Adding L-3-(2-Naphthyl)alanine to the Genetic Code of E. coli
Lei Wang,† Ansgar Brock,‡ and Peter G. Schultz*†‡§

Department of Chemistry, University of California at Berkeley, Berkeley, California 94720, Genomics Institute of the Novartis Research Foundation, 3115 Merryfield Row, San Diego, California 92121, and Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received October 8, 2001

The ability of chemists to rationally manipulate the structure and function of proteins is quite limited in comparison to that for smaller organic molecules, despite the central role of these macromolecules in nature. The development of methods that make it possible to increase the number of genetically encoded amino acids in living organisms beyond the common 20 would provide a tremendous opportunity to better understand and possibly enhance protein (and perhaps organismal) function. To this end, we have developed a general strategy in which additional components including a novel tRNA—codon pair, an aminoacyl-tRNA synthetase, and an amino acid are added to the translational machinery of the cell. 1 This new set of components functions orthogonally to the counterparts of the common 20 amino acids; that is, the orthogonal synthetase (and only this synthetase) aminoacylates the orthogonal tRNA (and only this tRNA) with the unnatural amino acid only, and the resulting acylated tRNA inserts the unnatural amino acid in response to only the unique codon. Using this strategy, we have shown recently that it is possible to augment the protein biosynthetic machinery of Escherichia coli to accommodate the additional genetically encoded amino acid O-methyl-L-tyrosine with fidelity close to that of the common amino acids. We now report the site-specific incorporation of an orthogonal synthetase with unnatural amino acid specificity. Preliminary results suggest that alkyl-, aryl-, acyl-, and azido-substituted amino acids can also be selectively incorporated.2

An amber stop codon and its corresponding orthogonal amber suppressor tRNA, mutRNA\textsubscript{CUA}, were selected to encode the unnatural amino acid. 3 The Methanococcus jannaschii TyrRS tyrosyl-tRNA synthetase (TyrRS) was used as the starting point for the generation of an orthogonal synthetase with unnatural amino acid specificity. This TyrRS does not aminoacylate any endogenous E. coli tRNAs 4 but aminoacylates the mutRNA\textsubscript{CUA} with tyrosine. 5 L-3-(2-Naphthyl)alanine was chosen for this study since it represents a significant structural perturbation from tyrosine and may have novel packing properties. To change the amino acid specificity of the TyrRS so that it charges the mutRNA\textsubscript{CUA} with 1-3-(2-naphthyl)-alanine and not any common 20 amino acids, a library of M. jannaschii TyrRS mutons was generated and screened. On the basis of an analysis of the crystal structure of the homologous TyrRS from Bacillus stearothermophilus, 6 five residues (Tyr\textsuperscript{12}, Asp\textsuperscript{158}, Ile\textsuperscript{159}, Leu\textsuperscript{162}, and Ala\textsuperscript{167}) in the active site of M. jannaschii TyrRS that are within 7 Å of the para position of the aryl ring of tyrosine were mutated (Figure 1). 7 To reduce the wild-type synthetase contamination in the following selection, these residues (except Ala\textsuperscript{167}) were first mutated to alanine. The resulting inactive Alas

Figure 1. The active site of TyrRS. Residues from B. stearothermophilus TyrRS are shown. Corresponding residues from M. jannaschii TyrRS are Tyr\textsuperscript{12} (Tyr\textsuperscript{25}), Asp\textsuperscript{158} (Asp\textsuperscript{176}), Ile\textsuperscript{159} (Phe\textsuperscript{177}), Leu\textsuperscript{162} (Leu\textsuperscript{180}), and Ala\textsuperscript{167} (Gln\textsuperscript{189}) with B. stearothermophilus TyrRS residues in parentheses. Randomly mutated residues are shown in yellow. TyrRS gene was used as a template for polymerase chain reaction (PCR) random mutagenesis with oligonucleotides bearing random mutations at the corresponding sites.

The mutant TyrRS library was first passed through a positive selection based on suppression of an amber stop codon at a nonessential position (Asp\textsuperscript{112}) in the chloramphenicol acetyltransferase (CAT) gene. Cells transformed with the mutant TyrRS library and the mutRNA\textsubscript{CUA} gene were grown in minimal media containing 1 mM L-3-(2-naphthyl)alanine and 80 μg/mL chloramphenicol. Cells can survive only if a mutant TyrRS aminoacylates the mutRNA\textsubscript{CUA} with either natural amino acids or L-3-(2-naphthyl)alanine. The surviving cells were then grown in the presence of chloramphenicol and the absence of the unnatural amino acid. Those cells that did not survive must encode a mutant TyrRS that charges the mutRNA\textsubscript{CUA} with L-3-(2-naphthyl)alanine and were picked from a replica plate supplied with the unnatural amino acid. After three rounds of positive selection followed by a negative screen, four TyrRSs were characterized using an in vivo assay based on the suppression of the Asp112TAG codon in the CAT gene (Table 1). In the absence of L-3-(2-naphthyl)alanine, cells expressing the selected TyrRS and the mutRNA\textsubscript{CUA} survived in 25–35 μg/mL chloramphenicol on minimal media plates containing 1% glycerol and 0.3 mM leucine (GMML plate); in the presence of L-3-(2-naphthyl)alanine, cells survived in 100–120 μg/mL chloramphenicol on GMML plates. Compared to the IC\textsubscript{50} value in the absence of any TyrRS (4 μg/mL chloramphenicol), these results indicate that the selected TyrRSs accept L-3-(2-naphthyl)alanine, but also still charge natural amino acids to some degree.

To further reduce the activity of the mutant TyrRS toward natural amino acids, one round of DNA shuffling was carried out using the above four mutant genes as templates. The resulting mutant TyrRS library was passed through two additional rounds of positive selections and negative screens. One mutant TyrRS (SS12-TyrRS) was evolved, whose activity for natural amino acids was greatly reduced (IC\textsubscript{50} = 9 μg/mL chloramphenicol) while its activity toward...
L-3-(2-naphthyl)alanine was enhanced (IC50 = 4 µg/mL of chloramphenicol) accumulation of mouse DHFR protein under different conditions. Table 1.

**Table 1. In Vivo Chloramphenicol Acetyltransferase Assay of Mutant TyrRS***

<table>
<thead>
<tr>
<th>mutant TyrRS</th>
<th>IC50 (µg/mL of Chloramphenicol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no TyrRS</td>
<td>240</td>
</tr>
<tr>
<td>wt TyrRS</td>
<td>240</td>
</tr>
<tr>
<td>After Selection</td>
<td></td>
</tr>
<tr>
<td>S1-TyrRS</td>
<td>30</td>
</tr>
<tr>
<td>S2-TyrRS</td>
<td>30</td>
</tr>
<tr>
<td>S3-TyrRS</td>
<td>25</td>
</tr>
<tr>
<td>S4-TyrRS</td>
<td>35</td>
</tr>
<tr>
<td>SS12-TyrRS</td>
<td>9</td>
</tr>
</tbody>
</table>

* A pYC-J17 plasmid was used to express the mutRNA_Tyr gene and the chloramphenicol acetyltransferase gene with an amber stop codon at Asp12. A pHK plasmid was used to express TyrRS, and was cotransformed with pYC-J17 into E. coli DH10B. Cell survival on GMML plates was titrated in the presence of different concentrations of chloramphenicol.

Figure 2. Accumulation of mouse DHFR protein under different conditions. (A) Silver-stained SDS-PAGE gel of purified mouse DHFR. Expression conditions are notated at the top of each lane. The very left lane is molecular weight marker. (B) Western blot of gel in (A).

An L-3-(2-naphthyl)alanine mutant of mouse dihydrofolate reductase (DHFR) was generated and characterized to confirm the ability of the mutRNA_Tyr/SS12-TyrRS pair to site-specifically incorporate L-3-(2-naphthyl)alanine in response to an amber stop codon. The Tyr163 codon of the mouse DHFR gene was mutated to TAG, and a His6 tag was added to the COOH-terminus of DHFR. The Tyr163 codon of the mouse DHFR gene was mutated to TAG, and a His6 tag was added to the COOH-terminus of DHFR.

Most residues are mutated to amino acids with hydrophobic side chains, which are expected to favor binding of L-3-(2-naphthyl)alanine. Efforts to solve the crystal structure of the wild-type *M. jannaschii* TyrRS and the evolved SS12-TyrRS are under way.

In summary, the cell growth, protein expression, and mass spectrometry experiments demonstrate that the mutRNA_Tyr/SS12-TyrRS pair is capable of selectively inserting L-3-(2-naphthyl)alanine into proteins in response to the amber codon with fidelity rivaling that of the natural amino acids. This result, which involves an amino acid that is structurally distinct from tyrosine, suggests that the above methodology should be applicable to a variety of unnatural amino acids. We are currently evolving synthetases that have specificities for unnatural amino acids with novel chemical and physical properties, as well as expanding the methodology to eukaryotic cells and four-base codon–anticodon pairs.

**Acknowledgment.** We acknowledge support of this work by NIH (GM62159).

**References**


(7) No synthetases specific for L-3-(2-naphthyl)alanine were selected from the mutant TyrRS library reported in ref 1a. JA012307J

**Figure 3.** Tandem mass spectrum of the tryptic peptide LLPEK*TGVL-SEVQEEK (X* = L-3-(2-naphthyl)alanine). The sequence can be read from the annotated b (red) or y (blue) ion series; even so, b7 and y13 are not observed. The base peak 821.7 (100%) assigned to the doubly charged y14 ion is truncated for clarity.