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Properties of Tyrosine-Inhibitable 3-Deoxy-D-Arabinoheptulosonic Acid-7-Phosphate Synthase from *Salmonella*

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Tyrosine-inhibitable 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) synthase was purified to homogeneity without significant loss of sensitivity to inhibition by tyrosine from an operator-constitutive strain (*tyrO*^{c}) of *Salmonella*. The enzyme had an apparent molecular weight of 76,000 by gel filtration and a subunit molecular weight of 40,000 by sodium dodecyl sulfate-gel electrophoresis and by reaction with dimethyl suberimidate. It had an isoelectric point of 4.68. Inhibition by L-tyrosine showed a Hill coefficient of 1.8 at pH 7.0, suggesting cooperative interaction between tyrosine-binding sites, and was competitive with phosphoenol pyruvate and noncompetitive with erythrose-4-phosphate.

Tyrosine-inhibitable 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase (EC 4.1.2.15) has been extensively purified from an operator-constitutive (*tyrO*^{c}) strain of *Salmonella typhimurium* (13). Since gel electrophoresis of this preparation indicated minor impurities (10), we undertook a further purification of the enzyme and developed a procedure for preparing it in homogeneous form. We also studied its allosteric properties and subunit structure.

(This report is in partial fulfillment of the requirements for the Ph.D. degree, submitted by C. Y. H., 1974.)

MATERIALS AND METHODS

Materials. Barium DAHP was prepared according to Sprinson et al. (24) and was converted to the potassium salt. Monocyclohexylammonium phosphoenolpyruvate (PEP) was prepared according to the method of Clark and Kirby (5) and used directly. Monocyclohexylammonium phosphoenol α-ketobutyrate was prepared according to Bondinell and Sprinson (3). Barium chorismate was prepared according to Gibson (12). Erythrose-4-phosphate (E4P) was prepared according to the method of Ballou and MacDonald (2). Hemoglobin was a gift from R. Benesch. Barium prephenate was isolated from accumulation media of strain *tyrA19* according to the method of Dayan and Sprinson (9). Dimethyl suberimidate was prepared according to the method of Davies and Stark (7). The following compounds were gifts: monocyclohexylammonium (*Z*)-phosphoenol-3-fluoropyruvate and α-(dihydroxyphosphinyl methyl)-acrylic acid from G. L. Kenyon (25); and fosfomycin from D. Hendlin (4, 15). All other materials used were obtained commercially and used without further purification.

Tyrosine and DAHP Sepharose. The method of Cuatrecasas (6) as modified by Rosenberry et al. (22) was used with 3,3'-imino-*bis*-propylamine and *O*-nitrophenylsulfenyl tyrosine. The tyrosine-substituted Sepharose was washed with 1 volume of dimethyl formamide-water (2:1) and washed extensively with water until absorbance at 280 and 420 nm was not detected in the washings. Coupled *O*-nitrophenylsulfenyl tyrosine was calculated to be 3.1 μmol/ml from the amount of unreacted ligand in the gel washings as measured by absorbance at 280 and 420 nm. The Sepharose was washed with 100 ml of 1 N NaOH for 10 min at room temperature and then with 1 liter of water. The *O*-nitrophenylsulfenyl protecting group was removed by a 2-h treatment with 100 ml of 0.02 M acetate buffer (pH 5.0) containing 0.2 M $Na_2S_2O_3$ (18), and the Sepharose was washed extensively with water.

Barium DAHP (50 μmol) was added to 30 ml of 1,12-diaminododecane Sepharose in 45 ml of dimethyl formamide-water (2:1), the pH was adjusted to 4.7 with 1 N HCl, and (3-dimethylamino)propylethyl carbodiimide (38 mg) was added. The solution was kept at pH 4.7 for 1 h at room temperature and allowed to stand for another 22 h. The DAHP-substituted Sepharose was washed extensively with water, and DAHP in the washings was assayed as described below. The amount of DAHP coupled was calculated to be 0.7 μmol/ml.

Assays. Specific activity was defined as micromoles of substrate used or product formed per hour per milligram of protein. DAHP synthase was determined according to DeLeo et al. (10) with 1.7 mM PEP, 0.85 mM E4P, 0.1 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.0, and 0.6 mg of bovine serum albumin in 0.6 ml (unless otherwise indicated). Inhibition by tyrosine was measured at 10^{-3} M unless otherwise stated. PEP was determined as described by Korn-

berg and Pricer (17). E4P was determined by conversion to DAHP in the presence of an excess of PEP, and the DAHP formed was assayed as described above. Protein concentration was determined by the method of Lowry et al. (19), with bovine serum albumin as standard, or from absorbance at 280 and 260 nm.

Purification of DAHP synthase. Unless otherwise stated, all operations were performed at 2 to 4°C. Strain SG12 (13) was grown at 25°C in two flasks containing 1 liter of minimal medium with vigorous shaking, or in 40 liters in a New Brunswick 130-liter fermentor, and harvested in late log phase (optical density at 660 nm = 1.4) by centrifugation at 4,000 × *g*. The cells were washed once with 0.03 M potassium phosphate buffer, pH 7.0, collected by centrifugation, and stored overnight at −18°C. Cell extracts were prepared as described previously (10) and absorbed at 60 ml/h under the pressure of a peristaltic pump on a column (2.5 by 63 cm) of Whatman DE-52 cellulose equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was washed with 1 column volume of 0.03 M buffer A (KPO_4 buffer, pH 7.0, containing 1 mM PEP and 0.1 mM tyrosine). The column was eluted with a programmed gradient controlled by an LKB 11300 Ultragrad: (i) 0.048 M buffer A for 9 h to remove most of the inactive protein; (ii) 0.08 M buffer A for 2 h; and (iii) a linear gradient from 0.08 to 0.15 M buffer A for 8 h, during which 20-ml fractions were collected. The peak of enzyme activity appeared at 0.08 M buffer A.

The above enzyme solution (28 volumes) was diluted with 0.001 M HEPES buffer, pH 7.0, containing 0.1 mM PEP (72 volumes) by programming with an LKB Ultragrad and applied simultaneously at the top of a tyrosine Sepharose column (2 by 16 cm) that had been equilibrated with 0.02 M HEPES buffer. The column was eluted with 50 ml of 0.02 M buffer B (HEPES buffer, pH 7.0, containing 1 mM PEP and 0.1 mM tyrosine) and then with a linear gradient from 250 ml of 0.02 M buffer B to 250 ml of 0.2 M buffer B containing 0.2 M KCl at 0.5 ml/min (10-ml fractions were collected). Peak enzyme activity appeared at about 0.1 M buffer B containing 0.1 M KCl, and fractions with specific activity higher than 600 were pooled.

The solution was absorbed at 15 ml/h on a column (1.5 by 8.5 cm) of hydroxyapatite (Bio-Rad HT) previously equilibrated with 0.005 M phosphate buffer, pH 7.0, in 10% glycerol. The column was washed with several column volumes of the same buffer mixture and eluted with a linear gradient prepared from 0.1 liter of 0.005 M buffer A containing 10% glycerol and 0.1 liter of 0.2 M buffer A containing 10% glycerol at a flow rate of 15 ml/h. Fractions of 2 ml were collected. Enzyme appeared at about 0.02 M phosphate, and fractions with activity inhibited more than 80% by tyrosine were pooled (24 ml).

The enzyme solution was diluted threefold with 0.001 M buffer C (HEPES buffer, pH 7.0, containing 1 mM PEP, 0.1 mM tyrosine, and 10% glycerol) with an LKB Ultragrad and applied simultaneously at 0.4 ml/h on a Whatman DE-52 column (1.5 by 8.5 cm) that had been equilibrated with 0.02 M buffer C. The column was washed with 1 column volume of 0.02 M buffer C and was eluted with a linear gradient prepared from 100 ml of 0.02 M buffer C and 100 ml of 0.2 M buffer C containing 0.2 M KCl. Fractions with activity inhibited more than 85% by tyrosine were combined. Peak enzyme activity appeared at about 0.1 M HEPES and 0.1 M KCl.

The enzyme was diluted eightfold with 0.002 M HEPES buffer containing 10^{-5} M PEP, 10^{-6} M tyrosine, and 10% glycerol and applied on a DAHP affinity column (1.5 by 9 cm) that had been washed thoroughly with the same buffer solution. The column was washed with some of this buffer, and the enzyme was eluted with 0.2 M buffer C. Fractions with DAHP synthase activity were pooled and concentrated by ultrafiltration (Diaflo model 52) with a PM-10 membrane. The concentrated enzyme solution was diluted with an equal volume of glycerol and stored at −18°C.

Polyacrylamide gel electrophoresis and measurement of subunit molecular weight. Standard analytical disc gel electrophoresis was performed with tris(hydroxymethyl)aminomethane-glycine, pH 9.5, and gels were stacked at pH 8.9 (8, 21). The separating gel, prepared as described in the Canalco manual (Canalco Inc., Rockville, Md.), had a final concentration of 7%. Proteins (0.04 mg) in 50% glycerol were applied to the stacking gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 20 μg of protein by the procedure of Weber and Osborn (26) in 5% acrylamide gel. A standard curve was prepared by plotting mobilities of standard proteins against the log of their molecular weight: 68,000 for bovine serum albumin, 35,000 for pepsin, 43,000 for ovalbumin, and 25,700 for chymotrypsinogen A.

Cross-linking of DAHP synthase with dimethyl suberimidate. Dimethyl suberimidate (melting point, 215 to 216°C) was prepared from suberonitrile according to the method of Davies and Stark (7). Enzyme (200 μg of protein in 0.5 ml) was diluted with 0.5 ml of 0.2 M triethanolamine hydrochloride buffer, pH 8.5, concentrated fourfold with a Minicon A-25 (Amicon Corp.) macrosolute concentrator, made up to 1 ml with the same buffer, concentrated to 40 μl, and treated with dimethyl suberimidate (final concentration, 4 mg/ml) as described by Davies and Stark (7).

Measurement of molecular weight of native enzyme (1). A standard column (2.5 by 82 cm) of Sephadex G-150 was washed and equilibrated with 0.02 M KPO_4 buffer (pH 7.2), and the molecular weight was calculated according to the manufacturer's instructions (Pharmacia Fine Chemicals, Rockville, Md.). Elution was carried out at 4°C with 0.02 M KPO_4 buffer, pH 7.2, at 15 ml/h and a pressure of 20 cm of water. Fractions of 5.5 ml were collected. The void volume of the column was determined with blue dextran 2000 and was checked each time that the elution volume of protein was determined. Gamma globulin (Cohn fraction II, molecular weight 205,000), and ovalbumin (molecular weight 43,000) were determined by absorbance at 280 nm. Lactate dehydrogenase (molecular weight 150,000) was assayed by the method of Kornberg and Pricer (17). Hexokinase (molecular weight 102,000 for the tetramer and

51,000 for the dimer) in 0.02 M phosphate buffer was assayed by the method of Kornberg and Horecker (16). Enolase (molecular weight 82,000) was assayed by the method of Kornberg and Pricer (17).

Electrofocusing. A continuous 0 to 60% gradient of glycerol containing 2% ampholine (LKB), pH 2 to 10, was prepared in an LKB column (2.5 by 40 cm), and proteins were electrofocused with an LKB 7900 Uniphor according to the manufacturer's instructions. A solution of 5 mg of enzyme (specific activity, 335) and 10 mg of hemoglobin (as internal standard) in 2 mM KPO_4 buffer (pH 7.0), containing 30% glycerol and 2% ampholine, was introduced at the center of the gradient. Electrophoresis was carried out at 4°C. Fractions of 3.0 ml were collected, the pH was determined at 25°C in a Radiometer model M 63 pH meter, and enzyme activity was assayed.

Inhibition studies. Incubation times were chosen within the range of linear rates. Reaction mixtures (1 ml) containing PEP, E4P, inhibitor, 0.1 M HEPES buffer, pH 7.0, and 1 mg of bovine serum albumin were incubated at 37°C for 5 min. The reaction was initiated by adding 10 μl of appropriately diluted enzyme (about 0.03 to 0.1 μg). After 8 min at 37°C, the reaction was stopped by the addition of 0.2 ml of 30% trichloroacetic acid and DAHP was assayed as described above, except that protein was not removed and 0.2 ml of sodium metaperiodate and 1.0 ml of sodium arsenite solutions (14) were added to the whole reaction mixture.

In applying the Hill equation to allosteric inhibitors, inhibition was assumed to be 100% (20): $\log [V/(V_0 - V)] = \log K - n' \log I$, where V and V_0 refer to initial velocity in the presence and absence of inhibitor, respectively, n' is the interaction coefficient, I is the inhibitor concentration, and K is a constant. Since maximum inhibition by tyrosine was 90%, the $V/(V_0 - V)$ term was modified as follows:

$$\frac{V - 0.1\,V_0}{(V_0 - 0.1\,V_0) - (V - 0.1\,V_0)}, \text{ or } \frac{V - 0.1\,V_0}{V_0 - V}$$

RESULTS

Molecular weight and subunit structure of DAHP synthase. The procedure developed in this investigation consistently afforded tyrosine-inhibitable DAHP synthase of high purity (Table 1). It was found necessary to add PEP to all buffers used in chromatography and to reduce exposure to phosphate after the first chromatographic step.

Gel electrophoresis patterns of enzyme from the pooled fractions of the DAHP affinity column in standard polyacrylamide gel electrophoresis showed that the enzyme had one major band and two very faint bands. After concentration by ultrafiltration, the enzyme showed one sharp band accounting for most of the activity and a broad, faint protein band. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol showed a symmetrical band (Fig. 1), which represented at least 98% of the total protein as shown by densitometer tracings. Amidination of the enzyme with the bifunctional reagent dimethyl suberimidate (7) produced predominately intramolecular crosslinkage. Disc gel electrophoresis in the presence of sodium dodecyl sulfate resolved the modified protein into two bands, the molecular weight of the covalently linked species being twice that of the faster-moving monomer (Fig. 1). Gel filtration on Sephadex G-150 (Fig. 2) indicated an apparent molecular weight of 76,000, whereas sodium dodecyl sulfate-gel electrophoresis showed a subunit molecular

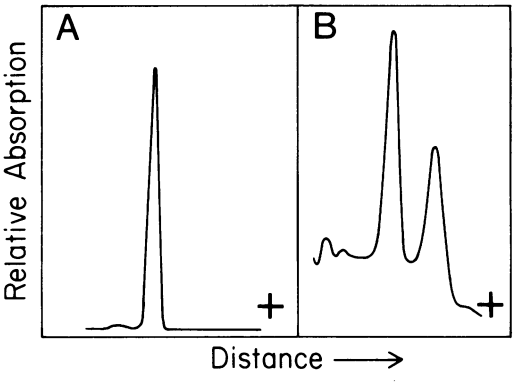

FIG. 1. *Polyacrylamide gel electrophoresis with 5% gel and 0.1% sodium dodecyl sulfate of (A) purified DAHP synthase (tyr) and (B) enzyme treated with dimethyl suberimidate.*

TABLE 1. *Purification of tyrosine-inhibitable DAHP synthase*

Fraction	Vol (ml)	Protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Inhibition by 1.0 mM tyrosine (%)	Yield (%)	Purification (fold)
Crude extract	360	7,100	92,500	13	84	100	
First DE-52	220	240	80,300	335	95	87	26
Tyrosine affinity	100	42	34,500	820	95	37	63
Hydroxyapatite	20	21	25,300	1,180	81	27	91
Second DE-52	33	13	21,200	1,600	89	23	123
DAHP affinity	10	8.6	19,300	2,250	89	21	173

[a] Amount of enzyme catalyzing synthesis of 1 μmol of DAHP per h.

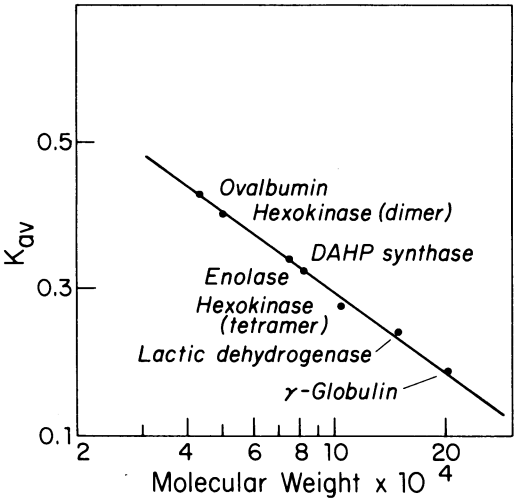

FIG. 2. *Estimation of apparent molecular weight of DAHP synthase (tyr) on Sephadex G-150.*

weight of 40,000 (Fig. 3). Thus the enzyme had an apparent molecular weight of close to 80,000 and was composed of two subunits of identical or very similar molecular weight. Isoelectrofocusing was attempted several times to aid in purifying the enzyme (see Materials and Methods), but it was unstable under our conditions. However, it was possible to show that active DAHP synthase was focused at pH 4.68.

Stability of purified enzyme. The purified enzyme was unstable in the absence of PEP at −20°C, 80% of the initial activity being lost compared with enzyme stored with 1 mM PEP. However, it remained completely active and inhibitable by tyrosine even at room temperature for 24 h if stored in HEPES buffer containing 50% glycerol, 1 mM PEP, and 0.1 mM tyrosine. Under assay conditions, the enzyme was active only in the presence of bovine serum albumin. There was no activity if the enzyme was incubated with bovine serum albumin only or with 1 mM PEP and bovine serum albumin lower than 1 μg/ml. In the presence of PEP, the enzyme was most active and the results were reproducible only when the concentration of bovine serum albumin was higher than 500 μg/ml. Incubation of enzyme for 30 min with PEP at 37°C in HEPES buffer without bovine serum albumin inactivated the enzyme, but addition of 1 mg of bovine serum albumin per ml reactivated it.

Allosteric properties. In the following experiments, careful measurements were carried out to ensure initial velocity conditions. D-Tyrosine, L-tyrosine, L-phenylalanine, L-tryptophan, prephenic acid, chorismic acid, *p*-hydroxyphenylpyruvic acid, and shikimic acid were tested as inhibitors. Only L-tyrosine inhibited the enzyme; all other compounds tested did not inhibit DAHP synthase significantly. At 0.7 mM PEP, 0.6 mM E4P, and 0.1 M HEPES buffer containing 1 mg of bovine serum albumin per ml, the plot of inhibition against tyrosine concentration was sigmoidal. Since maximum inhibition by tyrosine was 90%, a modified Hill equation was used (see Materials and Methods). A logarithmic plot of the modified velocity ratio versus tyrosine concentration gave a line with slope $n' = 1.8$ for DAHP synthase at pH 7.0 (Fig. 4). In the absence or presence of a constant concentration of tyrosine, the enzyme exhibited regular Michaelis-Menten kinetics when either substrate concentration was varied independently. Thus cooperative effects occurred only between inhibitor-binding sites. In double-reciprocal plots of $1/V$ against $1/S$, inhibition by tyrosine was competitive with respect to PEP (Fig. 5) and noncompetitive with respect to E4P (Fig. 6).

Inhibition by PEP analogues. (*Z*)-phosphoenol 3-fluoropyruvate, phosphoenol α-ketobutyrate, fosfomycin, and α-(dihydroxyphosphinyl methyl)-acrylic acid were tested as inhibitors of DAHP synthase at 10^{-2} M (Table 2). Only phosphoenol 3-fluoropyruvate was inhibitory (30% at 10^{-4} M). Inhibition by phosphoenol 3-fluoropyruvate with nonsaturating concentrations of substrates for fixed PEP and variable E4P and for fixed E4P and variable PEP showed, as expected, that this compound

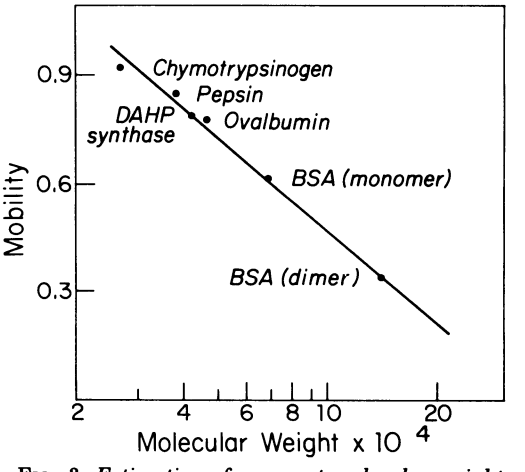

FIG. 3. *Estimation of apparent molecular weight of DAHP synthase (tyr) subunits by sodium dodecyl sulfate-gel electrophoresis.*

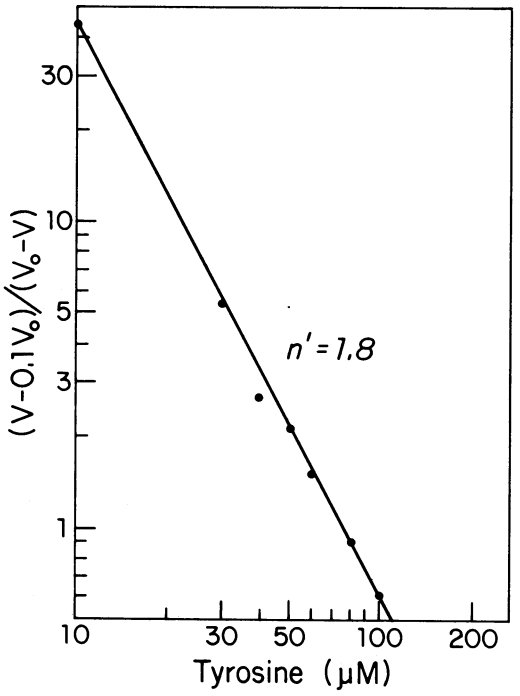

FIG. 4. *Hill equation plot of tyrosine inhibition of DAHP synthase (tyr).*

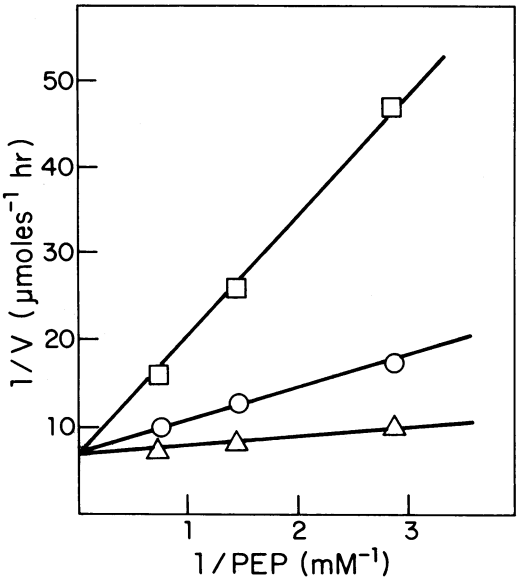

FIG. 5. *Double-reciprocal plots of initial velocity as a function of PEP concentration at 0 (△), 0.03 (○), and 0.05 (□) mM tyrosine.*

was a competitive inhibitor against PEP and noncompetitive against E4P. P. F. Pilch and R. L. Somerville (Fed. Proc. 35:1398, 1976) have recently reported that phosphoenol 3-fluoropyruvate can act as pseudosubstrate in the DAHP synthase reaction.

DISCUSSION

In wild-type *Salmonella*, tyrosine-inhibitable DAHP synthase accounts for approximately 10% of total DAHP synthase activity (11). In the operator-constitutive mutant SG12 grown at 25°C (13), activity of DAHP synthase is twice as high as found at 37°C (10), and activity of DAHP synthase (tyr) is 85% of the total (Table 1). The levels obtained were about 35-fold higher than in wild-type cells grown in minimal medium and approximated those of DAHP synthase (phe) found by Simpson et al. (23) in *Escherichia coli* by incorporating the

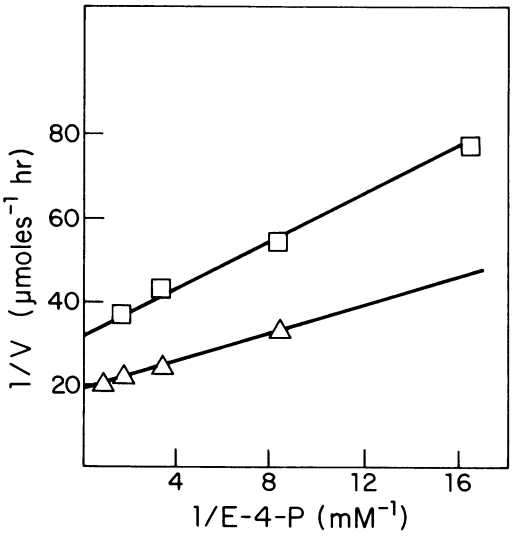

FIG. 6. *Double-reciprocal plots of initial velocity as a function of E4P concentration at 0 (△) and 0.05 (□) mM tyrosine.*

TABLE 2. *Effect of analogues of PEP on DAHP synthase (tyr)*[a]

Inhibitor	Optical density at 549 nm	Inhibition (%)
None	0.169	
(*Z*)-phosphoenol 3-fluoropyruvate	0.031	82
Phosphoenol α-ketobutyrate	0.114	32
Fosfomycin[b]	0.129	24
α-(Dihydroxyphosphinyl methyl)-acrylic acid	0.158	6.4

[a] Pure enzyme (0.1 μg) was added to the regular assay mixture containing PEP and 10 mM inhibitor. The reaction was started by adding E4P.

[b] (−) (1R,2S)-1,2-epoxypropylphosphonic acid.

structural gene for the phenylalanine isoenzyme into the genome of specialized transducing phage lambda.

We used the procedure described previously (10) as a starting point for purifying DAHP synthase (tyr) to homogeneity. We found that the use of phosphate buffer beyond the first column chromatography resulted in appreciable loss of inhibition by tyrosine. Efforts to restore this inhibition failed. These included addition of sulfhydryl reagents, ethylenediaminetetraacetic acid, Co^{2+}, and aromatic amino acids and changes in buffer, ionic strength, temperature, and pH. Frozen insensitive enzyme lost catalytic activity on repeated thawing. Since lost activity could be restored by incubation with PEP and tyrosine, PEP was added to all buffers during purification. After the first step, HEPES buffer was substituted for phosphate buffer in all steps except in chromatography on hydroxyapatite. Although a small loss of sensitivity to tyrosine occurred in this step, some of the insensitive enzyme was separated in the subsequent step on DE-52. Specific activity of the purest preparations was twice as high as reported previously (10) and essentially equal to that reported by Simpson et al. (23) for DAHP synthase (phe) from *E. coli*. The purification procedure represented a 4,500-fold increase in specific activity over wild-type levels.

In standard polyacrylamide gel electrophoresis at pH 9.5, the purest preparations of enzyme showed two very faint bands in addition to the major band. However, 98% of total protein was banded at molecular weight 40,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis at pH 7.2. Concentration of the enzyme by ultrafiltration resulted in considerable changes in the band pattern in standard gel electrophoresis. The apparent nonhomogeneity observed in standard gel electrophoresis, especially after concentration by ultrafiltration, may be due to a change in quaternary structure of the enzyme. The enzyme reacted with dimethyl suberimidate to give two bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the faster-moving band having a molecular weight about one-half that of the slow-moving band. Since an apparent molecular weight by gel filtration of about 76,000 was shown by the native enzyme, it would appear to consist of two similar or identical subunits.

DAHP synthase (tyr) showed cooperative kinetics for feedback inhibition by tyrosine, with an interaction coefficient of 1.8, indicating positive cooperative interaction between tyrosine-binding sites. Inhibition by tyrosine was competitive with PEP and noncompetitive with E4P, as was inhibition by the analogue 3-fluoro-PEP. The role of PEP resembles that of a positive effector. It reactivates inactive enzyme, binds with a dissociation constant of 4.7 μM (10) (probably lower than intracellular levels of PEP), and interacts competitively with respect to the allosteric inhibitor tyrosine to modulate the activity of the enzyme.

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