A Mutant Escherichia coli Tyrosyl-tRNA Synthetase Utilizes the Unnatural Amino Acid Azatyrosine More Efficiently than Tyrosine.

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Running Title

Efficient utilization of azatyrosine by a mutant *E. coli* TyrRS
Summary

Alloproteins, proteins which contain unnatural amino acids, have immense potential in biotechnology and medicine. Although various approaches for alloprotein production exist, there is no satisfactory method to produce large quantities of alloproteins containing unnatural amino acids in specific positions. The tyrosine analogue azatyrosine, L-β-(5-hydroxy-2-pyridyl)-alanine, can convert the ras-transformed phenotype to normal, presumably by its incorporation into cellular proteins. This provided the stimulus for isolation of a mutant tyrosyl-tRNA synthetase (TyrRS) capable of charging azatyrosine to tRNA. A plasmid library of randomly mutated *E. coli* tyrS (encoding TyrRS) was made by PCR techniques. The desired TyrRS mutants were selected by screening for *in vivo* azatyrosine incorporation of *E. coli* cells transformed with the mutant tyrS plasmids. One of the clones thus isolated, R-6-A-7, showed a 17-fold higher *in vivo* activity for azatyrosine incorporation than wild-type TyrRS. The mutant tyrS gene contained a single point mutation resulting in replacement of phenylalanine by serine at position 130 in the protein. Structural modeling revealed that position 130 is located close to Asp182 which directly interacts with tyrosyladenylate. Kinetic analysis of aminoacyl-tRNA formation by wild-type and the mutated F130S TyrRS enzymes showed that the specificity for azatyrosine, measured by the ratios of $k_{\text{cat}}/K_m$ for tyrosine and the analog, increased from 17 to 36 as a result of the F130S mutation. Thus, the high discrimination against azatyrosine is significantly reduced in the mutant enzyme. These results suggest that utilization of F130S TyrRS for *in vivo* protein biosynthesis may lead to efficient production of azatyrosine-containing alloproteins.
Introduction

We previously reported that the tyrosine analogue azatyrosine, (L-β-(5-hydroxy-2-pyridyl)-alanine), has the unique property of converting c-Ha-ras, c-raf or c-erbB-2 transformed NIH3T3 cells to apparently normal phenotype, when it is added to the tissue culture medium (1). The same phenomenon was also observed with the human pancreatic cancer cell line, PSN-1; these cells have an activated c-Ki-ras mutation (G12R), a p53 mutation and c-myc amplification (1). However, NIH3T3 cells transformed by hst or src were not converted to the normal phenotype on treatment with azatyrosine (1). When c-erbB-2 transformed NIH3T3 cells were treated with azatyrosine, phosphorylation of c-Raf and c-Jun were inhibited, while the amount of GTP-bound Ras p21 and phosphorylation of c-ErbB-2 were not altered (2). These results suggested that the azatyrosine effect is somewhere in the ras signal transduction pathway. Several indirect lines of evidence indicated that the effect of azatyrosine on conversion of the transformed phenotype to normal is due to its incorporation into cellular protein (3). Radioactive azatyrosine was incorporated into a small amount of cellular protein, and the amount of azatyrosine incorporated decreased upon addition of tyrosine to the tissue culture medium, concomitant with the inability to convert the transformed phenotype to normal (3).

The concentration of azatyrosine needed for phenotype conversion was quite high (250-500 µg/ml) (1-3). This hampered the further study of the crucial protein(s) involved in phenotype conversion by azatyrosine incorporation. Previously we showed that a chemically synthesized EGF fragment containing azatyrosine in place of tyrosine is not efficiently phosphorylated by EGF receptor kinase (4). It is tempting to speculate that the activity of a protein important in the ras signal transduction pathway is affected by having azatyrosine instead of tyrosine. To aid further analysis of azatyrosine function a mutant tyrosyl-tRNA synthetase (TyrRS)\(^1\) was desired that would charge azatyrosine efficiently to tRNA. For this purpose we used *E. coli*, because of the ease of genetic manipulation and tyrS mutant selection. Furthermore, if such a mutant tyrS became available, it could be used (together with the *E. coli* tRNA\(^{Tyr}\) gene) for transfection of mammalian cells to bring about selective incorporation of azatyrosine into a particular protein positions because *E. coli* TyrRS and tRNA\(^{Tyr}\) do not recognize the mammalian TyrRS and tRNA\(^{Tyr}\) and thus not cross-charge (5-7).
Experimental Procedures

**Strains and Plasmids** — A plasmid pBR322 (ΔNdeI-HindIII) was constructed by removing a 2.3 kbp NdeI-HindIII fragment from pBR322. The plasmid pYRS was constructed by introducing the 1.5 kbp *SspI* fragment of the wild-type (wt)¹ *E. coli* TyrRS gene (8) including flanking regions into pBR322 (ΔNdeI-HindIII) at blunted *EcoRI/SspI* sites. The plasmid pF130S was constructed by introducing the *E. coli* TyrRS (F130S) gene obtained by a modified PCR method into pYRS instead of the wild type TyrRS gene as described below. The plasmids pQE-YRS and pQE-F130S were constructed by introducing the wt TyrRS gene and F130S gene, respectively, downstream of the histidine tag gene of the pQE-30 vector (QIAGEN GmbH, Germany) for purification.

**Substrates** — Azatyrosine, L-β-(5-hydroxy-2-pyridyl)-alanine, was prepared in our institute from the culture broth of *Streptomyces* sp. SE-1346 (FERM BC-1536) by the method of Inoue et al. (9). The purity of this azatyrosine preparation was shown to be more than 99.9% by high performance liquid chromatography. L-[Methylene-³H]-azatyrosine was obtained from Amersham (UK).

**Random Mutagenesis of tyrS** — Two oligonucleotides were synthesized. Primer 1 (5’-ATGGCAAGCAGTAACTTGA-3’) was located near the 5’ end of the TyrRS gene (8). Primer 2 (5’-GAATACTCATACTCTTCCT-3’) was located in the pBR322 gene near the *SspI* cloning site. *E. coli* tyrS was amplified and mutagenized by a modified PCR method using the synthesized primers as described by Leung et al. (10). The PCR products were digested with *MunI*, 25 bp from the 5’-end of the TyrRS gene and *SspI*, and inserted into pYRS instead of the wt TyrRS gene at the *SspI/MunI* site to generate a mutant library.

**Screening Mutant tyrS clones** — The library of the recombinant randomly mutated plasmids was introduced into *E. coli* JM109. Single colonies of the transformants were picked and transferred to wells of the 96-well plates with 100 µl of M9 minimum medium. The plates were incubated at 37°C for 4 h, and 20 µl of cultures were transferred to filtration plates (MHVB N45 plate, Millipore) with 100 µl of M9 medium containing L-[³H]-tyrosine or L-[³H]-azatyrosine at final concentrations of 0.5 µM. After incubation at 37°C
for 30 min, 50 µl of 5% trichloroacetic acid (TCA) was added into each well of the filtration plates. The precipitates were filtered and washed 3 times with 5% TCA. Then the filtration plates were dried and 50 µl of liquid scintillator (MICROSCINT-0, PACKARD) was added to each well. Radioactivities were measured with a microplate liquid scintillation counter (TopCount, PACKARD). The transformant in which the incorporation ratio of azatyrosine to tyrosine was higher than that in E. coli JM109 transformed by pYRS (JM109/pYRS) was selected.

*Incorporation of Radiolabeled Amino Acids into E. coli Proteins in Liquid Culture* — JM109/pYRS and E. coli JM109 transformed by pF130S (JM109/pF130S) were inoculated into M9 minimum medium, and incubated at 37ºC. After the A600 reached about 0.6, 20 µl portions of cultures were transferred to MHVB N45 plates with 100 µl of M9 medium containing L-[3H]-tyrosine or L-[3H]-azatyrosine at a final concentration of 50 µM. After further incubation for 10 min, 50 µl of 5% TCA was added. The precipitates were filtered and radioactivities were measured with a microplate liquid scintillation counter.

*Purification of E. coli wt and F130S TyrRS* — JM109/pQE-YRS or JM109/pQE-F130S were cultured in 2 L of LB medium at 37ºC and harvested when A600 reached 0.8. The expression of histidine-tag fused wt TyrRS (His-wt TyrRS) or histidine-tag fused F130S (His-F130S) was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, followed by incubation with vigorous shaking at 37ºC for 3.5 h. The cells were harvested by centrifugation at 6,000 g for 15 min. The cell pellets were washed twice with 0.85 % NaCl. The cell pellets were then resuspended in buffer A (50 mM potassium phosphate (pH 7.0), 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride and 10 % glycerol) and lysed by sonication. PEG8000 was added to the lysates at a final concentration of 7.5% and the lysates were centrifuged at 30,000 g for 20 min. The supernatants were applied to a Ni-NTA column (QIAGEN), and proteins were fractionated using a 0-500 mM imidazole gradient in buffer A. Fractions containing His-wt TyrRS or His-F130S were pooled and dialyzed against 50 mM potassium phosphate (pH7.0), 10 mM 2-mercaptoethanol, 0.1 mM PMSF and 50 % glycerol.

*Aminoacylation Assay* — Using purified His-wt TyrRS or His-F130S, aminoacylation assays were
performed at 30°C for 15, 30, 45, 60, 75, 90, 105, or 120 seconds in 40 µl of reaction mixture containing 40 mM Tris-HCl (pH 7.5), 1.6 mM ATP, 3.2 mM KCl, 0.8 mM MgCl₂, 5-50 mg/ml E. coli unfractionated tRNA, 1-50 nM of His-wt TyrRS or 5-50 nM of His-F130S TyrRS and 1-60 µM L-[³⁵H]-tyrosine or 3-125 µM L-[³⁵H]-azatyrosine. The reaction mixtures were spotted on Whatman 3MM filter disks presoaked in 10 % TCA. The disks were then washed with 10 % TCA, and radioactivity was measured by scintillation counting. The initial rate was determined at various tyrosine or azatyrosine concentrations from the time course. Kinetic parameters were obtained from Lineweaver-Burk plots according to the procedures as described previously (11,12). The values of Km and kcat of E. coli wt TyrRS for tyrosine were in good agreement with those previously published by Buorocore et al (13).

**Fractionation of E. coli Proteins Labeled with Radioactive Tyrosine or Azatyrosine** — JM109/pYRS and JM109/pF130S were inoculated into M9 minimum medium, and grown to 0.5 A₆₀₀ at 37°C. Then 200 µl of cultures were inoculated into 1 ml of M9 medium containing L-[³⁵H]-tyrosine or L-[³⁵H]-azatyrosine at a final concentration of 50 µM, incubated with vigorous shaking at 37°C for 10 min, and centrifuged at 8,000 g for 10 min. The cell pellets were resuspended in 20 µl of loading buffer (50 mM Tris-HCl (pH 6.8), 700 mM 2-mercaptoethanol, 2 % sodium dodecylsulfate (SDS), 0.01 % bromophenol blue and 1 % glycerol) and boiled for 10 min. Aliquots of these crude lysates (15 µl) were developed by 10 % polyacrylamide gel electrophoresis (PAGE), and proteins were visualized by silver staining. For localization of radiolabelled proteins, the gel was dried at 80°C, exposed to an imaging plate (Fuji Film, Japan) for 4 days and then analyzed with BAS2000 (Fuji Film, Japan).

**Structural Modeling** — A three-dimensional structure model of E. coli TyrRS was constructed on the basis of the X-ray crystal structure of B. stearothermophilus TyrRS complexed with tyrosyl adenylate in the Protein Data Bank (14), entry 3TS1 (15). This X-ray crystal structure lacks residues of a disordered C-terminal domain (319 to 418), but the region is far from the binding pocket of tyrosyl adenylate. Therefore, the model of E. coli TyrRS does not have residues corresponding to 319-418 of B. stearothermophilus TyrRS. Protein modeling was performed on a workstation NEC EWS 4800/350 using the program of...
BIOCES(E)(NEC Co., Japan). The backbones and side chains of the insertion regions and the side chains of the substituted amino residues were minimized on a Silicon Graphics Power Indigo2 using CHARMM force field (16). A distance-dependent dielectric constant was used. The docking of tyrosyl adenylate into the model was performed manually on the basis of the structure of the *B. stearothermophilus* TyrRS and tyrosyl-adenylate complex.
Results

Screening for mutant E. coli tyrS genes — For allopolyprotein synthesis in vivo we wanted to generate an E. coli TyrRS mutant which can recognize azatyrosine efficiently. This is not an easy task, because with few exceptions (17,18) aminoacyl-tRNA synthetases recognize their natural counterpart more efficiently than unnatural amino acids (19). At first we attempted a directed approach inspired by the Bacillus stearothermophilus TyrRS crystal structure (15). We realized that the amino acid residues in the tyrosine binding site (YCGFD at positions 37 to 41) are conserved in E. coli TyrRS and reasoned that mutagenesis of these residues might be the best way of changing the substrate specificity of TyrRS. However, extensive cassette mutagenesis failed to generate any mutant TyrRS with the desired properties (unpublished results). Possibly changes in these amino acids causes too much debilitation of amino acid recognition (20,21). Therefore we attempted random mutagenesis of the whole tyrS gene followed by large-scale screening. Error-prone, efficient PCR mutagenesis (10) was used. After re-cloning the mutant plasmid population was transformed into E. coli cells and the incorporation of radioactive azatyrosine was measured. The chromosomal wt tyrS copy in the E. coli transformants did not interfere in the assay as its gene product was much less (about 1%) expressed than the mutant genes on the plasmids (data not shown). Using this screening method, we selected one positive clone (R-6-A-7) among 1400 transformants analyzed.

In Vivo Incorporation of Tyrosine or Azatyrosine by the E. coli R-6-A-7 Plasmid — To examine in detail azatyrosine incorporation in vivo of the R-6-A-7 clone, we measured incorporation of [3H]-azatyrosine or [3H]-tyrosine into the TCA-insoluble fraction of the JM109/pYRS and R-6-A-7 clones. The amount of tyrosine incorporated into protein in the R-6-A-7 transformed strain was 4.9-fold lower than that of JM109/pYRS (Fig. 1). In contrast, the amount of azatyrosine incorporated by the R-6-A-7 clone was 3.6-fold higher than that by JM109/pYRS. Thus, the R-6-A-7 clone had a 17-fold higher ability to incorporate azatyrosine, compared to tyrosine, into protein.

Isolation and Characterization of the Mutant TyrRS — Plasmid DNA was purified from A-6-A-7 cultures
and its DNA sequence was determined by dideoxy sequencing. Analysis revealed that the mutant tyrS gene had two changes: at residues 389 (from T to C) and 903 (from T to C). The former change results in an amino acid replacement of Ser instead of Phe at position 130, while the second mutation is silent (third position in a tyrosine codon). Position 130 is not located in the tyrosine binding site as defined by the B. stearothermophilus TyrRS crystal structure (15). This mutant was designated F130S and clone R-6-A-7 was renamed JM109/pF130S.

The mutant TyrRS is temperature-sensitive. Preincubation of the pure enzyme at 50ºC for 10 min almost completely inactivated F130S, while this treatment did not affect wt TyrRS (data not shown). However, the enzyme was stable enough to be purified and analyzed. As the wt enzyme had the same specific activity either in unmodified or His-tagged form, we purified the His-tagged versions of the wt and F130S enzyme by Ni-NTA column chromatography and determined the Km and kcat values for tyrosine and azatyrosine (Table 1). The Km of F130S for tyrosine was almost 20-fold higher than that of wt TyrRS, showing that affinity of F130S to tyrosine was greatly reduced. On the other hand, the Km of F130S for azatyrosine was only increased 2-fold over that of wt TyrRS. The specificity against azatyrosine can be determined from the ratios of kcat/Km for the two amino acids: the specificity constant (22,23) is 36 for wt TyrRS, while it is only 19 for the F130S mutant. Clearly, the mutant has a much reduced discrimination against azatyrosine. Combined with the fact that its Km for tyrosine is very much above physiological tyrosine levels (24), it is understandable why E. coli transformed with the F130S plasmid incorporates much azatyrosine into protein (Fig. 1).

In vivo Incorporation of [3H]-Azatyrosine into Cellular Protein — To confirm that azatyrosine is incorporated into some nascent polypeptide chain without the interruption of peptide elongation, we cultured E. coli cells with [3H]-azatyrosine or [3H]-tyrosine and fractionated the lysate of the cells by SDS/PAGE. As shown in Fig. 2, many high molecular weight proteins were detected in the lysate of E. coli cultured with [3H]-azatyrosine, and JM109/pF130S had higher activity for incorporation of azatyrosine into protein in vivo than E. coli having wt TyrRS (lane 6 and 8). It is of interest that the density and the pattern of azatyrosine-containing proteins are different from those of tyrosine-containing proteins. Possibly azatyrosine is not
evenly incorporated into all proteins, but preferentially into particular ones.

Structure Modeling of *E. coli* TyrRS — To find out more of how the F130S substitution affects aminoacylation of azatyrosine and tyrosine, we examined the crystal structure of *B. stearothermophilus* TyrRS complexed with tyrosyl adenylate (15). As there is a great sequence similarity (56% amino acid identity) between *E. coli* and *B. stearothermophilus* we felt that this would be meaningful. The mutation site (position 130) is located near the attachment site for tyrosyl adenylate (Fig. 3, a and b); amino acid 130 is close to D182 which directly interacts with the aminoacyl adenylate. Possibly the F130S replacement influences the conformation of the attachment site through its interaction with D182, thus affecting enzymatic specificity. *E. coli* TyrRS and *B. stearothermophilus* TyrRS are homodimeric enzymes (24). Position 130 is also relatively close to the dimer interface (Fig. 3, a). As proper dimerization is crucial for correct amino acid binding, it is also possible that the F130S mutation might affect dimerization and subsequently the environment of the amino acid binding site (20,21).
Discussion

Aminoacyl-tRNA synthetase mutants with altered amino acid specificity would be of great interest. Very few studies on their generation have been reported (22,23); they are certainly toxic \textit{in vivo} due to the presence of misacylated tRNAs, which may be corrected or further altered by editing mechanisms (25). A fine example of switching the amino acid specificity of human glutaminyl-tRNA synthetase to glutamate charging was based in inspired sequence/structure comparisons (23). The amino acid specificity of \textit{B. stearothermophilus} TyrRS was also modified by structure-based amino acid changes in the amino acid binding site (22). We show here the generation of an \textit{E. coli} TyrRS mutant that, compared to wt TyrRS, displays less discrimination against the tyrosine analogue azatyrosine. As TyrRS is not known to have an editing mechanism (27) this would provide the explanation of the increased azatyrosine incorporation into proteins observed in \textit{E. coli} strains harboring the F130S \textit{tyrS} gene (Fig. 1). Ibba \textit{et al.} (19) reported that mutation of A294 located in the amino acid binding site of \textit{E. coli} phenylalanyl-tRNA synthetase resulted in acceptance of $p$-chlorophenylalanine, suggesting that change of the amino acid binding pocket size influences amino acid specificity. Contrary to this, the site of the mutation of our \textit{E. coli} mutant TyrRS was found to be near the amino acid binding site; residue 130 interacts with D182 at the binding site (Fig. 3, a and b). In addition, the models of wt TyrRS with azatyrosyl adenylate, F130S TyrRS with tyrosyl adenylate, and that with azatyrosyl adenylate were constructed in the same manner as described (see Experimental Procedures). With these models, we calculated the binding energy of each substrate against each TyrRS. We found that mutant TyrRS with tyrosyl adenylate is energetically comparable with that with azatyrosyl adenylate, while normal TyrRS with tyrosyl adenylate was energetically more stable than that with azatyrosyl adenylate.\footnote{This result is consistent with the difference of $K_m$ of each substrate against each TyrRS.} This result is consistent with the difference of $K_m$ of each substrate against each TyrRS. This energy calculation may be useful to identify which residues should be mutated to obtain mutant TyrRS enzymes with greater specificity for azatyrosyl adenylate.

An \textit{in vivo} screen for isolation of a mutant aminoacyl-tRNA synthetase was reported recently by Liu and Schultz (28). Since this method does not require radioactive amino acids, it may be useful for isolation of a
mutant aminoacyl-tRNA synthetase that can activate a large number of unnatural amino acids. As we focused only on one amino acid, we adopted a simple but rather laborious procedure in which incorporation of radioactive azatyrosine into protein was measured in vivo without previous selection. Clearly, a high-throughput screening procedure is desirable for the isolation of more mutant TyrRS enzymes. In this regard it should be mentioned that growth of E. coli strains that harbor the pF130S plasmid is retarded by addition of azatyrosine to the culture medium (unpublished results).

The TyrRS:tRNA$_{Tyr}$ system differs from those of other aminoacyl-tRNA synthetases in the sense that prokaryotic tRNA$_{Tyr}$ possesses a unique structural feature distinct from that of eukaryotic tRNA$_{Tyr}$ (29). Thus, TyrRS from prokaryotes and eukaryotes only recognizes cognate tRNA from the same species (5-7). This property of TyrRS may provide an excellent opportunity to produce azatyrosine-containing alloproteins. Transfection of the E. coli F130S mutant tyrS gene (endowed with an appropriate promoter) together with the E. coli tRNA$_{Tyr}$ gene into oncogene-transformed mammalian cells should clarify the mechanism of action of azatyrosine for converting the transformed phenotype to normal. In addition, the use of an E. coli amber suppressor tRNA$_{Tyr}$, in place of the normal tRNA$_{Tyr}$, should allow the exclusive insertion of azatyrosine into a specific positions in proteins by introducing the corresponding gene which contains the UAG codons in the positions where azatyrosine should be inserted. The mutated TyrRS can also be used for large-scale production of azatyrosyl-tRNA$_{Tyr}$ by in vitro protein biosynthesis (30).


Footnote

1 The abbreviations used are: tyrosyl-tRNA synthetase, TyrRS; wild-type, wt; sodium dodecyl sulfate, SDS; polyacrylamide gel electrophoresis, PAGE; trichloroacetic acid, TCA

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Fig. 1  Incorporation of radiolabeled amino acids into the TCA-insoluble fraction in vivo.  
E. coli JM109 transformed by pYRS with the wt TyrRS gene (JM109/pYRS) and E. coli clone R-6-A-7 were cultured with L-[³H]-tyrosine or L-[³H]-azatyrosine.  Protein fractions were precipitated with TCA and the radioactivities of the fractions were measured in a liquid scintillation counter.

Fig. 2  In vivo incorporation of L-[³H]-tyrosine or L-[³H]-azatyrosine into cellular protein of E. coli JM109 transformed by pYRS with the wt TyrRS gene and E. coli J109 transformed by pF130S with the F130S gene were cultured with L-[³H]-tyrosine (Y) or L-[³H]-azatyrosine (AY).  Crude cell lysates were subjected to SDS/PAGE.  Proteins were located by silver staining (lane 1-4).  Radiolabeled proteins were located by autoradiography (lane 5-8).  Y and AY indicate results with tyrosine and azatyrosine, respectively.

Fig. 3  Structural model of E. coli wild-type TyrRS with the ligand tyrosyl adenylate.

a. Ribbon diagram of backbone of TyrRS.  The positions of residues Asp182 (red) and Phe130 (green) near tyrosyl adenylate (purple) are indicated.

b. Model of binding of tyrosyl adenylate at the active site of TyrRS.  Asp182 and Phe130 are displayed boldly and tyrosyl adenylate as a ball and stick.
Table 1. Aminoacylation kinetics of normal and mutated TyrRSs with tyrosine or azatyrosine

<table>
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<td></td>
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<td>Tyrosine</td>
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Fig. 1

Incorporation (pmol / well / O.D.)

- Tyrosine
- Azatyrosine

JM109/pTRS-S4
R-6-A-7
A mutant Escherichia coli tyrosyl-tRNA synthetase utilizes the unnatural amino acid azatyrosine more efficiently than tyrosine

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