Ancient Adaptation of the Active Site of Tryptophanyl-tRNA Synthetase for Tryptophan Binding†

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ABSTRACT: The amino acid binding domains of the tryptophanyl (TrpRS)- and tyrosyl-tRNA synthetases (TyrRS) of *Bacillus stearothermophilus* are highly homologous. These similarities suggest that conserved residues in TrpRS may be responsible for both determining tryptophan recognition and discrimination against tyrosine. This was investigated by the systematic mutation of TrpRS residues based upon the identity of homologous positions in TyrRS. Of the four residues which interact directly with the aromatic side chain of tryptophan (Phe5, Met129, Asp132, and Val141) replacements of Asp132 led to significant changes in the catalytic efficiency of Trp aminoacylation (200−1250-fold reduction in k_{cat}/K_M) and substitution of Val141 by the larger Glu side chain reduced k_{cat}/K_M by 300-fold. Mutation of Pro127, which determines the position of active-site residues, did not significantly effect Trp binding. Of the mutants tested, D132N TrpRS also showed a significant reduction in discrimination against Tyr, with Tyr acting as a competitive inhibitor but not a substrate. The analogous residue in *B. stearothermophilus* TyrRS (Asp176) has also been implicated as a determinant of amino acid specificity in earlier studies [de Prat Gay, G., Duckworth, H. W., and Fersht, A. R. (1993) *FEBS Lett. 318*, 167−171]. This striking similarity in the function of a highly conserved residue found in both TrpRS and TyrRS provides mechanistic support for a common origin of the two enzymes.

Aminoacyl-tRNAs consist of small RNAs (typically 76 nucleotides in length) to which an amino acid has been esterified at the 3′-end. The principal function of aminoacyl-tRNAs is to pair, via their anticodons, with complementary codons in mRNA and then act as substrates for ribosomal protein synthesis. The fidelity with which the genetic information contained in messenger RNA is translated into the corresponding sequence of amino acids in a protein is partly dependent (*1*) on the existence in the cell of a full complement of cognate aminoacyl-tRNAs, i.e., tRNAs exclusively 3′-esterified with an amino acid corresponding in identity to their anticodon. The majority of aminoacyl-tRNAs are synthesized by direct aminoacylation of a particular tRNA with the corresponding amino acid in a reaction catalyzed by a family of enzymes collectively known as the aminoacyl-tRNA synthetases (AARS;[1] reviewed in refs *2* and *3*). The AARSs can be divided into two mutually exclusive classes (I and II) defined by the presence of distinct sequence and structural motifs (*4*−*6*). Within classes I and II, the AARSs can be further divided into subclasses which constitute proteins showing a higher degree of sequence and structural homology compared to other members of the same class (*7*, *8*). The class I and II AARSs are clearly divided into three subclasses each (*7*), whose individual members are believed to be more recently related to each other in evolutionary terms than they are to members of the other subclasses (e.g., *9*). Such groupings among the AARSs are presumed to reflect common ancestries for the various subgroups and ultimately for all members of a particular class. In a few cases these inferred evolutionary relationships have been supported by experimental approaches. For example, in subclass 1a analogous sequence motifs in the methionyl- and isoleucyl-tRNA (IleRS) synthetases were found to determine the specificity of tRNA recognition (*10*) and IleRS and valyl-tRNA synthetase share functionally homologous editing domains (*11*, *12*). It has also been shown that the amino acid specificity of the subclass 1b enzyme glutaminyl-tRNA synthetase (GlnRS) can be partially changed to that of the other subclass 1b member, glutamyl-tRNA synthetase (GluRS, *13*). Taken together, such results provide

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[1] Abbreviations: AARS, aminoacyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; $tRNA^{Trp}$, transfer RNA specific for tryptophan; TrpRS, tryptophanyl-tRNA synthetase; TyrRS, tyrosyl-tRNA synthetase.

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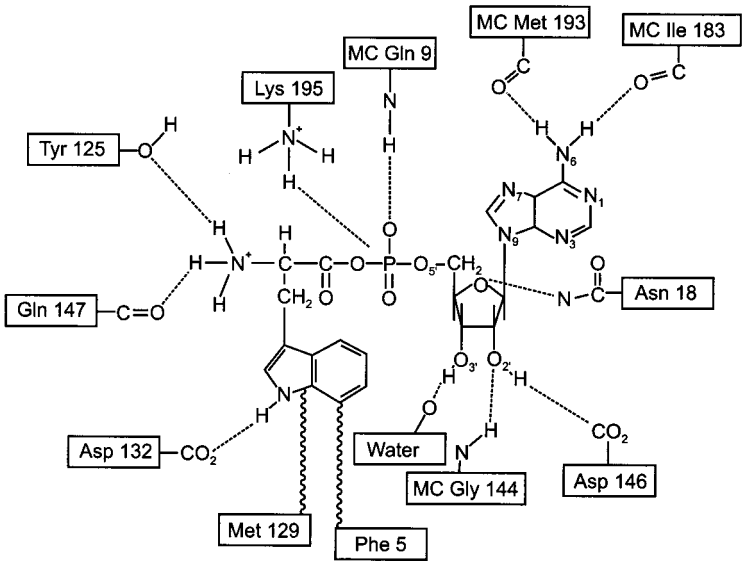

FIGURE 1: Active-site−ligand interactions in the TrpRS·Trp−5′AMP complex (*16*). Dashed lines indicate hydrogen bonds, wavy lines hydrophobic interactions.

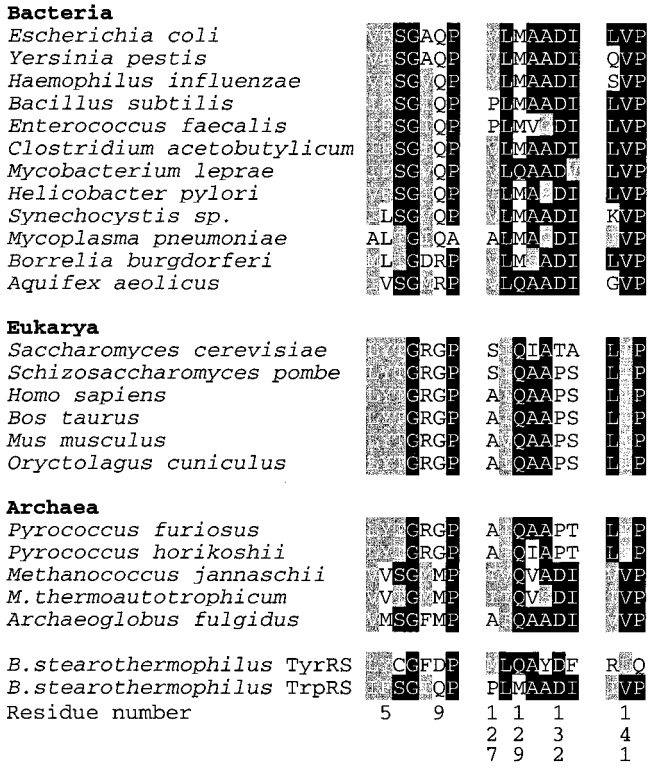

FIGURE 2: Partial alignment of TrpRS amino acid sequences showing the regions investigated in this study. The structure-based alignment of *B. stearothermophilus* TyrRS (*16*) is also shown. Residue number corresponds to *B. stearothermophilus* TrpRS.

strong support for common ancestry among subclass members.

Phylogenetic (*14*) and structural (*15*) studies have previously indicated that the class 1b enzymes glutaminyl- (GlnRS) and glutamyl-tRNA synthetase (GluRS) are the most closely related of all the aminoacyl-tRNA synthetases in terms of their catalytic domains. Based upon comparisons between the active sites of GlnRS and GluRS, a mutant GlnRS was constructed which showed a 25,000-fold increase in specificity for glutamic acid (*13*), providing biochemical support for the close relationship between the two enzymes. Solution of the three-dimensional structures of the subclass 1c enzymes tryptophanyl-(TrpRS) and tyrosyl-tRNA synthetase (TyrRS) from *B. stearothermophilus* revealed a great degree of homology between their active site architectures in terms of amino acid recognition (*16*). This suggested that, as with GlnRS and GluRS, TrpRS and TyrRS may have recently arisen from a common ancestor. However, phylogenetic studies have suggested that TrpRS and TyrRS are more distantly related than are GlnRS and GluRS (*17*, *18*).

To investigate the evolutionary relationship between TrpRS and TyrRS, residues in or near the active site of *B. stearothermophilus* TrpRS were mutated based upon the identity of the corresponding positions in the active site of *B. stearothermophilus* TyrRS. Residues predicted to contribute to amino acid specificity were selected for mutation by comparison of the crystal structures of *B. stearothermophilus* TrpRS (*16*) and TyrRS (*19*, *20*) complexed with their respective cognate aminoacyl-adenylates (Figure 1) and examination of sequence alignments (Figure 2). The resulting mutant TrpRS-encoding genes were then investigated for in vivo activity and used to produce and subsequently purify the corresponding mutant proteins. In vitro kinetic characterization showed that while the predicted residues are critical for tryptophan binding, they only modestly contribute to substrate specificity. This supports the suggestion from phylogenetic analyses that TrpRS and TyrRS are specifically related, albeit distantly.

MATERIALS AND METHODS

General. Oligonucleotides for PCR and sequencing were kindly provided by G. O'Neill, Merck Frost Centre for Therapeutic Research (Canada) or purchased from the William M. Keck Foundation for Biochemical Research, Yale University. L-[5-^{3}H]tryptophan (27 Ci/mmol), L-[3-^{14}C]-tryptophan (53.8 mCi/mmol), and [^{32}P]pyrophosphate (25.87 Ci/mmol) were from NEN (Boston, MA).

Media, Strains, and Plasmids. Escherichia coli rich (LB) and minimal media (M9) were made as described by Sambrook et al., (*21*). One liter of rich medium used for growth of *B. stearothermophilus* contained 10 g of Bacto

Tryptone (Difco), 5 g of Bacto Yeast Extract (Difco), 5 g of NaCl, 1.25 mL of a 1 M NaOH solution, 10 g of glucose, and 1 mL of a 40 mM $FeSO_4$ solution. The *B. stearothermophilus* strain NO1063 (kindly provided by Professor P. Moore, Yale University) was grown at 55 °C and used for preparation of genomic DNA for isolation of the $tRNA^{Trp}$ encoding DNA and for Southern blot analysis to determine the number of $tRNA^{Trp}$ encoding genes in *B. stearothermophilus*. The *E. coli* strain BL21(DE3) (*22*) was used as the host for TrpRS production. The *E. coli* strains *trpS*42C and *trpS*4040 (*23*, *24*) were used in the complementation analysis of the *B. stearothermophilus* TrpRS mutants. The plasmid pSK-TrpRS, which carries the wild-type *trpS* gene of *B. stearothermophilus*, was constructed by ligating *Sma*I digested pBluescript II SK (Stratagene) with a *Pvu*II fragment containing the *trpS* gene obtained from pCC282 (*25*).

Isolation of Chromosomal DNA from B. stearothermophilus. Chromosomal DNA from *B. stearothermophilus* strain NO1063 was isolated as described below. Cells were grown overnight in rich media at 55 °C, washed with 10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1 mM EDTA, 20% glucose, and protoplasts were made by incubating cells for 30 min at 37 °C in 10 mL of the above-described buffer containing 5 mg of lysozyme. The solution was cooled to room temperature before 5 mg of proteinase K was added and the solution incubated at 37 °C for 30 min. Phenol/chloroform/isoamyl-alcohol extraction was done three times upon addition of 0.53 g of NaCl followed by ethanol precipitation of DNA. The pellet was resuspended in 0.5 mL of TE pH 8.0 and treated with 20 μg of ribonuclease A for 15 min at 37 °C. The solution was again extracted with phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation and resuspension of DNA pellet in 300 μL of TE pH 7.5.

Cloning of the $tRNA^{trp}$ Gene of B. stearothermophilus. The *B. stearothemophilus* $tRNA^{Trp}$ gene was isolated by PCR using *B. stearothermophilus* NO1063 genomic DNA as template and oligonucleotides matching the surrounding sequences of the $tRNA^{Trp}$ gene from the closely related bacteria *Bacillus subtilis* as primers. The PCR product was subsequently cloned into the bluescript vector pUC18 and sequenced. Initial PCR experiments used as primers oligonucleotides matching the 5′-end and 3′-ends of the $tRNA^{Trp}$ gene from *B. subtilis* (5′-nnnnnaggggcatagtt and 5′-nnnnntg-gcaggggc-3′). To obtain sequence data for the 5′- and the 3′-ends of the *B. stearothermophilus* tRNA gene, oligonucleotides were constructed that matched the upstream and the downstream regions of the $tRNA^{Trp}$ gene of *B. subtilis*. $tRNA^{Trp}$ from *B. subtilis* is located in a tRNA gene cluster on the genome and is surrounded by $tRNA^{Tyr}$ at the 5′-end and $tRNA^{His}$ at the 3′-end (*26*). Thus, the oligonucleotides used matched the coding regions of the $tRNA^{Tyr}$ and $tRNA^{His}$ genes of *B. subtilis* (5′-nnnnngcggactgtaaatccgc-3′ and 5′-nnnnnccagagccacaatctgg-3′). The *B. stearothermophilus* $tRNA^{Trp}$ gene was finally subcloned into the plasmid pBTT (*27*) which is a pUC18 based in vitro and in vivo transcription vector containing the T7-promoter and a transcription termination sequence. The pBTT plasmid normally harbors a $tRNA^{Gln}$ gene, which was substituted with the $tRNA^{Trp}$ gene by digesting pBTT with *Eco*RI and *Bam*HI and ligating the vector fragment with a *Bam*HI/*Eco*RI digested PCR amplified $tRNA^{Trp}$ fragment containing the T7-promoter (primers, 5′-cgatcgccggaattctaat acgactcactataagggcatagtttaatggtagaac-3′ and 5′-atcgttcgcggatcctggcaggggcagtaggaatcgaac-3′; template, pUC18$tRNA^{Trp}$). The modified portion of the resulting plasmid (pT7TT) was sequenced and used in this work for in vitro transcription of *B. stearothermophilus* $tRNA^{Trp}$. Southern blot analysis performed with genomic DNA digested with a variety of restriction enzymes suggests that only one gene encodes $tRNA^{Trp}$ in *B. stearothermophilus*.

In Vitro tRNA Transcription. pT7TT digested with *Bst*NI served as DNA template for in vitro transcription of $tRNA^{Trp}$ (*28*). The procedure for in vitro transcription, purification, and refolding of the transcript is described elsewhere (*29*). Sixty percent of the in vitro transcribed $tRNA^{Trp}$ could be aminoacylated (1080 pmol/A_{260}).

Construction of TrpRS Active-Site Mutants. Mutants of TrpRS were made by site-directed mutagenesis using a plasmid containing the wild-type *B. stearothermophilus trpS* gene (pSK-TrpRS) as template together with appropriate oligonucleotide primers. The mutations were verified by sequencing. Parts of the mutant alleles were amplified by PCR with oligonucleotides that allow introduction of a 5′-terminal *Nde*I site. The remainder of the alleles were isolated from the vector pKS-TrpRS. A three fragment cloning procedure was then used in order to subclone the mutant TrpRS alleles into the *Nde*I and *BamH*I restriction sites of the expressions vectors pET11a or pET3a. The relevant parts of the pET11a and pET3a constructs were subsequently sequenced.

Overexpression and Purification of TrpRS. *E. coli* strain BL21 (DE3) was used as host for production of wild-type and mutant TrpRS enzymes produced from pET11a and pET3a expression vectors at 30 °C under control of the T7-RNA polymerase. Expression of the T7-RNA polymerase gene was achieved by adding IPTG (400 μM) to the growth medium. After one to 2 h induction cells were spun down, washed, and sonicated. After ultracentrifugation, proteins were precipitated by adding $(NH_4)_2SO_4$ to the supernatant to 75% and dialyzed against buffer A (20 mM diethanolamine, 100 mM KCl, 5 mM DTT, 5% glycerol, pH8.5). This and all subsequent steps were performed at 4 °C. Protein extracts were applied on three consecutive chromatographic columns before electrophoretic homogeneity was obtained judged by silver straining. First, dialyzed extracts were applied to a Mono Q column (Pharmacia) and developed with a pH gradient of pH 8.5−5.4 (pH 8.5; buffer A, pH 5.4; buffer B, 20 mM Bis-Tris, 100 mM KCl, 5 mM DTT, 5% glycerol, pH 5.4). TrpRS-containing fractions were then pooled, dialyzed and applied to a Mono S column (Pharmacia) which was developed using a gradient of 2−200 mM KCl in buffer C (10 mM Hepes/KOH pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5 mM DTT, 10% glycerol, pH 7.5). Appropriate fractions were again pooled and applied to a Mono Q column which was then developed using a gradient of 2 to 500 mM KCl in buffer D (10 mM Tris/HCl, 10 mM KCl, 1 mM $MgCl_2$, 5 mM DTT, 10% glycerol, pH 7.6). The pure protein was dialyzed against storage buffer (100 mM potassium phosphate, pH 7.0, 10 mM KCl, 5 mM $MgCl_2$, 4 mM DTT, and 50% glycerol) and stored at −20 °C. TrpRS P127S, V141Q, P127S/V141Q, Q9R, and D132T did not bind the Mono S column under the conditions described above. Therefore, flow through material was collected which yielded enzymes which were ∼98−99% electrophoretically pure except D132T which was 70−80% pure. Wild-type and

mutant proteins showed comparable production levels (0.2−0.5 mg mL^{-1}), except for the D132S variant which was produced at a significantly reduced level (0.03 mg mL^{-1}). Enzyme concentrations were estimated by active-site titration as previously described (*24*) and by determination of protein concentration using coomassie brilliant blue G-250 staining with bovine serum albumin as standard (Biorad, Hercules, CA). For wild-type, F5L, F5Y, Q9R, P127S, and M129Q TrpRS excellent correlation was observed between active-site titration and protein concentration with values varying by less than 10%. For P127S/V141Q, D132N/S/T, and V141Q TrpRS active site titration was not observed and enzyme concentrations were solely based upon protein determination, and additionally upon electrophoretic purity in the case of D132T TrpRS.

Aminoacylation Assay. Aminoacylation assays were done as described (*24*), except that potassium bicine (pH 8.0) was used instead of sodium cacodylate (pH 7.0). All assays were performed at 37 °C. Saturating concentrations were 2 μM for $tRNA^{Trp}$, 2 mM for ATP, and 10−60 μM for Trp. The kinetic parameters were determined using concentrations of Trp or ATP that varied over the range of 0.2−5 times the K_M values. The enzyme concentrations varied from 1 to 200 nM. Examination of Tyr inhibition of aminoacylation was investigated in the presence of 500 μM Tyr. The further processing and scintillation counting of samples were done as previously described (*24*, *28*).

Pyrophosphate Exchange Reaction. The pyrophosphate exchange reaction was performed as previously described (*24*) using the same conditions as detailed above for aminoacylation in the presence of 10 mM KF and 2 mM tetrasodium pyrophosphate (specific activity 1 cpm/pmol) and 16 μM Trp or 2.5−5 mM Tyr. The samples were processed as described previously (*24*) except that the quenching solution contained 300 mM tetrasodium pyrophosphate.

RESULTS

B. stearothermophilus $tRNA^{Trp}$ Is Highly Similar to $tRNA^{Trp}$ from B. subtilis. Cloning and sequencing of the gene encoding $tRNA^{Trp}$ from *B. stearothermophilus* revealed a nearly identical sequence to the *B. subtilis* $tRNA^{Trp}$ gene. In the 76 bp long sequences only one difference was observed, the sequence of *B. stearothermophilus* $tRNA^{Trp}$ containing a U in position 16 instead of the C found in the equivalent position in *B. subtilis* $tRNA^{Trp}$.

In Vivo Activity of the TrpRS Variants. The residues chosen for mutation in *B. stearothermophilus* TrpRS were Phe5, Gln9, Met129, and Asp132, all of which directly interact with the substrate tryptophan; Pro127, which is involved in positioning Asp132; and Val141, provides space to accommodate the indole ring of the substrate tryptophan. To investigate whether alignment-guided mutagenesis of these residues in TrpRS gave rise to enzymes with lost or severely reduced tryptophanylation activity, complementation analysis using auxotrophic *E. coli trpS* mutant strains was carried out. It has earlier been shown that wild-type TrpRS from *B. stearothermophilus* is able to restore growth of an *E. coli* strain deficient for TrpRS (*25*). Since sufficient production of wild-type and mutant TrpRS takes place when expressed under uninduced conditions from pET3a and pET11a vectors in *E. coli*, the respective pET-*trpS* constructs were used to transform the *E. coli trpS* mutant strains *trpS*42c and *trpS*4040 (*23*, *24*). The strain *trpS*42c carries a point mutation in the *trpS* gene which leads to a temperature sensitive tryptophan auxotrophic phenotype. At 42 °C, the strain is unable to grow. At 30 °C, the strain grows slowly in the absence of tryptophan, but is able to grow as wild-type in the presence of exogenous tryptophan. In contrast, the strain *trpS*4040 displays a tryptophan auxotrophic phenotype at 30 °C.

Table 1: In Vivo and in Vitro Activity of Wild-Type and Mutant TrpRS Enzymes

	in vivo activity[a]		
TrpRS	*trpS*42c	*trpS*4040	in vitro activity[b]
wild-type	++	++	+
F5L	++	++	+
F5Y	++	++	+
Q9R	-	±	+
P127S	+	++	+
M129Q	++	++	+
D132N	-	-	+
D132S	-	-	+
D132T	-	-	+
V141Q	-	-	+
P127S/V141Q	-	±	+
pET11a	-	-	−

[a] Complementation of *E. coli trpS* mutant strains *trpS*42c and *trpS*4040. Mutants were transformed with the pET11 vectors containing *B. stearothermophilus* wild-type or mutant *trpS* genes. The transformants were tested for growth at 30 °C (*trpS*4040) and 42 °C (*trpS*42c) on M9 plates without tryptophan. (++) Growth after 1 day; (+) growth after 2 days; (±) growth after 4 days; (−) no growth after 4 days. [b] Activity of wild-type and mutant TrpRS were assayed after partial purification. (+) activity; (−) no activity.

The complementation analysis (Table 1) showed that changing Phe5 to either Leu or Tyr and Met129 to Gln restored growth after 1 day of incubation. This is similar to the growth phenotypes of *trpS*42c and *trpS*4040 producing wild-type *B. stearothermophilus* TrpRS. Upon changing Pro127 to Ser growth is visible after 1 day with *trpS*4040 and 2 days with *trpS*42C. Of the remaining TrpRS variants, only Q9R and the P127S/V141Q double mutant were able to restore growth of *trpS*4040, but not *trpS*42c, after 4 days. This difference may reflect reduced stability or expression of the Q9R and P127S/V141Q mutants at the elevated temperature necessary for complementation analysis with the *trpS*42c strain. None of the remaining mutants were able to restore growth of either *E. coli* mutant strain.

In Vitro Activity of the TrpRS Variants. The in vitro activity of the TrpRS mutants was investigated after partial purification of the proteins. The fractions from the first anion exchange column were examined for activity by the aminoacylation assay. Activity was found for all mutants, including V141Q, and D132N/S/T, none of which could complement the strains *trpS*42c or *trpS*4040.

Kinetic Analysis of Tryptophanylation by Wild-Type and Mutant TrpRS. Aminoacylation assays were performed in order to determine the kinetic parameters for purified wild-type and mutant TrpRS enzymes. Of the TrpRS mutants which show both in vivo and in vitro activity, F5L, F5Y, P127S, and M129Q all have catalytic efficiencies (k_{cat}/K_M) for Trp within 1 order of magnitude of the values of the wild-type TrpRS enzyme (Table 2). The other mutants with

Table 2: Aminoacylation Kinetics of TrpRS with Trp and ATP

TrpRS	K_M (μM Trp)	k_{cat} (s^{-1})	k_{cat}/K_M (R)[a]
Trp			
wild-type	1.6 ± 0.1	1.1 ± 0.03	1
F5L	0.7 ± 0.06	0.36 ± 0.02	0.8
F5Y	7.3 ± 0.4	1.1 ± 0.03	0.2
Q9R	5.1 ± 0.6	0.19 ± 0.008	0.05
P127S	0.3 ± 0.01	0.083 ± 0.0006	0.4
M129Q	4.3 ± 0.3	0.69 ± 0.02	0.2
D132N	12.1 ± 1.6	0.0067 ± 0.0003	8×10^{-4}
D132S	17.8 ± 2.3	0.055 ± 0.004	5×10^{-3}
D132T	8.6 ± 1.4	0.011 ± 0.0008	4×10^{-3}
V141Q	11.6 ± 1.6	0.028 ± 0.002	3×10^{-3}
P127S/V141Q	12.7 ± 2.4	0.067 ± 0.006	7×10^{-3}
ATP			
wild-type	18.7 ± 4.0	0.53 ± 0.06	1
F5L	18.2 ± 4.3	0.39 ± 0.028	0.8
F5Y	336 ± 60	1.5 ± 0.1	0.2
Q9R	18.6 ± 3.9	0.083 ± 0.006	0.05
P127S	10.9 ± 4.2	0.12 ± 0.01	0.4
M129Q	67.6 ± 13	0.69 ± 0.06	0.4
D132N	385 ± 46	0.0081 ± 0.0006	8×10^{-4}
D132S	430 ± 46	0.056 ± 0.003	5×10^{-3}
D132T	1176 ± 196	0.0089 ± 0.0006	3×10^{-4}
V141Q	551 ± 110	0.022 ± 0.002	1×10^{-3}
P127S/V141Q	350 ± 81	0.056 ± 0.006	5×10^{-3}

[a] Relative increase compared to wild-type.

both in vivo and in vitro activity (Q9R and P127S/V141Q) showed significant reductions in catalytic efficiency for Trp (20−140-fold lower k_{cat}/K_M) compared to wild-type, in agreement with their weak complementation phenotype. These data indicate a reasonable correlation between changes in k_{cat}/K_M for Trp and complementation of *E. coli trpS*4040, and suggest that a threshold of approximately 1% of wild-type activity is necessary to rescue the auxotrophic phenotype.

The mutants which were not active in the in vivo test, V141Q and D132N/S/T, all showed significant reductions in catalytic efficiency (300−1250-fold lower k_{cat}/K_M than wild-type). This primarily resulted from changes in k_{cat} (20−170-fold reduction compared to wild-type), although less pronounced changes in K_M were also observed (5−11-fold increase compared to wild-type). All 10 mutants showed a comparable pattern of variation in k_{cat}/K_M for ATP (Table 2) to that observed with Trp (above), suggesting that none of the mutations has a specific effect on ATP binding. Double-mutant-cycle analysis (*30*) of P127S/V141Q TrpRS and the corresponding single mutants showed cooperativity between the two residues with respect to both Trp (∼1 kcal mol^{-1}) and ATP (∼1.5 kcal mol^{-1}) binding.

Effect of Tyrosine on Aminoacylation and Pyrophosphate Exchange by Wild-Type and Mutant TrpRS. The kinetics of the inhibition of aminoacylation in the presence of Tyr were investigated for wild-type and mutant TrpRS (Table 3). Tyr was found to function as a competitive inhibitor of Trp-tRNA synthesis by wild-type TrpRS and the mutants P127S and D132N. For P127S TrpRS the K_i for Tyr compared to wild-type was reduced 7-fold. However, a comparable change was observed in the K_M for Trp resulting in little change in the ratio of apparent dissociation constants for Trp and Tyr compared to wild-type (Table 3). A significant difference was observed in the ratio of apparent dissociation constants

Table 3: Aminoacylation Kinetics of TrpRS with Trp in the Presence and Absence of Tyr

TrpRS	K_M (μM Trp)	K_i (mM Tyr)	K_M/K_i (R)[a]
wild-type	1.6 ± 0.1	3.5 ± 0.3	1
P127S	0.3 ± 0.01	0.46 ± 0.02	1.4
D132N	12.1 ± 1.6	1.6 ± 0.3	17

[a] Relative increase in ratio of K_M (Trp) to K_i (Tyr) compared to wild-type.

for Trp and Tyr with the mutant D132N (17-fold decrease compared to wild-type). To investigate if Tyr acted as a substrate for TrpRS, the pyrophosphate exchange reaction (which measures aminoacyl-adenylate synthesis) was performed in the presence of tyrosine with the wild-type, P127S and D132N proteins. In all cases, pyrophosphate exchange was observed in the presence of Trp but not Tyr.

DISCUSSION

Mutational analysis of the TrpRS active site was undertaken with the goal of changing its amino acid specificity from tryptophan toward the related amino acid, tyrosine. The results in Table 3 suggest that this goal was only partially achieved; two mutants were more sensitive than wild-type enzyme to competitive inhibition by tyrosine, but none of the mutants gained the ability to activate tyrosine under conditions that should detect as little as 10^{-4} of the wild-type activity for tryptophan. In view of the relative ease with which a similar alteration of amino acid specificity was achieved for GlnRS (*13*), it is worthwhile to examine possible structural explanations for the modest changes observed.

Effects of Mutations on the Stability and Activity of TrpRS. A major concern during site-directed mutagenesis of any protein is whether the resulting mutants display global or local structural changes compared to the wild-type. Such concerns are of particular importance in a dimeric enzyme such as *B. stearothermophilus* TrpRS where interactions between the two monomers optimize the conformation of the active sites (*24*) by an induced fit mechanism (*31*). While the extent of any changes can only be precisely determined by structural methods, it is nevertheless of interest to probe the effects of mutations on stability as an estimate of global change. To address these issues, active-site titration and in vivo activity were investigated for the TrpRS mutants described in this work. Active-site titration, which in the method used here is dependent on the stability of the TrpRS·Trp-AMP intermediate, was observed for wild-type and the F5L, F5Y, Q9R, P127S, and M129Q mutants. In addition, all of these mutants were expressed at comparable levels to wild-type TrpRS and showed in vivo activity as assessed by complementation. None of the remaining mutants displayed detectable active-site titration and only P127S/V141Q was active in the complementation assay (lack of complementation could also result from reduced production in the case of D132S TrpRS). These results raise the possibility that some of these mutations could have allosteric effects on the structure of TrpRS; for D132T and V141Q, the likelihood of such global changes in structure is further supported by the observed changes in their chromatographic behavior during purification. While the methods employed

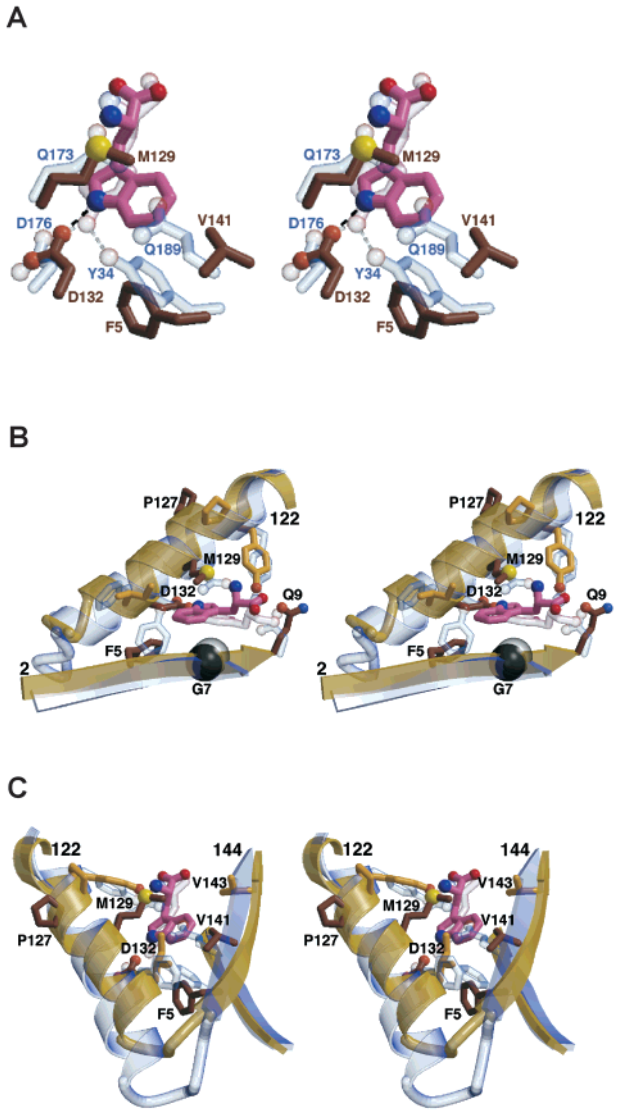

FIGURE 3: Active-site superpositions of TrpRS (full color) and TyrRS (transparent). (A) Amino acids interacting directly with the amino acid side chains in the two enzymes. The TrpRS side-chain identifications are in brown, tyrosine and TyrRS are transparent and their identifications in blue. (B) "Side" view of the active-site cavities formed between the initial β-strand of the Rossmann fold and the specificity-determining helix. G7 (black sphere) provides a platform for the face of the aromatic side chain. It is invariant in TrpRS and TyrRS sequences (Figure 2). (C) "Top" view of side chain binding pocket, showing secondary structures present in panel B together with the parallel β-strand following the specificity-determining helix. All TrpRS residues are numbered except I133 (TyrRS residue F177). Residues mutated in this study are shown in dark brown.

can only provide at best an indication of overall changes in protein structure, taken together our data would seem to suggest that at least in the cases of D132T and V141Q mutation resulted in significant disruption of the overall structure of TrpRS.

Role of Active Site and Proximal Residues in Tryptophan Recognition. Amino acids were selected for mutation following the active-site comparison given previously (*16*) and which is illustrated in Figure 3A. Each of the four illustrated side chains interacts with the substrate tryptophan and is structurally homologous with the corresponding residues in TyrRS, which are illustrated as transparent blue in Figure 3A. One of the three, M129, occurs as glutamine in several other bacterial TrpRSs, as well as in TyrRS, suggesting that methionine in this position does not contribute to specific amino acid recognition (Figure 2). Consistent with this supposition, the substitution M129Q does not affect TrpRS activity with tryptophan. The remaining side chains in Figure 3A appear capable of contributing directly to specific amino acid recognition in ways that should be testable by mutation. The superposition shows that V141 provides additional space to allow for the six-membered ring of indole, whereas Q189 in TyrRS would clearly pose a stereochemical conflict with indole. The homologue of F5, Y34 in TyrRS, makes an additional hydrogen bond to the phenolic oxygen of tyrosine, which is not possible in the case of the indole nitrogen. These two residues might have been expected to increase the tolerance for tyrosine independently of other mutations. Results from both in vivo and in vitro functional tests are in only modest accord with this expected behavior. While the V141Q and P127/V141Q mutants both have substantially reduced acylation activity with tryptophan as a substrate, in neither case was any improvement in tyrosine recognition observed. Thus, it appears to be far easier to abolish tryptophan activation than it is to replace it by tyrosine activation. Specific amino acid recognition is therefore substantially more robust to mutation than our earlier structure-based predictions suggested.

Asp132 as a Determinant of Substrate Specificity. Asp132 of TrpRS is almost, but not quite, structurally superimposable with Asp176 in TyrRS. The locations of the two residues relative to the respective substrates differ coordinately with differences between the location of the hydrogen bonding partner on the substrate (the Nϵ1 nitrogen in indole and the Oη oxygen in tyrosine). Thus, although Asp132 appears to be pivotal in determining substrate specificity, any contribution to specificity must originate elsewhere in the structure in the forces that determine its position, relative to the substrate. We hypothesized that residue 132 (176 in TyrRS) was positioned in part by the occurrence of proline at positions 126 and 127 in TrpRS, which appeared to alter the course of the specificity-determining helix, αD, 122−135. We tested this possibility by two alternative strategies, first by mutating P127 to serine in an attempt to redirect the course of the specificity-determining helix and hence the position of the D132 carboxylate, and second, by mutating D132 to asparagine, to serine, and to threonine in an attempt to allow room for the phenolic oxygen while maintaining the hydrogen bonding capability.

The hypothesis that P127 would shift the position of Asp132 is not borne out by the functional behavior of the P127S mutant, which only shows a moderate reduction in catalytic efficiency with respect to tryptophan. Without further structural data on this mutant, the reasons for this behavior are unclear. Of the D132 mutants investigated in this study only D132N showed a significant reduction in discrimination against Tyr compared to Trp, indicating that only this mutant retained the predicted hydrogen bonding capability. However, the inability of Tyr to act as a substrate for the D132N mutant suggests that the α-carboxylate of the amino acid is not correctly positioned to allow nucleophilic attack at the α-phosphate of ATP and indicates that additional mutations may be necessary to accommodate Tyr as a substrate in the active site of TrpRS. Previous studies

(*32*) showed that perturbation of the homologous D176 in TyrRS both decreased Tyr recognition and improved recognition of Phe, resulting in a change in specificity of about 1 order of magnitude. The identity of this position is completely conserved in all known TyrRS sequences and in all TrpRSs except those of the eukaryotic type (Figure 2 and ref *33*). The striking parallels in the function of a highly conserved residue found in both TrpRS and TyrRS provide mechanistic support for a common origin of the two enzymes.

Evolution of Substrate Specificity in TrpRS. The resilience of the TrpRS active site to manipulation of its substrate specificity suggests that supramolecular factors operate to reinforce and/or coordinate the roles of side chains involved in specific amino acid recognition. Figure 3, panels B and C, illustrates possible aspects of the construction of the amino acid binding pocket that might contribute to specific recognition. The pocket is formed along one face of a helix previously described as the "specificity determining" helix that contains many of the amino acids mutated in this study. The aromatic side chains of both tryptophan and tyrosine perch atop the N-terminal β-strand of the Rossmann fold, in nearly the same position relative to an invariant glycine residue (Figure 3B). The depth of the pocket, and hence the fit to the respective substrates, is determined by the position of the β-strand immediately following the specificity-determining helix (Figure 3C). Thus, although the mutated residue V141 appears to determine the depth of the pocket, the actual depth is somewhat greater than that determined by the lengths of valine vs glutamine, because the β-strand is actually displaced away from the active site in TrpRS, relative to its position in TyrRS. This difference may also be correlated with the fact that the specificity-determining helix in TyrRS is longer by nearly a full turn than that in TrpRS. Thus, the pocket in the former is narrower, even aside from the fact that Gln189 is longer than Val141. These differences are potentially also correlated with the presence in TrpRS of an additional valine (Val143) further along the β-strand in a position where TyrRS has glycine, and by the presence in TyrRS of a phenylalanine at position 133, where TrpRS has isoleucine.

These considerations underscore the implication of the experimental data that amino acid specificity in TrpRS and TyrRS cannot be analyzed simply by a linear combination of the effects of single point mutations; at a minimum, multi-mutant cycles will be necessary in order to accomplish our original goal. Furthermore, the possibility that contemporary TrpRS and TyrRS are too far diverged to readily allow the switching of substrate specificities between the two, is supported by comparisons with GlnRS and GluRS, a much more closely related pair of AARSs which have only comparatively recently diverged from a common ancestor (*33*). Upon the basis of similarities between the active sites of GlnRS and GluRS, a mutant GlnRS was constructed which showed a decrease in the specificity of Gln versus Glu of 4 orders of magnitude (*13*), supporting a comparatively recent divergence of GlnRS from GluRS. Together with structural data, these studies of the class Ib (GlnRS and GluRS) and class Ic (TrpRS and TyrRS) aminoacyl-tRNA synthetases now provide strong experimental support for the common origin of the respective subclass members as originally suggested by their molecular phylogenies.

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REFERENCES

1. Carter, C. W. (1993) *Annu. Rev. Biochem 62*, 715−746.
2. First, E. A. (1998) In *Comprehensive Biological Catalysis* (Sinnott, M. L., Ed.) pp 573−607, Academic Press, London.
3. Ibba, M., and Söll, D. (2000) *Annu. Rev. Biochem 69*, 617−650.
4. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) *Nature 347*, 203−206.
5. Cusack, S., Härtlein, M., and Leberman, R. (1991) *Nucleic Acids Res. 19*, 3489−3498.
6. Burbaum, J. J., and Schimmel, P. (1991) *J. Biol. Chem. 266*, 16965−16968.
7. Cusack, S. (1995) *Nat. Struct. Biol. 2*, 824−831.
8. Arnez, J. G., and Moras, D. (1997) *Trends Biochem. Sci. 22*, 211−216.
9. Nagel, G. M., and Doolittle, R. F. (1995) *J. Mol. Evol. 40*, 487−498.
10. Auld, D. S., and Schimmel, P. (1995) *Science 267*, 1994−1996.
11. Lin, L., Hale, S. P., and Schimmel, P. (1996) *Nature 384*, 33−34.
12. Lin, L., and Schimmel, P. (1996) *Biochemistry 35*, 5596−5601.
13. Agou, F., Quevillon, S., Kerjan, P., and Mirande, M. (1998) *Biochemistry 37*, 11309−11314.
14. Siatecka, M., Rozek, M., Barciszewski, J., and Mirande, M. (1998) *Eur. J. Biochem. 256*, 80−87.
15. Nureki, O., Vassylyev, D. G., Katayanagi, K., Shimizu, T., Sekine, S., Kigawa, T., Miyazawa, T., Yokoyama, S., and Morikawa, K. (1995) *Science 267*, 1958−1965.
16. Doublie, S., Bricogne, G., Gilmore, C. and Carter, C. W., Jr (1995) *Structure 3*, 17−31.
17. Ribas de Pouplana, L., Frugier, M., Quinn, C. L., and Schimmel, P. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 166−170.
18. Brown, J. R., Robb, F. T., Weiss, R., and Doolittle, W. F. (1997) *J. Mol. Evol. 45*, 9−16.
19. Fersht, A. R. (1987) *Biochemistry 26*, 8031−8037.
20. Brick, P., Bhat, T. N., and Blow, D. M. (1989) *J. Mol. Biol. 208*, 83−98.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Labororatory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
22. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
23. Bohman, K., and Isaksson, L. A. (1978) *Mol. Gen. Genet. 161*, 285−289.
24. Sever, S., Rogers, K., Rogers, M. J., Carter, C. W. and Söll, D. (1996) *Biochemistry 35*, 32−40.
25. Carter, C. W., Jr. (1988) *J. Crys. Growth. 90*, 168−179.
26. Wawrousek, E. F., Narasimhan, N., and Hansen, J. N. (1984) *J. Biol. Chem. 259*, 3694−3702.
27. Kim, S. I., and Söll, D. (1998) *Mol. Cells 8*, 459−465.
28. Ibba, M., Sever, S., Prætorius-Ibba, M., and Söll, D. (1999) *Nucleic Acids Res. 27*, 3631−3637.

29. Curnow, A. W., Kung, F. L., Koch, K. A., and Garcia, G. A. (1993) *Biochemistry 32*, 5239−5246.
30. Carter, P. J., Winter, G., Wilkinson, A. J., and Fersht, A. R. (1984) *Cell 38*, 835−840.
31. Ilyin, V. A., Temple, B., Hu, M., Li, G., Yin, Y., Vachette, P., and Carter, C. W., Jr. (2000) *Protein Sci. 9*, 218−231.
32. de Prat Gay, G., Duckworth, H. W., and Fersht, A. R. (1993) *FEBS Lett. 318*, 167−171.
33. Woese, C. R., Olsen, G., Ibba, M., and Söll, D. (2000) *Microbiol. Mol. Biol. Rev. 64*, 202−236.

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