ser294	328	317

Table 2: PP_i Exchange and Aminoacylation Kinetics of PheRS with Phenylalanine[a]

PheRS	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
	PP_i Exchange[b]		
Ala294 (wild-type)	40	8280	207
Gly294	150	4920	33
Ser294	220	3420	16
	Aminoacylation[c]		
Ala294 (wild-type)	7	336	48
Gly294	17.5	84	4.8
Ser294	22.5	204	9.1

[a] Phenylalanine concentrations were varied in the following ranges: [b]20–1000 μM and [c]2–100 μM.

RESULTS

Purification of Wild-Type and Engineered PheRS Enzymes. Wild-type PheRS, Gly294PheRS, and Ser294PheRS were all purified to homogeneity as defined by the absence of any non-PheRS protein bands after SDS–PAGE and silver staining. The engineered proteins contained no wild-type PheRS since they were purified from suitably modified *E. coli* host strains (see above). The two putative PheRS bands, corresponding to the α and β subunits, were verified by Western blot analysis using polyclonal anti-*E. coli* PheRS serum (Hennecke & Böck, 1975; data not shown). The specific activity of the wild-type protein was comparable to previously determined values [for example, Baltzinger and Holler (1982)] while overproduction of this enzyme and of Gly294PheRS resulted in a 10–14-fold reduction in the required purification factor (Table 1).

Steady-State Kinetics for Phenylalanine. The kinetic parameters with respect to phenylalanine for wild-type and engineered PheRS were investigated using the PP_i exchange and aminoacylation reactions (Table 2). Values for the wild-type (Ala294) enzyme are in good agreement with previous work (Holler, 1980; Santi & Danenberg, 1971). Both Gly294- and Ser294PheRS exhibit higher K_m for PP_i exchange and aminoacylation than the wild-type enzyme. Despite Ser294PheRS activating phenylalanine more poorly than Gly294PheRS as indicated by a lower k_{cat}/K_m for PP_i exchange, it is the more efficient of the two in the aminoacylation reaction (Table 2).

Steady-State Kinetics for ATP. The kinetic parameters with respect to ATP were investigated as for phenylalanine above (Table 3). Kinetic parameters determined for Ala294PheRS (wild-type) were in good agreement with earlier reports [for example, Santi and Danenberg (1971)]. The K_m values for both reactions with Gly294PheRS were almost identical to those for wild-type PheRS, indicating that the alanine-to-glycine substitution is not disruptive to the overall

Table 3: PP_i Exchange and Aminoacylation Kinetics of PheRS with ATP[a]

PheRS	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
	PP_i Exchange[b]		
Ala294 (wild-type)	800	10920	14
Gly294	760	5400	7
Ser294	920	3960	4
	Aminoacylation[c]		
Ala294 (wild-type)	123	308	2.5
Gly294	142	87	0.61
Ser294	287	139	0.48

[a] ATP concentrations were varied in the following ranges: [b]200–4000 μM and [c]20–800 μM.

Table 4: Aminoacylation Kinetics of PheRS with $tRNA^{Phe}$ [a]

PheRS	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Ala294 (wild-type)	0.25	308	1232
Gly294	0.29	67	231
Ser294	0.25	133	600

[a] $tRNA^{Phe}$ concentrations were varied in the range 0.025–1 μM.

structure of PheRS and does not interfere with ATP binding. The K_m for ATP in the aminoacylation, but not the PP_i exchange, reaction with Ser294PheRS is significantly higher than for both other enzymes.

Steady-State Kinetics for tRNA. The kinetic parameters with respect to $tRNA^{Phe}$ for wild-type and engineered PheRSs were investigated using the aminoacylation reaction, with $tRNA^{Phe}$ replacing bulk tRNA (Table 4). The results for wild-type PheRS were in good agreement with recent reports (Peterson & Uhlenbeck, 1992). All three enzymes had similar K_m for $tRNA^{Phe}$ in the aminoacylation reaction, further indicating that the alanine-to-serine and alanine-to-glycine substitutions are nondisruptive.

Synthetic Phenylalanine Analogues as Substrates of PheRS. The ability of phenylalanine analogues to act as substrates for wild-type and engineered PheRSs was investigated. The aminoacylation reaction was carried out in the presence of different concentrations of the *p*-fluoro (0–250 μM), *p*-chloro (0–1 mM), *p*-bromo (0–1 mM), and *p*-iodo (0–500 μM) derivatives of L-phenylalanine. Aminoacylation by Ser294PheRS was not affected by the presence of any of the analogues. Aminoacylation by wild-type PheRS was inhibited exclusively by *p*-F-Phe whereas Gly294PheRS was inhibited to varying extents by all of the para-halogenated phenylalanine analogues. In all cases inhibition was competitive, the apparent K_m increasing with higher inhibitor concentration while V_{max} was unchanged (not shown). The K_i (Fersht, 1985) for the various analogues increased in proportion to the increasing van der Waals radii (Bondi, 1964) of the para-halogen atoms (data not shown). A similar pattern was observed when PP_i exchange was performed with phenylalanine analogues replacing phenylalanine. Wild-type PheRS could activate only *p*-F-Phe, but Gly294PheRS could activate all four analogues, the K_m increasing in proportion to the van der Waals radii of the para groups (Figure 1). Kinetic parameters could not be accurately determined for *p*-I-Phe activation by Gly294PheRS due to its relatively poor solubility. Ser294PheRS was unable to activate any of the analogues.

These results supported the idea that Gly294PheRS can charge $tRNA^{Phe}$ with Phe analogues such as *p*-Cl-Phe, as has been suggested by the earlier observations that strains carrying a plasmid-borne Gly294PheRS gene are sensitive to *p*-Cl-Phe (Kast, 1994) and S140 extracts from these strains can aminoacylate $tRNA^{Phe}$ at an increased rate (compared to wild-type) with *p*-F-Phe (Kast & Hennecke, 1991). This was now proven by the successful replacement of [^{14}C]Phe with *p*-Cl-[^{14}C]Phe in the aminoacylation reaction. The K_m for *p*-Cl-Phe was 187 μM (Table 5), which is almost identical to its K_i for aminoacylation, 195 μM. The catalytic efficiency (k_{cat}/K_m) of Gly294PheRS with *p*-Cl-Phe as substrate is about 5-fold lower than for Phe during both PP_i exchange and aminoacylation (Table 5, and compare with Table 2).

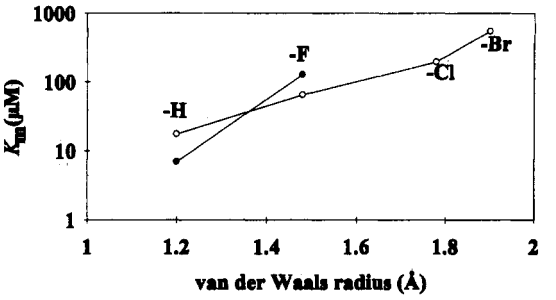

FIGURE 1: Effect of van der Waals radii of para groups of phenylalanine analogues on their K_m during PP_i exchange with wild-type PheRS (●) and Gly294PheRS (○).

Table 5: Steady-State Kinetic Parameters for Gly294PheRS with *p*-Cl-Phe as Amino Acid Substrate

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
aminoacylation	187	163	0.87
PP_i exchange	400	2640	6.6

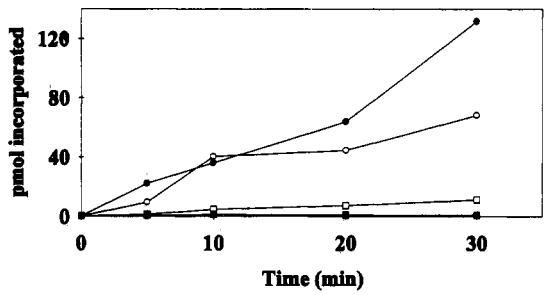

FIGURE 2: In vivo incorporation of [^{14}C]Phe and *p*-Cl-[^{14}C]Phe by *E. coli* strains producing different PheRSs: wild-type PheRS with phenylalanine (●); wild-type PheRS with *p*-Cl-Phe (■); Gly294PheRS with phenylalanine (○); Gly294PheRS with *p*-Cl-Phe (□).

In Vivo Incorporation of p-Cl-Phe into Proteins. The ability of Gly294PheRS to mediate the incorporation of *p*-Cl-Phe into cellular proteins *in vivo* was investigated using the strains KA3/pKSC-Gly294 and KA4/pKSC-Ala294 (the latter containing wild-type PheRS). Overnight cultures of both strains were grown, divided, and diluted, and either [^{14}C]-Phe or *p*-Cl-[^{14}C]Phe was added. The cultures were then incubated and sampled at 0, 5, 10, 20, and 30 min (Figure 2). Only KA3/pKSC-Gly294 incorporated *p*-Cl-Phe, in agreement with the *in vitro* $tRNA^{Phe}$ aminoacylation data. The level of incorporation was about 20% of Phe incorporation by the same strain.

Tyrosine as a Substrate of PheRS. The misactivation of tyrosine by wild-type and engineered PheRS was initially investigated by PP_i exchange kinetics. Activation was only measurable with Gly294PheRS, which gave a K_m of 2470 μM and a k_{cat} of 5100 min^{-1}. The aminoacylation reaction was also performed using Gly294PheRS in the presence of excess tyrosine (600 μM), but the formation of Tyr-$tRNA^{Phe}$ was not observed. In order to clarify the fate of tyrosine after activation by Gly294PheRS, we investigated the stability of various enzyme–amino acid–adenylate complexes. Enzyme–

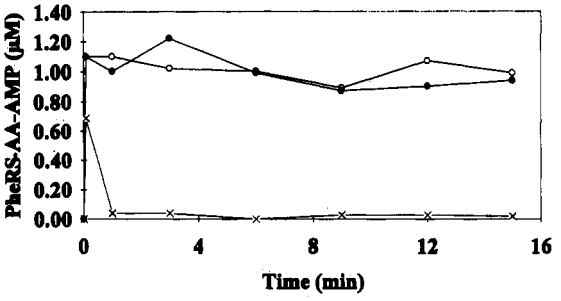

FIGURE 3: Formation of PheRS·AA(amino acid)~AMP complexes by Gly294PheRS with different amino acids: phenylalanine (●); *p*-Cl-Phe (○); tyrosine (×).

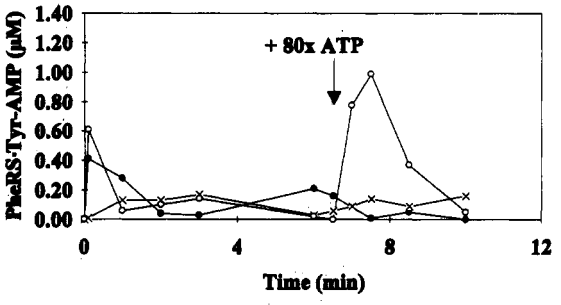

FIGURE 4: Formation of PheRS·Tyr~AMP complexes with different PheRSs: wild-type PheRS (●); Gly294PheRS (○); Ser294PheRS (×). ATP was added to all reactions after 6.5 min to give a final concentration 80-fold higher than the starting level.

amino acid–adenylate complexes were made as described for active site titration (see Materials and Methods). No complex was observed with tyrosine for wild-type PheRS or Ser294PheRS, while Gly294PheRS only appeared to form a transiently stable complex (Figure 3). The titration of excess ATP into the reaction resulted in renewed complex formation (Figure 4), indicating that Gly294PheRS enters an unproductive cycle of binding with tyrosine which eventually depletes ATP. By contrast, Gly294PheRS forms an apparently stable complex with *p*-Cl-Phe (Figure 3).

DISCUSSION

Amino Acid Binding Pocket Size as a Determinant of Specificity. When viewed collectively, the steady-state kinetic data for the natural substrates of PheRS clearly show that Ala294 in the α subunit of PheRS is involved in the process of phenylalanine binding. Alanine at this position apparently has the optimal volume to maximize the specificity of the binding pocket for phenylalanine: either increasing the void volume by the substitution with the smaller glycine (residue volume = 66 Å^3 compared to 92 Å^3 for alanine; Chothia, 1984) or decreasing the volume by the substitution with serine (99 Å^3) increases the K_m for phenylalanine. More conclusive evidence for the role of binding pocket size comes from the data for phenylalanine analogues as inhibitors and substrates of wild-type and engineered PheRS. Ser294PheRS has an even narrower substrate range than wild-type PheRS, being unable to activate *p*-F-Phe, which supports the role of binding pocket size as a determinant of specificity, although the possibility that charge–charge repulsion accounts for this inability cannot be excluded. On the other hand, Gly294PheRS displays a significantly broadened substrate range compared with that of wild-type PheRS, being able to activate *p*-F-Phe, *p*-Cl-Phe, *p*-Br-Phe, and perhaps even *p*-I-Phe. But rather than simply having lost its ability to discriminate against substrates which are similar to but are not phenylalanine, Gly294PheRS possesses a modified catalytic behavior strictly dependent on the size of the substrate. As the size of the para-position atom increases, so do both the K_i for aminoacylation and the K_m for PP_i exchange with the respective substrates. The sharp increase of both parameters for para substitutions larger than Cl suggests that access to the active site may itself become limiting in these cases. This dependence on binding pocket size for the determination of substrate specificity in PheRS is not surprising if one considers its *in vivo* specificity requirements; that is, that it should be able to discriminate against all other 19 amino acids. While in most cases profound differences in the structure of the amino acid's side chain compared with the phenyl group will make this relatively straightforward, the ability to discriminate against tyrosine is critical. One obvious way of achieving this is by means of a binding pocket which excludes tyrosine on the basis of its increased size compared with phenylalanine.

Tyrosine as a Substrate of Engineered E. coli PheRS. Given that Gly294PheRS displays a relaxed specificity toward phenylalanine analogues with enlarged para groups, one might also expect it to accept tyrosine as a substrate. This is indeed the case, tyrosine being activated by Gly294PheRS, but not by either wild-type or Ser294PheRS. Previous reports concerning the activation of tyrosine by wild-type *E. coli* PheRS are slightly contradictory, with either little (Gabius et al., 1983) or no activation (Santi & Danenberg, 1971) observed. These discrepancies may result from trace contaminants of phenylalanine in some commercial tyrosine preparations (Jakubowski & Goldman, 1992), and it is worth noting that, where no activation was observed, PP_i exchange-stimulating contaminants had been specifically removed. The unlabeled tyrosine used here had previously been shown to be phenylalanine-free in a separate enzymatic system (C. Appert, personal communication), and the ^{14}C-labeled compound was supplied as "phenylalanine-free".

While tyrosine is activated by Gly294PheRS, the resulting enzyme–Tyr–adenylate complex is rapidly hydrolyzed after its formation, a common diagnostic feature of proofreading in the presence of noncognate amino acids (Jakubowski & Goldman, 1992). This evidence for the presence of a proofreading mechanism against tyrosine in *E. coli* PheRS suggests that the wild-type enzyme may indeed activate tyrosine but the rate of hydrolysis is such that the complex cannot be readily observed. Interestingly, this proofreading is specific for tyrosine since the complex Gly294PheRS·*p*-Cl-Phe~AMP was of comparable stability to Gly294PheRS·Phe~AMP. Support for the existence of such a proofreading mechanism in *E. coli* comes from comparison with the *Saccharomyces cerevisiae* cytoplasmic PheRS, for which hydrolysis of noncognate complexes between PheRS and tyrosine has previously been reported (Lin et al., 1983). Notably, this PheRS naturally contains a glycine at the amino acid position analogous to position 294 in *E. coli* in contrast to all known bacterial PheRSs which contain an alanine there (Kast & Hennecke, 1991). Even though the activation of tyrosine by Gly294 PheRS is poor, one might nevertheless expect to observe the formation of some Tyr-tRNA^{Phe}. Lack of formation of this product may be a result of either the incompatibility of PheRS·Tyr~AMP with tRNA^{Phe} or the rapid hydrolysis of Tyr-tRNA^{Phe} upon formation, as reported for the *S. cerevisiae* cytoplasmic PheRS (Lin et al., 1984).

p-Cl-Phe as a Substrate of Gly294PheRS in Vitro—Mechanistic Implications. The results for the PP_i exchange and aminoacylation reactions with Gly294PheRS where *p*-Cl-Phe replaces phenylalanine as the substrate show that this

analogue is activated and can be attached to tRNA by the engineered enzyme *in vitro*. The k_{cat} for *p*-Cl-Phe was almost 2-fold higher than for Phe during aminoacylation but significantly lower during PP_i exchange. Similarly, Ser294PheRS performed more poorly during PP_i exchange with phenylalanine than Gly294PheRS, but better during aminoacylation. In both situations, the enzyme–amino acid–adenylate complex would be expected to be less tightly bound than the wild-type complex, which could give rise to a higher k_{cat} in the tRNA attachment reaction. This implies that *E. coli* PheRS has compromised between the rates of amino acid activation and tRNA attachment to obtain a maximal rate of aminoacylation. While the data presented here only show this indirectly, such a situation has clearly been demonstrated for tyrosyl-tRNA synthetase from *B. stearothermophilus* and thus would not be unexpected (Avis et al., 1993; Avis & Fersht, 1993).

p-Cl-Phe as a Substrate of Gly294PheRS in Vivo. The possibility that *p*-Cl-Phe-tRNA is unstable *in vivo* was excluded by the incorporation of the analogue into total cellular protein in a Gly294PheRS-containing *E. coli* strain. The absence of *p*-Cl-Phe incorporation in a wild-type PheRS background discounts the possibility that the observed incorporation is an artifact resulting from metabolic recycling of the labeled group of *p*-Cl-[^{14}C]Phe. Thus a strain containing the gene for the Gly294 mutant enzyme has the ability to incorporate *p*-Cl-Phe and probably other analogues such as *p*-Br-Phe into proteins in place of phenylalanine. This fulfills one of the requirements for the development of *in vivo* systems for the incorporation of synthetic amino acid analogues into proteins: the engineering of aminoacyl-tRNA synthetases to accept novel substrates.

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