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PURIFICATION AND PROPERTIES OF TYROSINE-SENSITIVE 3-DEOXY-D-ARABINO-HEPTOLOSONATE-7-PHOSPHATE SYNTHETASE OF *ESCHERICHIA COLI* K12

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Summary

1. The tyrosine-sensitive allosteric first enzyme of the aromatic amino acid biosynthetic pathway, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase (7-Phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose 4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15.) has been purified from a mutant strain of *Escherichia coli*.

2. The enzyme activity was inhibited to 50% at $2 \cdot 10^{-5}$ M tyrosine and to 90% at $2 \cdot 10^{-4}$ M tyrosine concentration. At tyrosine concentrations lower than $2 \cdot 10^{-5}$ M a cooperative interaction between tyrosine binding sites was observed.

3. Co^{2+} increased the enzyme activity about 2—2.5-fold. The presence of Co^{2+} ions stabilized the enzyme. EDTA inhibited the enzyme activity, and this inhibition was reversed by Co^{2+}. Tyrosine-sensitive DAHP synthetase seems to be a metal containing enzyme.

4. Kinetic experiments were carried out to study the catalytic action. Contrary to earlier suggestions it is concluded, that the reaction mechanism appears to be more complex — with either the ping-pong or sequential type predominating, depending on conditions.

Introduction

3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (7-Phospho-2-keto-3-deoxy-D-*arabino*-heptonate-D-erythrose 4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15) is the allosteric first enzyme of the aromatic amino acid biosynthetic pathway in bacteria, which catalyzes the formation of 3-deoxy-D-

Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate

arabino-heptulosonate 7-phosphate and P_i from phosphoenolpyruvate and erythrose 4-phosphate [1].

Three distinct isoenzymes (arom 1a, arom 1b and arom 1c) were detected in *Escherichia coli* [2—4], all of them catalyzing the same reaction. The enzyme activity of arom 1a, arom 1b and arom 1c is under the feedback control of the aromatic end-products, L-phenylalanine, L-tyrosine and L-tryptophan, respectively.

Kinetic properties of DAHP synthetase isoenzyme have been studied previously [5—7]. On the basis of kinetic measurements two types of reaction mechanisms were suggested: ping-pong and sequential mechanisms. The data presented in literature concerning the effect of EDTA and Co^{2+} on enzyme activity are also different and often conflicting [7—9].

In this paper we describe the purification of tyrosine-sensitive DAHP synthetase from a mutant strain of *E. coli* HfrH, some properties of the purified enzyme, and kinetic experiments concerning the mechanism of action of the purified enzyme.

Materials and methods

Organism and growth conditions

A deletion mutant strain of *E. coli* HfrH was used. In the strain H80c, the deletion involves the Gal operon, Aro G and Nad A loci [10]. We have found that it requires a high concentration of iron for growth. $H80cR_3$ was selected from this mutant; and it contains the tyrosine-sensitive DAHP synthetase on a highly derepressed level (the enzyme was constitutively derepressed about 25-fold). This strain was a good source of the tyrosine-sensitive DAHP synthetase, because it did not synthetise the phenylalanine-sensitive enzyme at all, and the third enzyme was present in a very low concentration.

Cells were grown on minimal medium A [11] containing 2 g yeast extract, 2 g casein hydrolysate and 0.1 g L-tryptophan in 1 l, and it was supplemented with $5 \cdot 10^{-6}$ M $FeCl_3$, 0.0002% B_1 vitamin, 0.001% nicotinic acid and 0.2% glucose. The cultures were grown at 37° C on a New Brunswick gyrotory shaker at 180 rev./min. Cells were harvested in the early stationary phase by centrifugation at 2° C. The sedimented cells were washed twice with 0.1 M potassium phosphate buffer (pH 7) by centrifugation and stored at —20° C until used.

Purification of tyrosine-sensitive DAHP synthetase

Step 1, Preparation of crude extract. Frozen cells (13—14 g) were suspended in 100 ml 0.1 M potassium phosphate buffer (pH 7) containing 1 mM mercaptoethanol and 0.1 mM Co^{2+}, and were disintegrated by a 3 min sonic treatment (M.S.E. 100 watt ultrasonic disintegrator). Cell debris was removed by centrifugation at 40 000 × *g* for 30 min at 2° C.

Step 2, Fractionation with $(NH_4)_2SO_4$. Ammonium sulfate solution saturated at 0° C and adjusted to pH 7 with ammonium hydroxide, was prepared. Precipitation was made at 35—55% saturation (with 0.1 mM phospho*enol*pyruvate, 1 mM mercaptoethanol and 0.1 M Co^{2+} in the solution. The precipitate was dissolved in 15—20 ml of 0.01 M potassium phosphate buffer (pH 6.8) con-

taining 1 mM mercaptoethanol and 0.1 mM Co^{2+}, and was dialysed overnight against the same buffer.

Step 3, Adsorption to and elution from calcium phosphate gel. Calcium phosphate gel was washed with 0.01 M potassium phosphate buffer (pH 6.8) containing mercaptoethanol and Co^{2+} as indicated before. 10 ml of the gel suspension (20 mg dry weight per ml) was added to 1 ml of dialysed protein solution. The suspension was stirred for 15 min and sedimented by centrifugation at 2° C. The gel was washed with 20 ml of buffer described above and centrifuged. The enzyme was eluted with successive 20 ml portions of 0.04 M potassium phosphate buffer (pH 7) containing mercaptoethanol and Co^{2+} as indicated before. The active fractions were collected and concentrated to about 10—20 ml by ultrafiltration (in a Diaflo ultrafiltration cell).

Step 4, DEAE-cellulose chromatography. DEAE-cellulose was equilibrated with 0.01 M potassium phosphate buffer (pH 7) containing mercaptoethanol and Co^{2+}. The enzyme-containing solution was adsorbed on a DE 32 column (1.5 × 7 cm) at 50 ml per h. After washing with 5 bed volumes of the same buffer, the enzyme was eluted with a linear gradient prepared from 90 ml of 0.01 M potassium phosphate buffer (pH 7) and 90 ml of 0.8 M potassium phosphate buffer (pH 7), each of them containing 1 mM mercaptoethanol and 0.1 mM Co^{2+}. Fractions of 2.5 ml were collected and tested for enzyme activity. A typical purification is summarized in Table I, and the elution profile of DEAE-cellulose chromatography is shown in Fig. 1.

Enzyme assay

The activity of the enzyme was determined by measuring the amount of DAHP formed [1].

Assay 1: During the preparation and in crude extracts the following standard reaction mixture was used: 100 μmol of potassium phosphate buffer (pH 7), 1 μmol of mercaptoethanol, 0.1 μmol of Co^{2+}, 0.5 μmol of phospho*enol*pyruvate and a rate-limiting amount of enzyme. These were preincubated for 10 min at 24° C. The reaction was initiated by the addition of 0.5 μmol of erythrose

TABLE I

SUMMARY OF PURIFICATION OF TYROSINE-SENSITIVE DAHP SYNTHETASE FROM *ESCHERICHIA COLI* H80cR_3

The activity of the enzyme was determined as it is described in Assay 1 in Materials and Methods.

Step	Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1	Crude extract	88	781	3040	3.9	100	
2	$(NH_4)_2SO_4$ fractionation 35—55%	21	399	3410	8.5	112	2
3	Calcium phosphate gel adsorption	17.7	33	934	28.2	30.7	7
4	DEAE-cellulose chromatography	2.5	1.3	175	136.5	6	35

4-phosphate (final volume of the reaction mixture: 1 ml). After 10 min at 24° C the reaction was stopped by the addition of 0.4 ml of 10% trichloroacetic acid and the precipitated protein was removed by centrifugation. The amount of DAHP formed was determined by the thiobarbituric acid method [1]. Molar extinction coefficient $E_{550\ nm} = 3.34 \cdot 10^4$ was used to calculate the amount of DAHP [3].

One unit of enzyme was defined as the amount that catalyzes the formation of 0.1 μmol of DAHP in 5 min. Specific activity was expressed as units per mg of protein.

Assay 2: The activity of the purified enzyme was determined as in Assay 1, with the difference that the reaction was started by the addition of the enzyme, and was measured at 37° C.

In determination of initial velocities the unit of initial rate of reaction, v_o, was defined as 1 μmol DAHP formed per 10 min per 1 mg of enzyme. The unit of initial velocity of the reaction in the presence of inhibitor, v_i, was defined in the same way.

Calcium phosphate gel was prepared by the method of Keilin and Hartree [12].

Protein was determined according to the method of Lowry et al. [13] using crystalline bovine serum albumin as a standard.

P_i was determined by the method of Eibl and Lands [14].

Polyacrylamide gel electrophoresis was conducted according to the method of Davis [15].

Chemicals

Monocyclohexylammonium phospho*enol*pyruvate was prepared according to the method of Clark and Kirby [16]. Cyclohexylammonium ion was exchanged for potassium ion by ion-exchange chromatography on Dowex 50x4 resin. D-erythrose 4-phosphate was prepared from D-glucose 6-phosphate according to the method of Ballou [17] and purified by ion-exchange chromatography [18].

Yeast extract and casein hydrolyzate were obtained from Difco laboratories, L-tryptophan, L-phenylalanine, L-tyrosine, vitamin B_1, HEPES and Triton X-100 were obtained from Sigma, thiobarbituric acid was purchased from Fluka, and DEAE-cellulose was Whatman DE 32.

All other reagents and chemicals were obtained from Reanal.

Results

Properties of the purified enzyme

The enzyme preparation obtained by the method described in Materials and Methods was partially purified. After pre-incubation with 1 mM tyrosine, a sample was scrutinized for purity by gel electrophoresis on a 7% polyacrylamide gel [15], in the presence of 1 mM tyrosine. One major and several faint bands could be detected on the gel.

As seen from Fig. 2a, $2 \cdot 10^{-5}$ M L-tyrosine caused 50% inhibition of enzyme activity, and 90% inhibition was obtained at $2 \cdot 10^{-4}$ M L-tyrosine concentration. L-phenylalanine in the same concentration range did not inhibit the enzyme activity at all, as it could be expected. The plot of inhibition, $i = 1 - v_i/v_o$, against tyrosine concentration appears to be sigmoidal.

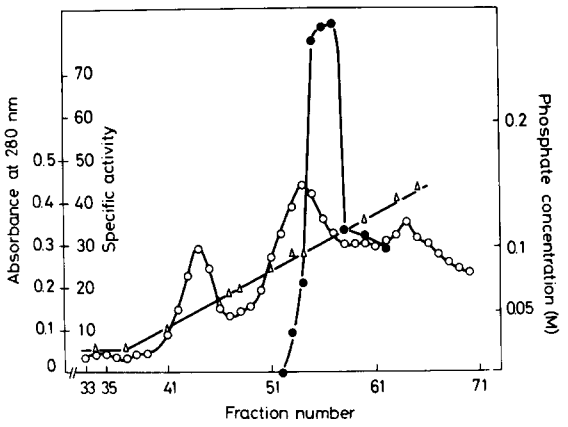


Fig. 1. Chromatography of the partially purified tyrosine-sensitive DAHP synthetase on a DEAE-cellulose column. A sample obtained in Step 3 (12 ml, concentration: 3.8 mg/ml) was applied to the column (1.5 × 7 cm) of Whatman DE 32 cellulose, equilibrated with 0.01 M potassium phosphate buffer (pH 7) containing 1 mM mercaptoethanol and 0.1 mM Co^{2+}. The enzyme was eluted with a linear potassium phosphate gradient at pH 7. 2.5-ml fractions were collected and tested for enzyme activity. ●-●-●, specific activity; ○-○-○, absorbance at 280 nm; △-△-△, phosphate gradient.

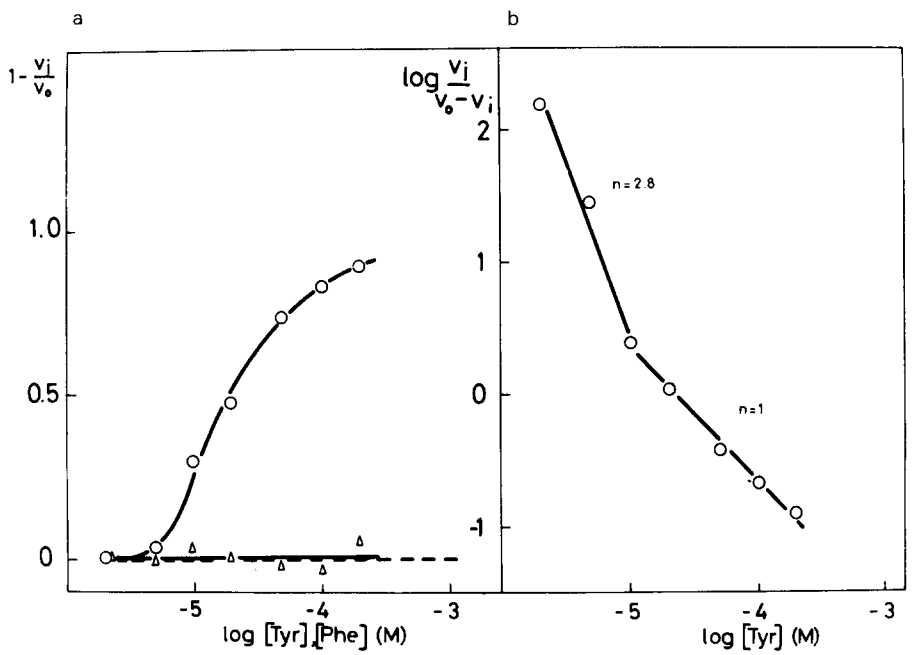


Fig. 2(a) Effect of tyrosine and phenylalanine on the activity of the purified enzyme. The standard reaction mixture (described in Assay 1 in Materials and Methods) contained 25 μg of protein and tyrosine or phenylalanine as indicated. v_0 and v_i refer to the initial velocity in the absence and presence of inhibitor. The unit of initial velocity was defined as 1 μmol DAHP formed per 10 min per 1 mg of enzyme. (b) Hill-plot of tyrosine inhibition. Data are the same as in Fig. 2a.

In Fig. 2b a plot of these data is shown in terms of the Hill equation [19]. The slope of the line is different below and above 50% inhibition. At lower inhibitor concentrations $n = 2.8$, suggesting cooperative interactions of tyrosine binding sites on the enzyme. At higher tyrosine concentrations, however, $n = 1$.

In the presence of 0.1 mM Co^{2+} enzyme activity is increased about 2—2.5-fold. Similar results were reported earlier [5,6,20,21]. Moreover, the enzyme is stabilized by the presence of Co^{2+}, as it is shown in Fig. 3. The horizontal line on the figure represents the control activity measured in the absence of Co^{2+} and at 112 μg/ml protein concentration. Curve 1 was measured at 37 μg/ml protein concentration in the presence of increasing amounts of Co^{2+} ions. Considering that all the data presented in the figure are calculated as the amount of DAHP formed by 1 mg protein in 10 min, all the points of curve 1 should be above the horizontal line (if Co^{2+} activates the enzyme) or on the line (if Co^{2+} does not influence the activity). The lower activity obtained shows that the decrease of the protein concentration presumably caused denaturation. As it is seen in curve 2, this denaturation van be overcome by preincubation of the enzyme with both Co^{2+} and phospho*enol*pyruvate (samples of curve 1 were preincubated with phospho*enol*pyruvate only). On increasing the protein concentration to 56 μg/ml, the activity is higher than that of the control even in the samples preincubated with phospho*enol*pyruvate only (curves 3 and 4). At 75 μg/ml protein concentration the difference between the two curves (5 and 6) disappears and at 0.2 mM Co^{2+} the enzyme activity is about 2.4-fold higher than that of the control.

It was previously found that EDTA inactivates DAHP synthetase and the inactivation is reversible [6,8,20]. According to other investigations DAHP synthetase was unaffected by EDTA [1,7]. In our experiments 0.1 mM EDTA caused 80% inactivation of enzyme activity under the conditions of Assay 1 (see Materials and Methods). After EDTA treatment activity can be restored by adding 0.2 mM Co^{2+} to the inactive enzyme.

Substrate saturation and initial velocity measurements

The presence of phosphate stabilized the enzyme and ensured the appropriate ionic strength at pH 7, therefore substrate saturation and initial velocity were measured in 0.1 M potassium phosphate buffer.

The dependence of initial velocity of DAHP formation on phospho*enol*pyruvate concentration (Fig. 4) and on erythrose 4-phosphate concentration (Fig. 5) in the presence of different fixed concentrations of erythrose 4-phosphate and phospho*enol*pyruvate, respectively, follows the regular Michaelis-Menten kinetics. Therefore under such conditions no kinetic evidence for subunit interaction can be detected in the substrate binding process.

As shown in Fig. 4, the Lineweaver-Burk plot of the data (inset) gives a set of parallel lines. Such initial velocity pattern can be obtained, if the reaction mechanism is ping-pong, and it can be described by the equation:

$$\frac{1}{v} = \frac{K_{m_a}}{V}\left[\frac{1}{A}\right] + \frac{1}{V}\left[1 + \frac{K_{m_b}}{B}\right] \quad (1)$$

where V is the maximal velocity of the reaction, A and B are the concentrations of varied and fixed substrates, respectively, and K_{m_a} and K_{m_b} are the Mich-

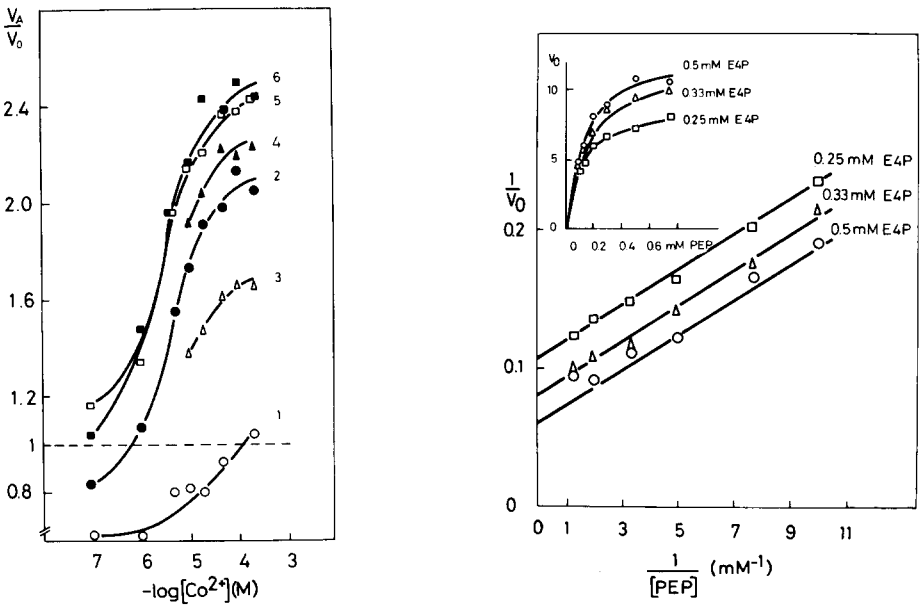


Fig. 3. Effect of Co^{2+} ions on the enzyme activity at different protein concentrations. The reaction mixture contained 100 μmol of potassium phosphate buffer (pH 7), 1 μmol of mercaptoethanol, 0.5 μmol of phospho*enol*pyruvate, 0.5 μmol of erythrose 4-phosphate, 37 μg protein at curves 1 and 2, 56 μg protein at curves 3 and 4, 75 μg protein at curves 5 and 6, and Co^{2+} as indicated in a volume of 1 ml. The samples of curves 1, 3 and 5 were preincubated with phospho*enol*pyruvate at 24° C. After 10 min Co^{2+} and erythrose 4-phosphate were added to the mixtures. The samples of curves 2, 4 and 6 were preincubated with phospho*enol*pyruvate and Co^{2+} for 10 min at 24° C, then erythrose 4-phosphate was added to initiate the reaction. v_A and v_0 are the initial velocities in the presence and absence of Co^{2+}, respectively, and are expressed as in Fig. 2a.

Fig. 4. The double reciprocal plot of initial velocity against phospho*enol*pyruvate concentration at different fixed erythrose 4-phosphate concentrations. The standard reaction mixture described in Assay 2. (see Materials and Methods) contained 38 μg protein and substrates as indicated. v_0 is expressed as in Fig. 2a.

aelis constants of the two substrates [22]. This type of reaction mechanism was observed previously by many authors [5,6,9,23].

The double reciprocal plot of the data of Fig. 5 gives intersecting lines (see inset). This result seems to indicate a sequential reaction given by the equation:

$$\frac{1}{v} = \frac{K_{m_a}}{V}\left[1 + \frac{K_{ia}K_{m_b}}{K_{m_a}B}\right]\frac{1}{A} + \frac{1}{V}\left[1 + \frac{K_{m_b}}{B}\right] \qquad (2)$$

where K_{ia} is the dissociation constant of the enzyme · A complex. Similar results were found by DeLeo, Dayan and Sprinson [7], but they could obtain intersecting lines only when they decreased the substrate concentrations approximately 10-fold. Nagano and Zalkin also reported [9], that in the Lineweaver-Burk plot no unequivocally parallel lines were obtained when $1/v_0$ was plotted against 1/erythrose 4-phosphate concentration.

The contradiction between the two results (ping-pong and sequential) can be resolved, if we consider that the difference between eqns. 1 and 2 is the pres-

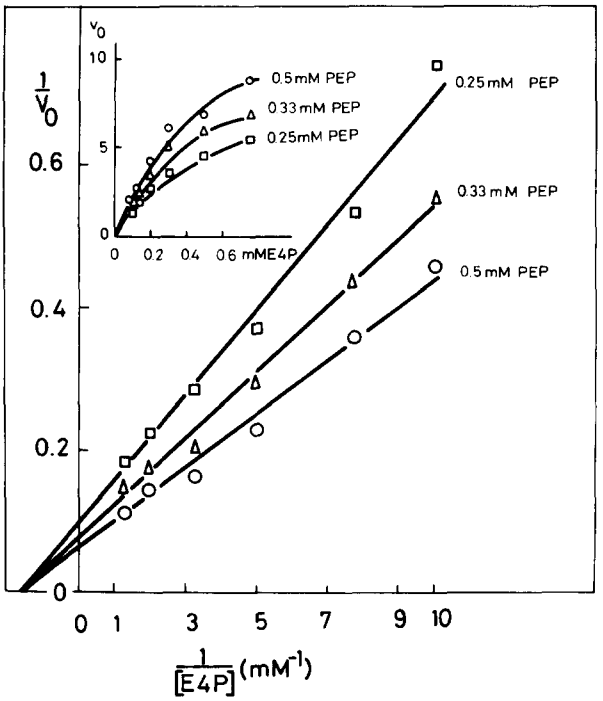


Fig. 5. The double reciprocal plot of initial velocity against erythrose 4-phosphate concentration at different fixed phospho*enol*pyruvate concentrations. The reaction mixture was the same as in Fig. 4.

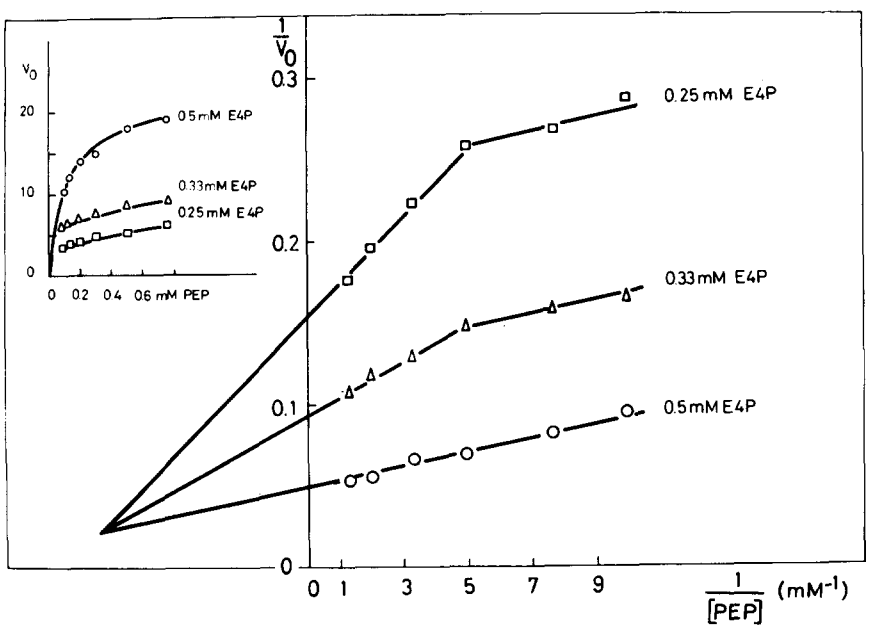


Fig. 6. The double reciprocal plot of initial velocity of DAHP formation against phospho*enol*pyruvate concentration at different fixed concentrations of erythrose 4-phosphate in HEPES buffer. The reaction mixture was the same as in Fig. 4, except that it contained 100 μmol of HEPES buffer (pH 7.2) instead of 100 μmol of potassium phosphate buffer (pH 7).

ence of the term $K_{ia}K_{m_b}/K_{m_a}B$ in the case of sequential reaction mechanism. When this ratio is small (i.e. the dissociation constant of the enzyme-first substrate complex is small, eqn. 2 approximates eqn. 1 and nearly parallel or parallel lines can be detected. Arranging the equation for the other substrate the ratio can not be neglected and intersecting lines can be obtained.

Measuring the dependence of initial velocity on substrate concentration in phosphate buffer, kinetic constants can not be calculated because of inhibition by P_i, the first product of enzyme action. Therefore the buffer was changed after the purification procedure using a Diaflo ultrafiltration system, and initial velocity measurements were made in 0.1 M HEPES buffer (pH 7.2) containing 1 mM mercaptoethanol and 0.1 mM Co^{2+}.

The plot of the reciprocal of the initial velocity of DAHP formation against the reciprocal of phospho*enol*pyruvate concentration at three different fixed concentrations of erythrose 4-phosphate gives a straight line only at the saturating concentration of the fixed substrate, while at lower concentrations of erythrose 4-phosphate it gives broken lines as shown in Fig. 6.

The plot of initial velocity of DAHP formation against the phospho*enol*pyruvate concentration at three fixed concentrations of erythrose 4-phosphate can be seen in Fig. 6a. Michaelis-Menten kinetics can be obtained only at the saturating concentration of erythrose 4-phosphate. If its concentration is below 0.5 mM, there is a deviation from the Michaelis-Menten kinetics, suggesting the probability of cooperative interaction between substrate binding sites at low concentration of phospho*enol*pyruvate. This effect can not be observed in phosphate buffer (see also Figs. 4 and 5, inset).

The effect of P_i on the initial velocity is presented in Fig. 7. With the enzyme originally in HEPES buffer, Michaelis-Menten kinetics can be measured even at a non-saturating erythrose 4-phosphate concentration when P_i is added to the

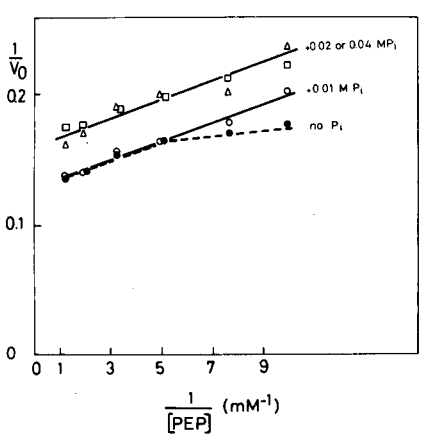


Fig. 7. Effect of P_i on the plot of initial velocity of the enzyme reaction against phospho*enol*pyruvate concentration at non-saturating erythrose 4-phosphate concentration. The reaction mixture contained 100 μmol of HEPES buffer (pH 7.2), 1 μmol of mercaptoethanol, 0.1 μmol of Co^{2+}, 33 μg of protein, 0.33 μmol of erythrose 4-phosphate, and phosphate and phospho*enol*pyruvate as indicated in a volume of 1 ml. v_0 is expressed as in Fig. 2a.

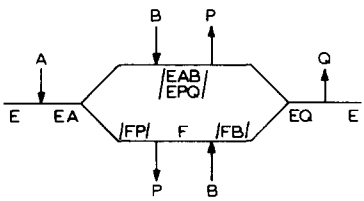
reaction mixture in 0.01 M concentration. Increasing the concentration of P_i to 0.04 M the value of the apparent V decreases, but the slope of the line does not change. Further increase of P_i concentration may cause undesirable increase of ionic strength.

Discussion

In some microorganisms (e.g. in *E. coli* [2—4], in *Salmonella typhimurium* [24,25] and in *Neurospora crassa* [26]) three DAHP synthetase isoenzymes catalyzing the same reaction were found, differing, however, in many other respects, first of all in type and sensitivity for feedback control, metal content, requirement for Co^{2+} ions, etc.

The experimental data concerning the role of Co^{2+} ions are contradictory. In the case when the enzyme activity was not influenced by EDTA, the DAHP synthetase was either independent of Co^{2+} for activity, or the presence of Co^{2+} ions inhibited the enzyme [1,7]. In other experiments Co^{2+} ions were reported to increase the activity about 2-fold [5,6,20,21], or an absolute requirement was found for Co^{2+} [9,27]. Our experimental data show that the presence of 0.1 mM Co^{2+} causes 2—2.5 fold increase in enzyme activity, and the activating effect of Co^{2+} depends upon protein concentration. This observation allows some conclusions about the possible role of Co^{2+}. On the basis of molecular weight measurements [5] it is supposed that the DAHP synthetase of *E. coli* is a tetrameric enzyme. The structure of the protein molecule appears to be very labile. As is shown in Fig. 3, dilution of the protein may cause denaturation, but the activity of the enzyme is restored by preincubation of diluted samples with Co^{2+}. Co^{2+} ions bound to the enzyme stabilize the oligomeric structure, presumably inhibiting the dissociation or unfolding of the molecule.

Conclusions supporting either the ping-pong [5,6,9] or the sequential [7] type of reaction medhanism have been drawn mainly on the basis of kinetic experiments. We suppose that theoretically the enzyme can act by the ping-pong or by the sequential mechanism with equal probability. The reaction of the enzyme may be the following:



where E is enzyme, F is a pyruvyl-enzyme intermedier, A is phosphoenolpyruvate, B is erythrose 4-phosphate, P is inorganic phosphate and Q is DAHP.

Which reaction mechanism predominates may depend on conditions. For example, the presence of inorganic phosphate can influence the reaction so that it shifts the catalytic action towards the sequential mechanism. At a high concentration of the first product, binding of the second substrate (erythrose 4-phosphate) is more likely than the cleavage of P_i (see Figs. 4 and 5). The small dissociation constant of the enzyme-phospho*enol*pyruvate complex, the concentration ratio of the substrates and the probability of cooperative interactions

between substrate binding sites at low substrate concentrations may also have an important role, as can be seen in Figs. 6 and 6a. On the basis of these experimental data it is concluded that the reaction of DAHP synthetase seems to be more complex than was proposed in earlier literature.

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