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Regulation of 3-Deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) Synthase of *Bacillus* sp. B-6 Producing Phenazine-1-carboxylic acid

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The 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase is the first enzyme of aromatic amino acid-, folic acid-, and phenazine-1-carboxylic acid biosynthetic pathways. DAHP synthase of *Bacillus* sp. B-6 that produces phenazine-1-carboxylic acid was feedback inhibited by two intermediary metabolites of aromatic amino acid biosynthetic pathways, prephenate and chorismate, but not by other metabolites, such as anthranilic acid, shikimic acid, *p*-aminobenzoic acid, and 3-hydroxyanthranilic acid. DAHP synthase of *Bacillus* sp. B-6 was not inhibited by end products, such as aromatic amino acids, folic acid, and phenazine- 1-carboxylic acid. The inhibition of DAHP synthase by prephenate and chorismate was non-competitive with respect to erythrose 4-phosphate and phosphoenolpyruvate. Prephenate and chorismate inhibited 50% of the DAHP synthase activity at concentrations of 2×10^{-5} M and 1.2×10^{-4} M, respectively. The synthesis of DAHP synthase of *Bacillus* sp. B-6 was not repressed by exogenous aromatic amino acids, folic acid, and phenazine 1-carboxylic acid, single or in combinations.

Keywords: 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, Phenazine-1-carboxylic acid biosynthetic pathway, Aromatic amino acids, Prephenate, Chorismate

A large amount of evidence indicates that shikimic acid is a precursor of naturally occurring phenazine compounds. This evidence is based on the incorporation of radioactivity from ^{14}C-shikimate into phenazine compounds in intact cells (Byng and Turner, 1977; Chang and Blackwood, 1968; Levitch and Stadtman, 1964). Since shikimate has long been known to be a precursor of aromatic amino acids in microorganisms, it would be of interest to determine what role phenazine- 1-carboxylic acid (Kim and Kim, 1998; Mavrodi *et al.*, 1998), which is end product of this same aromatic pathway, plays in the regulation of aromatic amino acids biosynthesis. Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi (Salcher and Lingens *et al.*, 1980; Smirnov and Kiprianova, 1990; Keith *et al.*, 1991; Slinger and Shea-Wilbur, 1995; Akowski and Bauerle, 1997). The discovery of regulatory isoenzymes (Baek *et al.*, 2000) in *Escherichia coli* (Kikuchi *et al.*, 1997; Schumilin *et al.*, 1999) that catalyze the first reaction of aromatic amino acid biosynthesis, 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, preceded investigation of the control of this pathway in a number of microorganisms as well as in higher plants. In a branched biosynthetic pathway (Sugimoto and Shio, 1985; Pereira and Livi, 1996), total feedback inhibition (Kwak *et al.*, 1999) by a single end product has the disadvantage of immobilizing the organism for the production of other end products. It also leads to the cessation of growth if these particular products are essential cell constituents. Another regulatory dilemma that confronts the organism with respect to a branched pathway is that of differential production of the end products. Channeling of intermediates by means of multienzyme complexes can afford enough control to account for the production, in varying amounts, of end products in a branched pathway. These considerations are of importance in phenazine 1-carboxylic acid producing *Bacillus* sp. B-6 (Kim and Kim, 1998), since the phenazine-1-carboxylic acid is produced in larger quantities than most of the other end products of the aromatic pathway. *Bacillus* sp. B-6 produced approximately 400 mg phenazine-1-carboxylic acid per liter (Kim, 2000). Cell synthesis during this period was approximately 4 to 5 g (wet weight) per liter. It is assumed that most amino acids represent no more than 20 to 30 mg each. The biosynthesis of aromatic amino acids starts with the synthesis of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) from D-erythrose 4-phosphate and phosphoenolpyruvate (Friedlich and Schlegel, 1975). A common pathway for aromatic amino acids-, folic acid biosynthesis, and a hypothetical scheme for phenazine-1-

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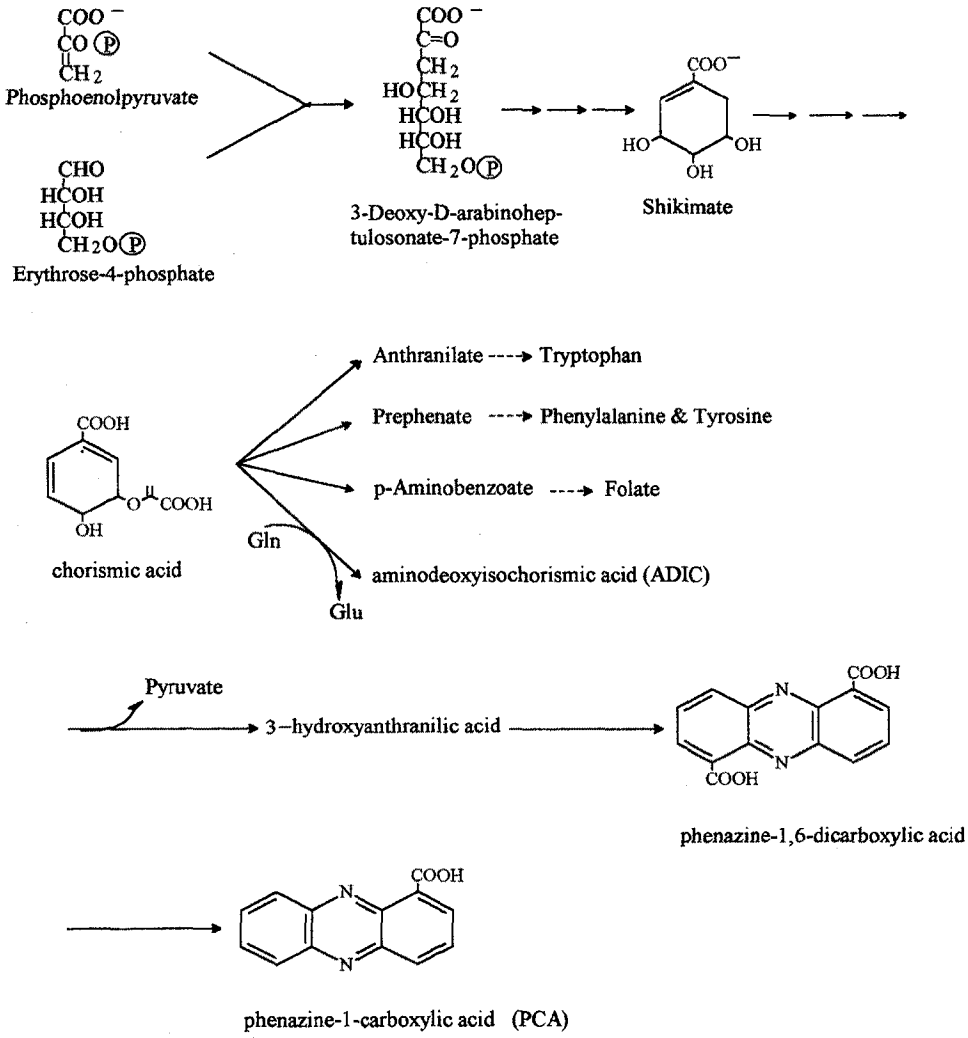

Fig. 1. Common pathway for aromatic amino acids-, folic acid biosynthesis, and hypothetical pathway for phenazine-1-carboxylic acid biosynthesis. 1; DAHP synthase, Gln; glutamine, Glu; glutamate

carboxylic acid biosynthesis are represented in Fig. 1. In this pathway, chorismate (Schnappauf *et al.*, 1998) is the common precursor of phenazines, folic acid and aromatic amino acids. We have now studied the regulatory pattern of DAHP synthase of *Bacillus* sp. B-6 that produces phenazine-1-carboxylic acid.

Materials and Methods

Bacterial stain The *Bacillus* sp. B-6 producing phenazine-1-carboxylic acid, which is used in this experiment, was described previously (Kim and Kim, 1996; Kim and Kim, 1998).

Enzyme preparation Cells were grown on a minimal medium (Kanner *et al.*, 1978) that contained the following: Na_2HPO_4, 0.4%, KH_2PO_4 0.15%, NH_4Cl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, $FeNH_4$-citrate, 0.0005%, glucose, 1%. The cells were harvested in the late exponential phase, centrifuged, and washed twice with a 0.02 M potassium phosphate buffer (pH 7.0). They were then suspended in the same buffer to which dithiothreitol (1 mM) had been added. Cells were disrupted by sonic oscillation, and the debris was removed by centrifugation for 10 min at 30,000 × *g*. Nucleic acid was then precipitated with 1.5% streptomycin sulfate and centrifuged. The supernatant fluid was fractionated with ammonium sulfate precipitation between 35% and 65% saturation. The enzyme fractions were desalted on a column of Sephadex G-25. The desalted enzyme fractions were used as a cell free extract. All of the operations with the cell free extracts were performed at 4°C.

Chemicals Erythrose-4-phosphate, phosphoenolpyruvate, prephenic acid, anthranilic acid, chorismic acid, *p*-aminobenzoic acid, and 3-hydroxyanthranilic acid were purchased commercially from the Sigma Chemical Co. (St. Louis, USA). Phenazine-1-carboxylic acid (PCA) was isolated from the culture supernatant of *Bacillus* sp. B-6, as described in a previous report (Kim and Kim, 1998). PCA was dissolved in 0.001 N sodium hydroxide, and the pH was then adjusted to 7.0 with a potassium phosphate buffer (pH 7.0). All of the other compounds were obtained as commercial products and used without further purification.

Table 1. % Inhibition of DAHP synthase activity of *Bacillus* sp. B-6 by aromatic amino acids, folic acid, and phenazine-1-carboxylic acid (PCA)

Additives	Concentration of each amino acid, PCA or folic acid		
	1 mM	0.5 mM	0.05 mM
L-Tyrosine (Tyr)	13	10	3
L-Phenylalanine (Phe)	5	3	0
L-Tryptophan (Trp)	10	8	2
PCA	4	0	0
Folic acid	0	0	0
Tyr + Phe + Trp +PCA	12	10	3
Tyr + Phe + Trp +PCA +Folic acid	10	8	2

The additives were added in the enzyme assay mixture. A cell free extract of *Bacillus* sp. B-6 was used. The assay was carried out as detailed in Materials and Methods.

Assay of DAHP activity DAHP synthase activity (Denenu and Demain, 1981) was assayed spectrophotometrically by monitoring the rate of DAHP formed at 549 nm using a Jasco UV-spectrophotometer. Standard 1.0 ml reaction mixtures contained a 20 μmol potassium phosphate buffer (pH 7.0), 0.4 μmol each of D-erythrose-4-phosphate (ERY-4-P) and phosphoenolpyruvate (PEP), 50 μmol $MnCl_2$, and 0.5 mg of protein. The DAHP formed was estimated by using 4.5×10^4 for the molar extinction coefficient at 549 nm. One unit was expressed as the activity of an enzyme forming 1 μmol of the DAHP product per min. When the amino acids or phenazine-1-carboxylic acid supplements were used, they are indicated in the text. The reactions were followed in 1 cm square cuvettes maintained at 30°C.

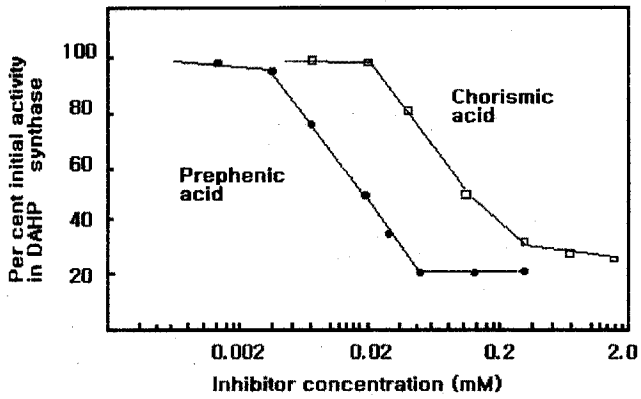

Fig. 2. Kinetics of feedback inhibition of DAHP synthase *in vitro* by chorismic acid and prephenic acid. A crude extract from *Bacillus* sp. B-6 was used (500 μg/ml, final protein concentration). Solutions of chorismic and prephenic acids were adjusted to a pH of 7.0 The activity value of the controls incubated in the absence of the inhibitors are arbitrarily given values of 100%. The reaction mixture was incubated at 30°C for 20 min.

Results and Discussion

Effect of end-products on DAHP synthase activity *in vitro* The addition of tryptophan, tyrosine, phenylalanine, folate, and phenazine-1-carboxylic acid to the cell free extract of *Bacillus* sp. B-6 singly, or in all possible combinations, caused no significant inhibition of DAHP synthase activity (Table 1.). These findings contrasted with the situation in *Alcaligenes eutrophus* H16, in which phenylalanine and tyrosine inhibited enzyme activity (Freidlich and Schlegel, 1975). The DAHP synthase of *Brevibacterium flavum* was also known to be feedback that was inhibited by phenylalanine and tyrosine (Sugimoto and Shiio, 1985). DAHP synthase in *Pseudomonas aureofaciens* was reported to be inhibited by L-tyrosine (Salcher and Linger, 1980). Several patterns of control in the branched biosynthetic pathway were described in bacterial systems (Whitaker *et al.*, 1982; Krappmann *et al.*, 1990; Jensen and Nester, 1966; Walker *et al.*, 1996; Sugramaniam *et al.*, 1998). The elaboration of isoenzymes provides a means by which different end products can inhibit independently a fractional portion of enzyme activity. For example, in *Escherichia coli* there are 3 DAHP synthase isoenzymes; phenylalanine-sensitive DAHP synthase (PHE); tyrosine-sensitive DAHP synthase (TYR); tryptophan-sensitive DAHP synthase (TRP). Different end products can control common enzymatic steps by a cumulative feedback inhibition. Thus, individual end products produce a partial inhibition; a combination of end products causes cumulative degrees of inhibition. The inhibition of enzyme activity (Lee *et al.*, 2000) by the synergistic interaction of end products has been called concerted feedback inhibition (Pittard, 1987). Since the end products showed no significant inhibition on the DAHP synthase activity of *Bacillus* sp. B-6, the intermediary metabolites of the aromatic amino acid-, those of phenazine-1-carboxylic acid (Tomashow and Weller, 1988) and that of the folic acid biosynthetic pathway, were tested as feedback inhibitors of DAHP synthase.

Repressibility of the DAHP synthase synthesis The repressibility of the DAHP synthase synthesis was tested by growing *Bacillus* sp B-6 in the presence of a 1 mM concentration of the aromatic amino acids, folic acid (Scott,

Table 2. % Repressibility of the DAHP synthase synthesis of *Bacillus* sp. B-6 by aromatic amino acids, folic acid, and phenazine-1-carboxylic acid (PCA)

Additives	Concentration of each amino acid, PCA or folic acid 1 mM
L-Tyrosine (Tyr)	0
L-Phenylalanine (Phe)	5
L-Tryptophan (Trp)	0
PCA	4
Folic acid	0
Tyr + Phe + Trp +PCA	5
Tyr + Phe + Trp +PCA +Folic acid	5

The additives were added in the culture of *Bacillus* sp. B-6 at the concentration of 1 mM. The assay was carried out as detailed in Materials and Methods.

Table 3. % Inhibition of DAHP synthase activity *Bacillus* sp. B-6 by intermediary metabolites.

Additives	Concentration of metabolites		
	1 mM	0.1 mM	0.01 mM
Anthranilic acid	5	0	0
ρ-aminobenzoic acid	0	0	0
3-hydroxyanthranilic acid	0	0	0
Shikimic acid	5	0	0
Chorismic acid	70	20	0
Prephenic acid	80	75	15

The additives were added in the enzyme assay mixture. A cell free extract of *Bacillus* sp. B-6 was used. The assay was carried out as detailed in Materials and Methods.

1999), or phenazine-1-carboxylic acid (Levitch and Stadtman, 1964; Pierson *et al.*, 1994; Pierson *et al.*, 1995), individually or in combinations added to the medium. There was no significant change in the specific activity of DAHP synthase in the presence of aromatic amino acids, folic acid, or phenazine-1-carboxylic acid, individually or in combinations (Table 2.). It is logical to assume that any regulation of the pathway would act at one of the later reactions. A similar result was obtained with the *Pseudomonas aureofaciens* (Salcher and Linger, 1980) and Hansenula polymorpha mutant (Denenu and Demain, 1981), in which DAHP synthase was not repressed by aromatic amino acids.

Demonstration of feedback inhibition of intermediary metabolites *in vitro* A number of intermediary metabolites (Hussein *et al.*, 1998; Urrestarazu *et al.*, 1998) of the aromatic amino acid pathway, those of the phenazine-1-carboxylic acid biosynthetic pathway and that of the folic acid biosynthetic pathway, were tested as potential feedback inhibitors (Table 3.). DAHP synthase from *Bacillus* sp. B-6 was feedback inhibited by prephenate and chorismate, but not by other

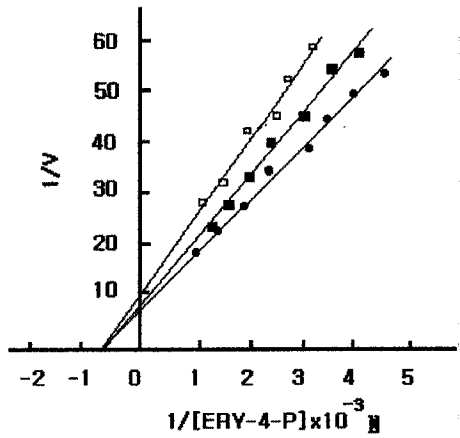

Fig. 3. Effect of substrate concentration upon kinetics of DAHP synthase inhibition by chorismate and prephenic acid; double reciprocal plot of ERY-4-P concentration and initial reaction velocity. The PEP concentration was 0.3 mM. Inhibitor concentrations; chorismic acid, 0.09 mM; prephenic acid, 0.02 mM. Enzyme solution from *Bacillus* sp. B-6 was used (500 μg/ml, final protein concentration). The velocity (V) is expressed as moles of DAHP/min/ml. -□-, prephenic acid; -■-, chorismic acid; -●-, no inhibitor.

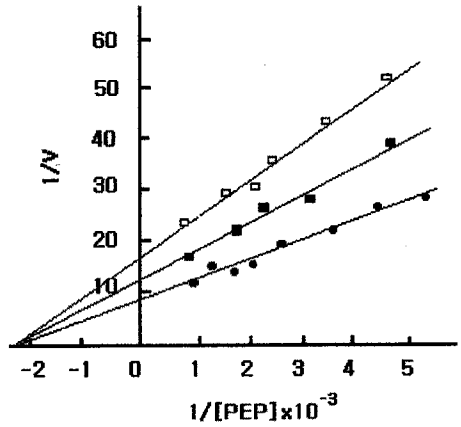

Fig. 4. Effect of substrate concentration upon kinetics of DAHP synthase inhibition by chorismate and prephenic acid; double reciprocal plot of PEP concentration and initial reaction velocity. The ERY-4-P concentration was 0.3 mM. Inhibitor concentrations; chorismic acid, 0.09 mM; prephenic acid, 0.02 mM. Other conditions are indicated in Fig. 3. -□-, prephenic acid; -■-, chorismic acid; -●-, no inhibitor.

metabolites, such as anthranilic acid, shikimic acid, ρ-aminobenzoic acid, and 3-hydroxyanthranilic acid. Prephenate is 6 times more inhibitory than chorismate at comparable levels. Prephenate and chorismate inhibited 50% of the DAHP synthase activity at concentrations of 2×10^{-5} M and 1.2×10^{-4} M, respectively (Fig. 2.). These findings were different from the situation in *Pseudomonas aureofaciens* (Salcher and Lingens, 1980), in which prephenate did not inhibit the DAHP synthase. It has been reported that each DAHP synthase isoenzyme in *Pseudomonas aeruginosa* was strongly

inhibited by an amino acid end product and weakly inhibited by an intermediary metabolite (Whitaker *et al.*, 1982).

Effect of substrate concentration upon kinetics of DAHP synthase inhibition by chorismate and prephenic acid

The effect of intermediary metabolites of the aromatic amino acids biosynthetic pathway (Joo and Kim, 1999), chorismate, and the prephenate (Dewick, 1998) concentration upon DAHP synthase of *Bacillus* sp. B-6 was investigated. The results of the kinetic analysis of chorismate and prephenate inhibition at varying concentrations of ERY-4-P and PEP were presented as Lineweaver-Burk plots in Fig. 3 and Fig. 4. Both chorismate and prephenate were non-competitive inhibitors of DAHP synthase with respect to both the ERY-4-P (Fig. 3) and PEP substrates (Fig. 4). These findings were different than the situation in *Pseudomonas aureofaciens*, in which DAHP synthase was inhibited competitively by ERY-4-P, but non-competitively by PEP (Salcher and Lingens, 1980).

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