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Modification of the amino acid specificity of tyrosyl-tRNA synthetase by protein engineering

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The amino acid specificity of *Bacillus stearothermophilus* tyrosyl-tRNA synthetase was studied by site-directed mutagenesis of residues close to the active site. X-ray crystallographic studies of the enzyme have suggested that Asp-176 is a major determinant of amino acid specificity, as its carboxylate is observed to make a hydrogen bond with the hydroxyl group of the substrate tyrosine. Previous efforts to test the importance of Asp-176 by site-directed mutagenesis led to inactive enzymes. We have now investigated the catalytic properties of enzymes altered, not at Asp-176 itself, but instead at two amino acids, Asn-123 and Trp-126, that appear in the crystallographic structure to form hydrogen bonds with Asp-176. Mutation of Trp-126 does not affect the kinetics of activation with respect to ATP but leads to modest increases in the K_m for tyrosine. Conversely, position Asn-123 mutants are strongly affected: 160-fold lower k_{cat} and 5-fold higher K_m for the Ala-123; and 17-fold decrease and 270-fold increase, respectively, of the same parameters for the Asp-123 mutation. The specificity against phenylalanine was determined from the ratios of k_{cat}/K_m for the amino acids in the pyrophosphate exchange reaction. The ratio of 1.2×10^5 for the wild-type enzyme decreases 4-fold on mutation of Asn-123 but increases 7-fold on the mutation of Trp-126→Phe and 2-fold on Trp-126→Leu. The wild-type enzyme has not reached the maximum limit of discrimination between tyrosine and phenylalanine.

Site-directed mutagenesis; Aminoacyl-tRNA synthetase; Recognition

1. INTRODUCTION

Fidelity in protein biosynthesis relies on a precise mechanism for amino acid selection by aminoacyl-tRNA synthetases and subsequent transfer to its cognate tRNA. Preferential binding of the cognate substrates amino acid and tRNA, as well as proof-reading mechanisms in some cases, give rise to this phenomenon [1]. The evolutionary pressure on the aminoacyl-tRNA synthetases to maximise their specificity leads to their being good models for probing the limits of such specificity. The *Bacillus stearothermophilus* tyrosyl-tRNA synthetase gives information on the specificity of binding -OH groups because it has to discriminate against phenylalanine [2]. This enzyme, the catalytic mechanism and structure of which has been extensively studied in the past, is a homodimer of 47,300 kDa per unit [3]. It catalyses a two-step reaction for the transfer of tyrosine to $tRNA^{TYR}$ [4]

$$\text{I} \quad E + Tyr + Mg \cdot ATP \rightleftarrows E \cdot Tyr\text{-}AMP + Mg \cdot PP_i$$

$$\text{II} \quad E \cdot Tyr\text{-}AMP + tRNA \rightleftarrows E \cdot Tyr \cdot tRNA + AMP$$

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The first step of the reaction is the stage at which the selection of the amino acid takes place. In most cases, the structure of the cognate amino acid is considerably different from its competitors, and, therefore, facilitates the discrimination. When the difference in binding energies between cognate and non-cognate amino acids is small, the selectivity is achieved by a proof-reading mechanism [5–8]. The specificity factor of tyrosyl-tRNA synthetase for tyrosine is 10^5 compared with phenylalanine [2]. In addition, the phenylalanine content in the bacterial cell is 60 to 70 times that of tyrosine [9]. These two factors combined give a rate of misincorporation comparable to other amino acid pairs [10] and shows that, in the absence of an editing mechanism, tyrosyl-tRNA synthetase can discriminate between tyrosine and phenylalanine by simple preferential binding [2].

The specificity site of the tyrosyl-tRNA synthetase is formed by two residues that interact directly with the tyrosine hydroxyl (substrate) by hydrogen bonding: Asp-176 and Tyr-34 [11,12]. These are also hydrogen bonded to other residues surrounding the specificity site in an hydrogen bonding network. Residues Trp-126 and Asn-123 are hydrogen bonded to Asp-176 as shown in Fig. 1. It is possible that Asp-176 is hydrogen bonded to its own main-chain amide nitrogen, a common arrangement for this residue. All four residues are conserved in the *E. coli* tyrosyl-tRNA synthetase. Mutation of Tyr-34 to Phe weakens the binding of tyrosine by 0.52

kcal·mol^{-1} and decreases the specificity for tyrosine against phenylalanine by a factor of 15 [13]. Asp-176, acting as hydrogen bond acceptor for the substrate hydroxyl, seems to be critical not only for tyrosine specificity in particular but also for substrate binding in general. As mentioned above, this residue is hydrogen bonded to Asn-123 and Trp-126 through Oδ1 and Oδ2 respectively. All attempts to mutate Asp-176 have yielded inactive enzyme (D.M. Lowe, this laboratory, unpublished data).

In the present work, we have mutated residues that do not interact directly with the tyrosine hydroxyl (substrate), but rather are hydrogen bonded with Asp-176. In this way we obtain subtle and measurable changes that affect amino acid specificity. We mutated Asn-123 to Ala and Asp, the former to investigate the effect of removing the hydrogen bond and the latter to perturb the Asp-176 by electrostatic repulsion. In addition, we replaced Trp-126 with Leu, to eliminate the hydrogen bond, and to Phe, to produce the same effect whilst retaining an aromatic side chain. Residue Gln-173 interacts indirectly through the hydrogen bond network with Tyr-34, one of the two residues that interact directly with the tyrosine (substrate) hydroxyl group. This residue is also hydrogen bonded with the α-amino group of the substrate through its carbonylic oxygen; it has been mutated to Ala in the past and found to affect K_m for tyrosine [14]. In this study, we mutated Gln-173 to Glu in order to introduce a negative charge with minimal steric alteration.

2. MATERIALS AND METHODS

2.1. *Materials*

Radiochemicals were purchased from Amersham International. All other reagents were of the highest purity available. Nitrocellulose discs (0.22 μm pore size, 2.5 cm diameter), were obtained from Sartorius. Glass microfibre filters (GF/C) were from Whatman and Duolite C225 (H) resin from BDH Laboratory Supplies. Water was deionized and twice distilled.

2.2. *Site-directed mutagenesis, expression and purification of mutant proteins*

The *B. stearothermophilus* tyrosyl-tRNA synthetase gene [15] was expressed using the pYTS5 construct (First, E.A., unpublished). Site-directed mutagenesis was performed on single-stranded DNA using the method of Kunkel et al. [16]. The following oligonucleotides were used to make the mutations (mutagenic bases are indicated by asterisks)

Trp-126→Leu 5′-CGG CCC GAT C*A*A GTC GTA GTT-3′
Trp-126→Phe 5′-CGG CCC GAT G*A*A GTC GTA GTT-3′
Asn-123→Ala 5′-A GTC GTA GG*C* GTT TTT G-3′
Asn-123→Asp 5′-A GTC GTA GT*C* GTT TTT G-3′
Gln-173→Glu 5′-ATC GTA TGC CTC* CAG CAT CAT-3′

Wild-type and mutant proteins were expressed in *E. coli* TG2 hosts (*recA*$^-$ form of TG1, [17]), and were purified as described by Wells and Fersht [18]. Once purified, enzymes were further heat treated at 55°C for 20 min to eliminate any traces of *E. coli* tyrosyl-tRNA synthetase and dialysed extensively against 1 mM Na_4PP_i to remove any residual adenylate from the tyrosyl-tRNA synthetase. Enzyme preparations were more than 98% pure as judged by SDS-PAGE.

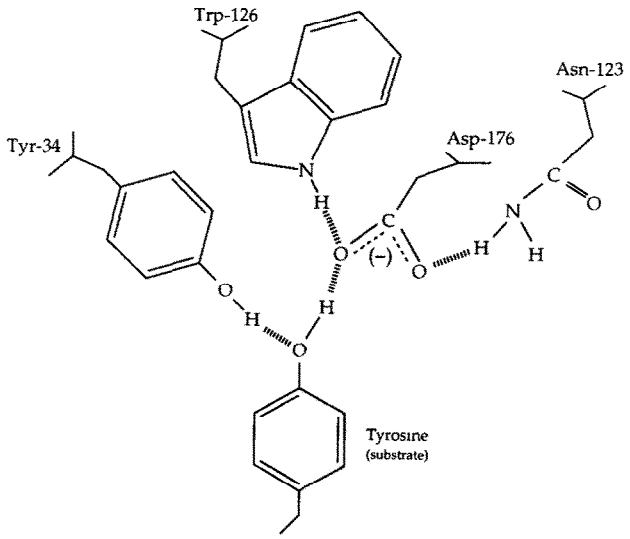

Fig. 1. Hydrogen bonding network of active site residue Asp-176.

2.3. *Treatment and purification of phenylalanine*

In order to remove tyrosine from commercial phenylalanine batches, amino acid solutions were treated with tetranitromethane and impurities eliminated prior to recrystallization as described earlier [2]. Duolite C225 (H^+ form) was used in place of Zerolit 225. The UV absorbance spectrum of the final solution showed no traces of tyrosine.

2.4. *Kinetic procedures and analysis*

Both pyrophosphate exchange kinetics methods and analysis were carried out as described by Wells et al. [19]. Pre-steady-state kinetics of ATP binding was measured as described by Fersht et al. [20], using Applied Photophysics stopped-flow equipment. Enzyme concentrations were determined by active site titration with ^{14}C-labeled tyrosine using nitrocellulose filters [21]. For low activity mutants, the concentration of protein were determined by the Bradford assay [22].

2.5. *tRNA charging*

$tRNA^{TYR}$ from *B stearothermophilus* was in vitro transcribed from its gene, purified and assayed as described [23].

3. RESULTS

3.1. *Mutation of Asn-123*

Mutation of this residue to Ala lowers k_{cat} by 160-fold, whereas the K_m is increased 5-fold for the tyrosine activation (Table I). The Asn-123 to Asp mutation gives a 270-fold increase of K_m with a decrease of 17-fold in k_{cat}. Mutation to Ala affects mainly k_{cat} and less significantly K_m. Mutation to Asp dramatically alters the binding of tyrosine with less effect on the k_{cat}. Both mutants form a very weak enzyme–adenylate complex when analysed by active-site titration (not shown), suggesting an important role of residue Asn-123 in the structure of the active site.

The data obtained for ATP binding (K_m) show no change for the Ala substitution and a small increase for

the Asp mutant; the k_{cat} is equally affected showing similar k_{cat}/K_m ratio (Table II). These results indicate the non-disruptive nature of the mutations on the structure of the protein.

3.2. *Mutation of Trp-126*

There is no significant effect upon replacement of Trp-126 with Leu or Phe in terms of k_{cat} for tyrosine (Table I). However, a 6-fold increase in K_m is observed for the Phe mutant. The mutations have little effect on the ATP kinetics both in pyrophophate exchange (Table II) and pre-steady-state (Table III). The pre-steady-state kinetics is followed by measuring a change in the tryptophan fluorescence of the enzyme on formation of the tyrosyl adenylate. This change still occurs after mutation of Trp-126 and, therefore, this residue is clearly not responsible for the fluorescence signal upon substrate binding despite its proximity to the active site.

3.3. *Mutation of Gln-173*

Substitution of the Gln by Glu in position 173 gives rise to a 50-fold decrease of k_{cat} for tyrosine with only a slight change of K_m, suggesting a role for Gln-173 amide nitrogen in transition state binding with a contribution of 3 kcal mol^{-1} (calculated from Table I). The same is true for ATP, where the decrease in k_{cat} is 22-fold with no change at all in K_m (Table II). Previous results showed a 58-fold increase in K_m for the Gln-173→Ala mutation due to the lack of the carbonyl group hydrogen bonding to the α-ammonium group of tyrosine [14]. Nonetheless, mutation of this residue does not affect the tyrosine/phenylalanine specificity ratio (Table V).

3.4. *Modification of specificity for phenylalanine*

The value for k_{cat}/K_m for activation may be measured with precision for mutants but the high values of K_m lead to inaccurate estimate of k_{cat} and K_m. It is the ratio k_{cat}/K_m, however, the important value for calculation of the specificity [1].

Table I

Pyrophosphate exchange kinetics of tyrosine activation by wild type and mutant tyrosyl tRNA synthetases[a,b]

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
Wild type	2.6	2.2	1.2×10^6
Trp126→Leu	1.5	3.5	0.42×10^6
Trp126→Phe	2.9	12.6	0.23×10^6
Asn123→Ala	0.016	9.9	1.6×10^3
Asn123→Asp	0.15	585[c]	2.6×10^2
Gln173→Glu	0.051	5.1	1.0×10^4

[a] Experiments were carried out in the presence of 2 mM Na_4PP_i, 2 mM ATP, 144 mM Tris-HCl pH 7.88, 10 mM $MgCl_2$, and 0.1 to 10 μM enzyme and proceeded as described by Wells et al., 1991.
[b] Standard errors are less than ± 10%, except [c] ± 15%.

Table II

Analysis of wild type and mutant tyrosyl tRNA synthetases by pyrophosphate exchange kinetics for ATP[a,b,c]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
Wild type	2.1	1.6	1300
Trp126→Leu	2.8	1.3	2200
Trp126→Phe	3.3	1.5	2200
Asn123→Ala	0.020	1.2	17
Asn123→Asp	0.054	4.3	13
Gln173→Glu	0.095	1.1	86

[a] Experiments were carried out as described in table I with 100 μM tyrosine.
[b] Standard errors are less than ± 10%.
[c] Concentrations higher than 10 mM ATP showed decreased rates due to competitive inhibition.

In contrast to tyrosine kinetics, both Trp-126 mutants are strongly affected in their phenylalanine activity. Mutation of Trp-126 to Leu yields a very similar k_{cat} value to wild type whereas in the Trp-126→Phe mutant the same kinetic constant is decreased approximately by 7-fold. In both cases, the values of K_m are difficult to measure accurately since they are higher than the solubility of phenylalanine in water. The specificity constants (k_{cat}/K_m) are 1.9 and 0.28 $s^{-1}\cdot M^{-1}$ for Trp-126→Leu and Trp-126→Phe, respectively. This results together with the ones in Table I are used to calculate an increase in the specificity factor for tyrosine against phenylalanine of ~2 and ~7-fold for Leu and Phe mutations respectively (Table V).

It was not possible to carry out any kinetic measurements on the Asn123→Asp mutant due to its low activity; however, the Asn-123→Ala mutant shows a 170-fold decrease in k_{cat}/K_m for phenylalanine and a subsequent ~4-fold decrease in the specificity for tyrosine against phenylalanine (Tables IV and V).

3.5. *Charging of $tRNA^{TYR}$*

Trp126→Leu and Trp126→Phe mutants were assayed for tRNA charging capacity and showed a similar behaviour to wild-type enzyme (Table VI).

Table III

Analysis of wild type and mutant tyrosyl tRNA synthetases by pre-steady-state kinetics for ATP[a,b]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
Wild type	27.8	1.8	15400
Trp126→Leu	17.9	2.4	7460
Trp126→Phe	21.9	4.3	5090

[a] Experiments were carried out in 144 mM Tris-HCl pH 7.88, 10 mM $MgCl_2$, 100 μM tyrosine and 0.3 μM enzyme.
[b] Standard errors are less than ± 10%.

Table IV

Pyrophosphate exchange kinetics of phenylalanine activation by wild type and mutant tyrosyl tRNA synthetases[a]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Wild type	0.59	62	9.5[b]
Trp126→Leu	~0.7	~340[d]	2.1[e]
Trp126→Phe	~0.08	~250[d]	0.28[e]
Asn123→Ala	~0.07	~1100[d]	0.057[e]
Asn123→Asp	n.m.[c]		
Gln173→Glu	~0.01	88	0.13

[a] Experiments were carried out as described in Table I using phenylalanine instead of tyrosine.
[b] Value well in agreement with previous (9.8, Fersht et al., 1980).
[c] n.m., not measurable.
[d] Standard errors are approximately ± 30% and
[e] the ratios k_{cat}/K_m were determined with precision from linear fitting of the low concentration points. Concentrations of phenylalanine were varied from 2 to 120 mM. Separation into k_{cat} and K_m is approximate because of the high values of K_m.

4. DISCUSSION

A series of mutations surrounding the primary specificity site of the tyrosyl-tRNA synthetase have been made to probe the discrimination between tyrosine and phenylalanine as substrates. Mutation of residue Asn-123 yields enzymes with low activity in the formation of tyrosyl adenylate. Asn-123→Ala is also affected in its phenylalanine activation properties, resulting in a decrease of specificity for tyrosine compared with phenylalanine. In contrast, mutation of Trp-126 decreases the rate of phenylalanine activation, more than that of tyrosine. This produces mutant enzymes with higher degrees of discrimination (higher specificity) for tyrosine against phenylalanine. This is a surprising result.

Specificity in biology is mediated in general by the binding of complementary surfaces. Very high specificity can be achieved by the avoidance of a) unsolvated charged residues at the active site and b) unfavourable steric interactions between the residues involved [13,24]. It is found that removal of a partner of a charged hydrogen bond weakens binding by ~4 kcal·mol^{-1} in many instances [13]. The binding of phenylalanine to the tyrosyl-tRNA synthetase does not provide a partner for the carboxylate of Asp-176. A desolvated charge is so unstable that either the carboxylate becomes protonated or, more likely, a water molecule remains between phenylalanine and the enzyme residues Asp-176 and Tyr-34 [13]. There is no change in the Tyr/Phe specificity factor between pH 7.9 and 6.0 for either wild type or Tyr-34 Phe mutant, suggesting that the protonation of Asp-176 is not required for binding of phenylalanine (J.P. Shi, this laboratory, unpublished results).

Mutation of Trp-126 and Asn-123 removes a hydrogen bonding partner that must be replaced by a solvent water molecule or an interaction elsewhere in the protein. In one case this weakens the binding of phenylalanine and in the other it strengthens it. A crystal structure is required to give the correct explanation.

The mutant Trp-126→Phe charges tRNA nearly as well as wild-type enzyme and is more specific. Thus, *B. stearothermophilus* tyrosyl-tRNA synthetase has not reached its maximum value for differential binding or discrimination of its substrate during evolution. Perhaps the preferential binding for tyrosine by wild-type enzyme is enough to cope with the different concentrations of the amino acids encountered in vivo and therefore there has been no selective pressure for further improvement.

Table V

Specificity of wild type and mutant tyrosyl tRNA synthetases for tyrosine compared to phenylalanine

Enzyme	Specificity factor[a]	Specificity variation
Wild type	1.2×10^5	
Trp126→Leu	2.2×10^5	1.8× increase
Trp125→Phe	8.2×10^5	6.8× increase
Asn123→Ala	0.28×10^5	4.3× decrease
Gln173→Glu	0.8×10^5	1.5× decrease

[a] The specificity factor is defined as $\{(k_{cat}/K_m)_{Tyr}\}/\{k_{cat}/K_m)_{Phe}\}$.

Table VI

tRNA charging by wild type and mutant tyrosyl t-RNA synthetases[a]

Enzyme	Initial rate mol aa-tRNA·mol enzyme^{-1}·s^{-1}	(%)
Wild type	0.46	100
Trp126→Leu	0.51	110
Trp126→Phe	0.50	108

[a] Conditions: 0.5 μM $tRNA^{TYR}$, 10 mM ATP, 100 μM tyrosine, 144 mM Tris-HCl pH 7.88, 10 mM $MgCl_2$, 20–40 nM enzyme.

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