Mutational Identification of an Essential Tryptophan in Tryptophanyl-tRNA Synthetase of Bacillus subtilis

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The strongly conserved single tryptophan residue, Trp\(^\text{p2}\), in Bacillus subtilis tryptophanyl-tRNA synthetase has been mutagenized via site direction singly into Gin, Ala, and Phe. All three mutant enzymes were inactive toward the catalysis of tRNA tryptophanylation. The Trp\(^\text{p2}\) → Phe mutant has been subcloned into the high expression plasmid pKK223-3 to yield the recombinant plasmid pKSW-F92. Growth of bacteria carrying the latter plasmid made possible the purification of the mutant TrpRS-F92 enzyme to homogeneity. This mutant enzyme was deficient in ultraviolet absorbance and fluorescence relative to the wild type enzyme and inactive in the partial reaction of Trp-activation as well as the overall reaction of tRNA tryptophanylation. Furthermore, unlike the wild type B. subtilis trpS gene, the mutant trpS-F92 gene upon transformation into Escherichia coli trpS 10343 failed to complement the temperature sensitive trpS mutation of the host cells. Trp\(^\text{p2}\) therefore represents an essential residue both in vitro and in vivo for the function of the tryptophanyl-tRNA synthetase.

The accuracy of protein synthesis is essential to the survival of any organism, and the fidelity of aminoacyl-tRNA synthetases represents a key requirement in this regard. Insight into the structure-function relationship of aminoacyl-tRNA synthetases is therefore a subject of intensive experimental investigation.

Tryptophanyl-tRNA synthetase (TrpRS),\(^1\) a dimeric enzyme, contains the smallest subunit chains among known aminoacyl-tRNA synthetases. Therefore it provides a particularly valuable system for structure-function analysis through mutagenesis. Of the prokaryotic/organelle TrpRS sequences that have been determined, that of Bacillus subtilis is therefore a subject of intensive experimental investigation.

Bacteria and Plasmids—Escherichia coli JM109 (5) was employed as host cell for expressing the B. subtilis trpS gene. The bacterial culture was grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 75 μg/ml ampicillin. E. coli trpF 10343, a tryptophan auxotroph containing a temperature-sensitive trpS gene (6), could grow in M9-glucose minimal medium at 32 °C but not at 42 °C. It thus furnished a basis for testing in vivo complementation to the E. coli trpS gene in this medium.

The trpS gene of B. subtilis encoding wild type TrpRS was carried in the pUC8-derived pTSQ2 plasmid (1). The plasmid pKK223-3, containing the highly efficient tac promotor (7), was used as the cloning vector to overexpress either the wild type or the mutated trpS gene.

Site-directed Mutagenesis and Expression of trpS Gene—Synthetic oligonucleotides 18 nucleotides in length were used to convert the codon for Trp\(^\text{p2}\) on the B. subtilis gene for TrpRS to that of Gin, Ala, or Phe. The mutagenesis procedure used in this study was based on that developed by Nakamaye and Eckstein (8). Since an initial cloning of the trpS-carrying fragment from pTSQ2 into pEMBLY (9) gave a low yield of single-stranded DNA, the gene was cloned instead into the M13 phage. The mutated M13 phage DNAs were used to transform E. coli JM109, and plaque-purified phages were employed for the preparation of single-stranded DNA. After the presence of a desired mutation within the trpS gene had been confirmed by sequencing the region of interest, the nucleotide sequence of the entire gene was determined to ensure the absence of any adventitious mutations. The yield of mutations with this method was 80-90%. Since the trpS gene for unidentified reasons was not significantly expressed in the M13 phage system, the mutated trpS-carrying fragment was recloned into pUC8 and propagated in JM109 for expression. One of the three mutant genes, which encodes Phe at position 92, was further subcloned into the high expression plasmid pKK223-3 (from Pharmacia LKB Biotechnology Inc.) to yield pKSW-F92 for overproduction of the gene product, as had been performed previously for the wild type trpS gene yielding the pKSW1 plasmid (10).

The over 8000-fold purification of native TrpRS from B. subtilis required a combination of four purification steps including two high performance liquid chromatography steps (11). On account of the high expression from E. coli JM109 cells bearing either pKSW1 or pKSW-F92, only a 2-fold purification employing chromatography on DEAE-Sepacel and hydroxylapatite sufficed to yield a homogeneous enzyme.\(^5\)

Enzyme Assays—The catalyzed tryptophanylation of tRNA was assayed based on the formation of [\(^{14}\)H]Trypt-tRNA, and the ATP-PP\(_i\) exchange reaction was assayed based on the incorporation of [\(^{32}\)P] pyrophosphate into ATP, both as described (11). Trp-hydroxamate formation was determined in accordance with Davie (12).

Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent from Pierce Chemical Co. (13). Ultraviolet absorption spectra were determined with a Kontron Uvikon 810 spectrophotometer, and fluorescence spectra with a Spex Fluorolog fluorometer.

RESULTS

Activities of Mutated Enzymes—When the three mutated trpS genes cloned into pUC8 were expressed in JM109, and

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the enzyme extracts assayed for tRNA tryptophanylation, all three of the mutants, bearing Ala\textsuperscript{92}, Gln\textsuperscript{92}, or Phe\textsuperscript{92}, respectively, displayed a practically complete loss of activity relative to the wild type enzyme (Table I).

Subcloning of the Trp\textsuperscript{92}Phe Mutant Gene—While the tryptophanylation assay was readily measured on enzyme extracts prepared from JM109 cells carrying the pTSQ2 plasmid, the fluorescence, ATP-PP\textsubscript{i} exchange, and Trp-hydroxamate formation studies required a purified enzyme. For this purpose, a high-level expression of the mutant TrpRS-F92 enzyme had to be sought utilizing the same system that yielded the high level expression plasmid pKSW1 for wild type TrpRS. Accordingly, as in the construction of pKSW1, the trpS-bearing 1.49-kilobase BamHI-EcoRI fragment derived from pTSQ2 was cloned into phage M13, and mutagenized to generate an EcoRI site flanking the very 5’ end of the trpS gene. Additionally, the TGG codon for Trp\textsuperscript{92} was converted to TTT, encoding for phenylalanine, before the mutated gene was cleaved by EcoRI and inserted into the EcoRI site of pKK223-3 and transformed into JM109. The mutant trpS gene recovered from the transformants could display one of two possible orientations with respect to the tac promotor on the vector. A transformant that carried the plasmid with the trpS gene aligned downstream to the tac promotor was designated pKSW-F92. Upon expression of the mutant trpS gene on pKSW-F92 in JM109, the yield of the mutant TrpRS-F92 enzyme was comparable with that of the wild type enzyme expressed from the pKSW1 plasmid, exceeding 50% of total proteins in the crude extract. Purification of the TrpRS-F92 so obtained readily yielded the homogeneous enzyme shown in Fig. 1.

Characterization of the TrpRS-F92 Enzyme—Compared with wild type TrpRS at the same protein concentration as

<table>
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<th>Enzyme</th>
<th>Relative activity %</th>
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<tr>
<td>Wild type</td>
<td>100</td>
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<tr>
<td>Trp\textsuperscript{92} \rightarrow Ala</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Trp\textsuperscript{92} \rightarrow Gln</td>
<td>&lt;0.2</td>
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<tr>
<td>Trp\textsuperscript{92} \rightarrow Phe</td>
<td>&lt;0.2</td>
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FIG. 2. Ultraviolet absorption spectra. Upper curve, wild type TrpRS; lower curve, TrpRS-F92. Both samples contained 0.73 mg/ml protein.

FIG. 3. Fluorescence spectra. A, emission spectra (with excitation at 280 nm); B, excitation spectra (emission measured at 325 nm). Solid curve, wild type TrpRS; dashed curve, TrpRS-F92; dotted curve, water.

determined by the BCA assay (13), which measures the peptide bond content of a protein sample, purified TrpRS-F92 displayed a greatly reduced ultraviolet spectrum (Fig. 2). Both the emission (Fig. 3A) and excitation (Fig. 3B) fluorescence spectra of the mutant enzyme were also diminished relative to wild type. These decreases in absorption and fluorescence are consistent with the loss of Trp\textsuperscript{92} from the mutant enzyme. In the case of the emission spectrum, the peak emission decreased from 334 nm for the wild type enzyme to 307 nm for the F92 enzyme. The fluorescence emission peak of free Tyr is known to be located at 303 nm, well below the peak for Trp at 348 nm (14). Since there are 14 Tyr residues in TrpRS, the spectra recorded in Fig. 3A point to a shift from the Trp-dominated fluorescence in the wild type enzyme to fluorescence dominated by the Tyr residues in the F92 enzyme, as
the mutant enzyme exhibited little observable activity.

The ATP-PPi exchange reaction depends on Step 1, while the tryptophanylation of tRNA depends on both Steps 1 and 2. The loss of tRNA tryptophanoylation by TrpRS-F92 could result from a loss of Step 1 or Step 2 or both. To assess the ability of TrpRS-F92 to catalyze Step 1, the ATP-PPi exchange reaction was measured according to Xu et al. (11). The F92 enzyme was found to be severely defective relative to the wild type (Fig. 5).

Another method for assessing the formation of Trp-AMP in Step 1 was to monitor the reaction of this intermediate with hydroxylamine to produce Trp-hydroxamate. On this basis, TrpRS-F92 was again found to be deficient, showing less than 1% of wild type activity (Table II).

Previously it was observed that the cloned wild type B. subtilis trpS gene on pKSW1 could complement the temperature-sensitive TrpRS of E. coli trpS 10343. When pKSW-F92 was used to transform E. coli trpS 10343 instead, all the ampicillin-resistant transformants recovered could only grow at 32°C on M9-glucose agar and not at 42°C. The mutant trpS-F92 gene therefore failed to function in vivo just as it was found to be inactive in vitro.

**Discussion**

The abolishment of enzyme activity by the replacement of Trp92 with Phe92, Gln92, or Ala92 clearly establishes the importance of this residue in B. subtilis TrpRS. Fig. 6 shows the positions of Trp residues in known TrpRS sequences. The enzymes from B. subtilis (330 amino acids), B. steartotherophilus (328 amino acids), and E. coli (334 amino acids) are comparable in length and similar in sequence. The B. subtilis enzyme exhibits 78.1% homology with the B. steartotherophilus enzyme and 55.8% homology with the E. coli enzyme (1). The yeast mitochondrial enzyme (374 amino acids) has a longer polypeptide chain and displays a more limited homology toward the bacterial enzymes (4). Even though the enzymes from B. steartotherophilus, E. coli, and yeast mitochondria contain 3, 2, and 4 Trp residues, respectively, only that Trp residue on these molecules which corresponds to Trp92 in B. subtilis is conserved. Sequence conservation around this residue, which extends to all four enzymes (Fig. 7), agrees with the conserved Trp fulfilling a central role in the catalytic mechanisms of this family of aminoacyl-tRNA synthetases.

The exact role of Trp92 remains to be determined. Since Trp is one of the most hydrophobic of amino acids, one possibility might be that Trp92 interacts with the tryptophan, ATP, or tRNA substrate of the enzyme through hydrophobic or stacking interactions. Previously, it has been found that substrate hydrophobicity among the fluorotryptophans is a key factor determining the selectivity of TrpRS toward these substrates (11). Recently, 2 Trp residues on a single-stranded DNA-binding protein also have been found to be important in complex formation with DNA through base stacking (15). Because Trp92 is required for the formation of Trp-AMP in the absence of tRNA no less than the formation of Trp-tRNA, its role cannot be restricted to one of interaction with tRNA alone.

Being the only tryptophan residue on the synthetase molecule, Trp92 is particularly open to observation by absorption, fluorescence, and NMR spectroscopies. Application of these and other physical measurements will undoubtedly throw further light on this crucial amino acid residue on the smallest...
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of polypeptide chains among the aminoacyl-tRNA synthetases.

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REFERENCES