APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 761–762 Vol. 63, No. 2
0099-2240/97/$04.00+0

Mutational Analysis of the Feedback Sites of Phenylalanine-Sensitive 3-Deoxy-D-*arabino*-Heptulosonate-7-Phosphate Synthase of *Escherichia coli*

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In *Escherichia coli*, *aroF*, *aroG*, and *aroH* encode 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase isozymes that are feedback inhibited by tyrosine, phenylalanine, and tryptophan, respectively. In vitro chemical mutagenesis of the cloned *aroG* gene was used to identify residues and regions of the polypeptide essential for phenylalanine feedback inhibition.

In bacteria and plants, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase catalyzes the first step in the pathway that leads to the biosynthesis of aromatic amino acids and vitamins. In *Escherichia coli*, three unlinked genes, *aroF* at 57 min, *aroG* at 17 min, and *aroH* at 37 min, encode three DAHP synthase isozymes that are sensitive to tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp), respectively (1, 2, 6). Carbon flow through the shikimate pathway is regulated initially through repression and feedback inhibition by these three amino acids, and the latter is quantitatively the major control mechanism in vivo (3, 5, 6). In wild-type cells grown in minimal medium, Phe-, Tyr-, and Trp-sensitive DAHP synthases comprise about 80, 20, and 1% of the total DAHP synthase activity, respectively (10). The three genes have been cloned, and the amino acid sequences of the encoded DAHP synthases have been deduced by nucleotide sequence analysis (4, 7, 9). The nucleotide sequences of any two of the three genes show 50 to 60% sequence similarity, and the degrees of similarity of the predicted amino acid sequences of their encoded enzymes are similar. Although several *aroF* and *aroH* mutations encoding feedback-insensitive enzymes have been reported (7, 12), little is known about *aroG* mutations. We report the nucleotide sequences of four *aroG* mutations encoding Phe-insensitive DAHP synthases.

Cloning of *aroG*. A 2.0-kb fragment containing an entire *aroG* gene and flanking sequences was amplified from chromosomal DNA of *E. coli* W3110 by the PCR using two primers (5′-GTATTTACCCCGTTATTGTC-3′ and 5′-ACTCCGCC GGAAGTGACTAA-3′) and digested with *Sal*I and *Eco*47III. This fragment was cloned into the *Sal*I-*Sma*I site of the low-copy-number vector pMW119 (Wako Pure Chemical Industries, Osaka, Japan), and the resulting plasmid was named pMGW.

Hydroxylamine mutagenesis. pMGW DNA was isolated with the Wizard Minipreps DNA Purification System (Promega), in accordance with the manufacturer's protocol. For hydroxylamine mutagenesis, 5 μg of purified plasmid DNA was incubated at 75°C for 2 h in 100 μl of sodium phosphate buffer (50 mM, pH 6.0) with 400 mM hydroxylamine hydrochloride and 1 mM EDTA, and treated DNA was purified with EASYTRAP (Takara Shuzo, Kyoto, Japan), in accordance with the manufacturer's protocol, and transformed into strain AB3257 (*aroF363 aroG365 aroH367 str-712 thi-1 his-4 proA2 argE3 ilv-7*), which is deficient in each DAHP synthase (11). The obtained transformants were screened for resistance to Phe (10 mM) on minimal agar containing ampicillin (50 μg/ml). Normally, only strains with an intact *aroG* gene (*aroF aroH* mutant) are prototrophic, but they are subject to growth inhibition by Phe, as Phe-mediated repression of *aroG* expression and feedback inhibition of Phe-sensitive DAHP synthase starve the cell of Tyr and Trp. Thus, each obtained transformant was expected to harbor a plasmid with a Phe-insensitive *aroG* gene. In order to eliminate mutations in the vector DNA, the *Eco*RI-*Hin*dIII fragment of each treated plasmid was excised and recloned into untreated pMW119, and finally, four Phe-resistant mutant plasmids (pAROG4, pAROG8, pAROG15, and pAROG29) were obtained.

Nucleotide sequence analysis. The *aroG* genes of the wild-type and mutant plasmids were sequenced by the dideoxy chain-termination method with a Sequenase version 2.0 sequencing kit (U.S. Biochemical Co.) with [α-^{35}S]dCTP (Amersham). Subsequent sequencing was carried out by creating primers based on a previously determined sequence to traverse the DNA, both strands of which were sequenced by this method. The nucleotide and the deduced amino acid sequences of *aroG* have been published (4). The determined wild-type sequence is identical to the previously determined one, except for one base change. This base is in the second position of a valine codon, at position 56 of AroG, and our sequence would encode an alanine rather than valine. Table 1 shows the amino acid sequence changes in four *aroG* mutations. Each mutation resulted from a single base change that changed a single amino acid in the primary sequence of *aroG* (Table 1).

Assay of DAHP synthase activity. The DAHP synthase activities of crude cell extracts from strain AB3257 carrying pMGW, pAROG4, pAROG8, pAROG15, and pAROG29

TABLE 1. Amino acid sequence changes in the *aroG* mutations

Mutation	Codon change[a]	Amino acid change
aroG4	CCA→CTA	150 Pro→Leu
aroG8	GCC→ACC	202 Ala→Thr
aroG15	GAT→AAT	146 Asp→Asn
aroG29	ATG→ATA	147 Met→Ile

[a] Changes are shown by underscoring.

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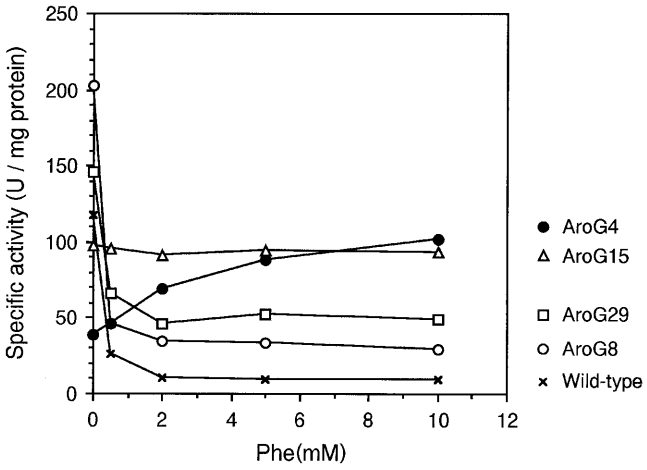

FIG. 1. Inhibition patterns of mutant Phe-sensitive DAHP synthase. One unit of enzyme activity was defined as that forming 1 μmol of DAHP from phosphoenolpyruvate and erythrose-4-phosphate at 37°C in 1 min. Specific activity is reported as units per milligram of protein.

were assayed for sensitivity to feedback inhibition by Phe by the method described by Schoner and Herrmann (8). The wild-type enzyme activity was inhibited 90% by 2 mM Phe (Fig. 1). The activity of AroG15, with a change at D146N, was completely resistant to Phe inhibition, and AroG4, with a change at P150L, was not only resistant to Phe inhibition but also activated by Phe (Fig. 1). The other mutants (AroG8, with a change at A202T, and AroG29, with a change at M147I) were partially resistant to Phe inhibition (Fig. 1). As reported previously, amino acid residues 147 through 149 appear to represent part of an aromatic amino acid-binding pocket common to Tyr- and Trp-sensitive DAHP synthase in that amino acid changes at positions Val-147 and Gly-149 yielded Trp-insensitive *aroH* products and a change at Pro-148 yielded a Tyr-insensitive *aroF* product (7, 12). Similarly, an amino acid change at positions Pro-150 (AroG4) and Asp146 (AroG15) yielded Phe-insensitive *aroG* products. However, AroG29 with an amino acid change at position Met-147 to Ile showed partial resistance to Phe inhibition. Met and Ile residues have similar hydrophobic characteristics and similar effects on protein secondary structure. Moreover, AroG8 with an amino acid change at position Ala-202 in a different region from residues 147 through 149 also showed partial resistance to Phe inhibition. Thus, the amino acid residues 146 through 150 in *aroG* products appear to represent part of an aromatic amino acid-binding site common to the other *E. coli* DAHP synthase isozymes AroF and AroH, and mutation in this region affected the allosteric regulation of Phe-sensitive DAHP synthase.

We thank B. J. Bachmann for supplying *E. coli* AB3257.

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