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SOME REGULATORY PROPERTIES OF PHOSPHO-2-KETO-3-DEOXYHEPTONATE ALDOLASE FROM THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS**

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SUMMARY

1. The activity of phospho-2-keto-3-deoxyheptonate aldolase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, also known as DAHP synthetase) in crude extracts of the blue-green alga *Anacystis nidulans* was feedback inhibited by L-tyrosine and L-phenylalanine. At a concentration of 2 mM tyrosine produced greater than 90% inhibition; phenylalanine usually produced 10–20% inhibition. On a molar basis tyrosine was at least 100 times more inhibitory than phenylalanine.

2. Partial purification of the enzymatic activity by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 filtration failed to resolve isoenzymes. The 14-fold purified preparation retained full sensitivity to both tyrosine and phenylalanine.

3. Kinetic studies carried out with the partially purified enzyme suggest that the presence of tyrosine promotes cooperative interactions in the binding of molecules of phosphoenolpyruvate but only weakly affects the binding of D-erythrose 4-phosphate.

4. Inhibition by tyrosine appears to be of the mixed type with regard to both substrates.

INTRODUCTION

Studies concerned with comparative aspects of feedback control of branched biosynthetic pathways have revealed that identical biochemical sequences in different organisms are often subject to different patterns of regulation[1]. The aromatic amino acid pathway provides an excellent example of this regulatory diversity[2]. The first step unique to the synthesis of phenylalanine, tyrosine and tryptophan is the condensation of phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate to give 3-

Abbreviations: PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

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deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) and inorganic phosphate. The reaction is catalyzed by the enzyme phospho-2-keto-3-deoxyheptonate aldolase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, also known as DAHP synthetase). In a number of enteric bacteria[3,4] and fungi[4,5] this enzyme exists in multiple molecular forms each of which is under the feedback control of a different endproduct. For example, *Escherichia coli*[3,6] possesses three phospho-2-keto-3-deoxyheptonate aldolases, one of which is inhibited by tyrosine, a second by phenylalanine and the third by tryptophan. In *Bacillus subtilis*, on the other hand, Jensen and Nester[7,8] have demonstrated the existence of a single phospho-2-keto-3-deoxyheptonate aldolase. The activity of this enzyme is not affected by any of the endproducts but is inhibited by two intermediates, chorismate and prephenate. Little information is presently available concerning phospho-2-keto-3-deoxyheptonate aldolase from photosynthetic organisms. In a survey of a number of green and blue-green algae Weber and Böck[9] reported that phospho-2-keto-3-deoxyheptonate aldolase in crude extracts of *Anacystis nidulans* was feedback inhibited by tyrosine and phenylalanine. In addition they indicated that the kinetic properties of the enzyme were similar to those exhibited by phospho-2-keto-3-deoxyheptonate aldolases from other organisms. In the present communication we present evidence that phospho-2-keto-3-deoxyheptonate aldolase of *A. nidulans* is a single enzyme and describe some of the regulatory properties of the partially purified activity.

MATERIALS AND METHODS

Organism

The blue-green alga *A. nidulans* No. 625 was obtained from the Indiana University Culture Collection[10] and had been purified prior to this study.

Media

The composition of the minimal medium used in this study is presented in Table I. Usually improved enzymatic activity could be obtained if the amount of nitrate was reduced so as to be exhausted by the end of the growth period. Presum-

TABLE I

COMPOSITION OF GROWTH MEDIA

The medium was made up in distilled water. The minimal medium had pH 7.5.

Minimal medium		*Trace elements*	
Constituent	*Concn (g/l)*	*Constituent*	*Concn (g/100 ml)*
$NaNO_3$	0.1–1.0	H_3BO_3	0.1
NaCl	0.5	$MnCl_2 \cdot 4H_2O$	0.2
$MgSO_4 \cdot 7H_2O$	0.15	$ZnSO_4 \cdot 7H_2O$	0.05
Sodium citrate	0.10	$CoCl_2 \cdot 6H_2O$	0.05
$FeSO_4 \cdot 6H_2O$	0.007	$CuSO_4 \cdot 5H_2O$	0.05
KH_2PO_4	0.4	$VOSO_4 \cdot 2H_2O$	0.005
$K_2HPO_4 \cdot 3H_2O$	3.5	$Na_2MoO_4 \cdot 2H_2O$	0.1
Trace elements	1.0 ml	$CaCl_2 \cdot 2H_2O$	1.5

ably, the onset of nitrogen starvation engendered some derepression of enzyme synthesis. Consequently the amount of nitrate used varied depending upon the size of the inoculum but was usually between 0.1 and 0.25 g/l. One g/l was used in the maintenance of stock cultures.

Growth of cells

Cultures were grown in either Erlenmeyer or Fernback flasks at 37 °C under constant illumination provided by cool white fluorescent lights. In addition they were gassed with sterile air and agitated with magnetic stirrers. Cultures were inoculated with a one-hundredth volume portion of a fully grown starter culture and allowed to grow to early stationary phase (6 days). Immediately prior to harvesting samples of all cultures were streaked onto a complex medium to check for bacterial contamination. Cultures which gave positive results were discarded.

Preparation of extracts for phospho-2-keto-3-deoxyheptonate aldolase

Cells were harvested by centrifugation and washed twice with 0.02 M potassium phosphate (pH 7.0) *plus* 1.0 mM $MgCl_2$. The pellet was resuspended in 0.04 M potassium phosphate (pH 7.0) *plus* 1.0 mM $MgCl_2$ at a concentration of 4.0 ml/g of wet packed cells. An equal volume of glass beads (0.11 mm) was added and the cells were disrupted in a Bronwill MSK cell homogenizer for about one minute. Cooling was provided by CO_2. The resulting homogenate was centrifuged (0–5 °C) for 5 min at 5000 rev./min to remove the glass beads. The supernatant was centrifuged for 20–30 min at 15 000 rev./min. The second supernatant was retained as a crude enzyme preparation. Prior to assay crude extracts were either filtered through a column of Sephadex G-25 equilibrated with 0.04 M potassium phosphate (pH 7.0) or dialyzed against the same buffer. Crude extracts were stored frozen (−15 °C) and used within one week.

Enzyme assay

Phospho-2-keto-3-deoxyheptonate aldolase was assayed according to the procedure of Srinivasan and Sprinson[11] as modified by Smith *et al.*[3]. The reaction mixture contained: potassium phosphate (pH 6.4), 50 μmoles; phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate, 0.5 μmole each (except when indicated otherwise) and enzyme in a total volume of 0.5 ml. The reaction was initiated by the addition of enzyme and terminated after 10 or 15 min at 37 °C by the addition of 0.1 ml of 10% trichloroacetic acid. The reaction velocity was linear with time for 25 min and to an absorbance of about 0.5. In the kinetic experiments the concentration of the fixed substrate was increased to 1.0 μmole (2 mM) in order to ensure saturation of the enzyme. Control tubes contained all of the components of the reaction mixture except that the enzyme was added after the trichloroacetic acid. Compounds examined as inhibitors were tested for interference in the assay for DAHP by adding them to a reaction mixture after the addition of the trichloroacetic acid. The amount of DAHP formed was calculated by the use of $4.5 \cdot 10^4$ as the molar extinction coefficient for DAHP at 549 nm[8]. One unit of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 nmole of DAHP per min. Specific activity is expressed as units/mg protein.

Protein determination

Protein was determined by a slight modification of the method of Lowry *et al.*[12]. Incubation of the samples with the copper reagent was for 30 min at 37 °C and with the phenol reagent for 20 min at room temperature. Crystalline bovine serum albumin was used as a standard. Photosynthetic pigments were not removed prior to determinations. Extracts were diluted sufficiently to reduce color to a minimum. In addition the alkaline copper reagent usually destroyed what little pigment remained. The absorbance of the final product was read at 750 nm. At this wavelength pigment absorption is low. Protein concentrations in column eluates were estimated by measuring the absorbance at 280 nm on a Coleman 124 spectrophotometer.

Purification of phospho-2-keto-3-deoxyheptonate aldolase

(I) $(NH_4)_2SO_4$ fractionation. Crude extracts were prepared as described above except that the cells were disrupted in 0.04 M potassium phosphate (pH 6.8) *plus* 0.5 mM EDTA. For 10.5 g of wet packed cells, the crude extract was diluted to a volume of 80.0 ml (protein concentration about 13.0 mg/ml) with the same buffer. All of the following operations were performed at 0–5 °C. 2 ml of a 2.0% protamine sulfate solution (made up in the buffer described above) was added dropwise with continuous slow stirring. Stirring was continued for 20 min, and then the precipitate was removed by centrifugation. To the resulting supernatant (78 ml) 12.8 g of solid $(NH_4)_2SO_4$ was added (30% saturation). The pH was monitored after half and complete addition of the $(NH_4)_2SO_4$ and adjusted to 6.8 with KOH if necessary. The same procedure was used to obtain cuts at 50% saturation (9.7 g of $(NH_4)_2SO_4$) and 65% saturation (7.7 g). The precipitates were dissolved in 0.04 M potassium phosphate (pH 6.8) and dialyzed against the same buffer for 6 h. Enzymatic activity was recovered in the 30–50% fraction.

(II) Sephadex G-100 filtration. A Sephadex G-100 column 2.4 cm × 53 cm was prepared according to the method of Andrews[13] and equilibrated with 0.04 M potassium phosphate (pH 6.8). 8–9 ml of a 30–50% $(NH_4)_2SO_4$ fraction was carefully layered on top of the gel. Elution was begun after the sample had entered the gel. Fractions of 2–3 ml were collected.

Chemicals

Potassium phosphoenolpyruvate, sodium D-erythrose 4-phosphate (75%), barium chorismate (60%), barium prephenate (75%) shikimic acid, L-tyrosine, L-tryptophan and L-phenylalanine were obtained from Sigma Chemical Co. Sephadex was purchased from Pharmacia. Other reagents were obtained from Fisher or Eastman. Barium prephenate and barium chorismate were converted to the potassium salts by the addition of excess K_2SO_4 and removal of the $BaSO_4$ precipitate by centrifugation. Weights of substrates used in enzyme assays were corrected according to the purity stated by the supplier.

RESULTS

Feedback inhibition of phospho-2-keto-3-deoxyheptonate aldolase activity in crude extracts

An initial survey was conducted to determine which metabolites of the aromatic pathway are inhibitors of phospho-2-keto-3-deoxyheptonate aldolase (Table II). At

TABLE II

PERCENT INHIBITION OF PHOSPHO-2-KETO-3-DEOXYHEPTONATE ALDOLASE ACTIVITY BY AROMATIC AMINO ACIDS

Crude extracts of strain 625 were used. Assay conditions were those described in Materials and Methods. The data are from several independent experiments.

Amino acid addition	*Inhibition (%) at a concentration of each amino acid of:*				
	2 mM	*1 mM*	*0.5 mM*	*0.1 mM*	*0.01 mM*
L-Tyrosine	91	88	82	62	16
L-Tryptophan	—	18	0	2	3
L-Phenylalanine	13	7	5	0	3
Tryptophan + tyrosine				59	
Phenylalanine + tryptophan				3	
Phenylalanine + tyrosine				62	
Tryptophan + tyrosine + phenylaline				60	

concentrations of 10 μM and 100 μM, tyrosine produced 16 and 62% inhibition respectively. Phenylalanine and tryptophan were without appreciable effect. At a concentration of 100 μM each, various combinations of the three amino acids were inhibitory only when tyrosine was included, and the extent of inhibition was similar to that produced by tyrosine alone. When higher concentrations of the endproducts were used, tyrosine was found to produce a maximal inhibition of slightly greater than 90% at 2 mM. At this concentration phenylalanine inhibited activity about 13%, whereas the addition of 1 mM tryptophan resulted in 18% inhibition. However, at concentrations greater than 0.5 mM tryptophan interferes in the assay for DAHP. Although a correction was made for this interference, apparent inhibitory effects by tryptophan at concentrations of greater than 0.5 mM should be regarded with some skepticism. On a molar basis the enzyme from *Anacystis* was inhibited at least 100 times more effectively by tyrosine than by phenylalanine. Inhibition by tyrosine was observed even at a concentration as low as 1 μM.

Since the single phospho-2-keto-3-deoxyheptonate aldolase of *B. subtilis* is inhibited by aromatic intermediates, the effects of shikimate, chorismate and prephenate on enzymatic activity was investigated. These intermediates were not inhibitory at concentrations of 0.1 and 0.5 mM.

Effects of the growth medium

In organisms which possess multiple phospho-2-keto-3-deoxyheptonate aldolases the contribution of each isoenzyme to the total activity can often be altered by the growth of cells in medium supplemented with the individual endproducts[4]. This alteration is observed as a change in the percent inhibition produced by saturating concentrations of each effector. Therefore, in an attempt to reveal the existence of minor isoenzymes, cultures were grown in minimal medium supplemented with the aromatic amino acids both singly and in combinations. Crude extracts were prepared and enzymatic activity examined for inhibition by saturating amounts of tyrosine and phenylalanine (Table III). 2 mM tyrosine inhibited 90–95% of the activity from both cells grown in minimal medium and cells grown in supplemented medium. Inhibition by 2 mM phenylalanine, when it occurred, was between 10 and 25%.

TABLE III

THE EFFECT OF SUPPLEMENTATION OF THE GROWTH MEDIUM ON THE PERCENT INHIBITION OF PHOSPHO-2-KETO-3-DEOXYHEPTONATE ALDOLASE ACTIVITY BY TYROSINE AND PHENYLALANINE

All supplementations were at a concentration of 1 mM. A low nitrogen minimal medium was used in all cases (0.13 g $NaNO_3$ per l). Growth of cells and assays were carried out as described in Materials and Methods.

Medium	*Inhibition (%) by:*	
	2 mM tyrosine	*2 mM phenylalanine*
a. Minimal + tyrosine	90.0	0.0
Minimal	95.5	9.5
b. Minimal + phenylalanine	95.7	14.6
Minimal	95.3	0.0
c. Minimal + tryptophan	91.0	19.0
Minimal	91.0	24.0
d. Minimal + tyrosine + phenylalanine	89.6	21.3
Minimal	92.6	19.0
e. Minimal + tyrosine + phenylalanine + tryptophan	93.3	*
Minimal	94.3	*

* Not estimated.

Activity from cells grown in the presence of tyrosine exhibited no inhibition by phenylalanine. However, since this ineffectiveness of phenylalanine has also been observed in extracts of cells grown in minimal medium, its significance is unclear.

Since phospho-2-keto-3-deoxyheptonate aldolase activity of *Anacystis* was almost completely inhibited by tyrosine and since supplementation of the minima medium failed to alter significantly this extent of inhibition, the existence of a single enzyme in this organism was tentively indicated.

Enzyme purification

In order to obtain additional evidence concerning the number of phospho-2-keto-3-deoxyheptonate aldolase present in *Anacystis*, a purification of the enzymatic activity was undertaken. A crude extract was subjected to $(NH_4)_2SO_4$ fractionation at the following percent saturations: 0–20, 20–35, 35–50, 50–65 and 65–80. When assayed for enzymatic activity about 93% of the total recovered appeared in the 35–50% fraction while the remaining 7% was found in the 20–35% fraction. An examination of the feedback properties of the activities present in the two fractions showed that both were inhibited by more than 90% by 2 mM tyrosine. Inhibition by 2 mM phenylalanine amounted to 31% for the 20–35% fraction and 18% for the 35–50% fraction. Thus the two activities appear to be identical. In subsequent purifications fractions of 0–30, 30–50 and 50–65% saturation were taken. Almost all of the activity was recovered in the 30–50% fraction.

Further purification of phospho-2-keto-3-deoxyheptonate aldolase activity was accomplished by the passage of the 30–50% $(NH_4)_2SO_4$ fraction through a column of Sephadex G-100. The enzymatic activity eluted as a single symmetrical peak (Fig. 1). Column fractions which contained phospho-2-keto-3-deoxyheptonate aldolase activity exhibited greater than 90% inhibition by 2 mM tyrosine and 10–20% inhibition by 2 mM phenylalanine. The preceeding results, therefore, are consistant

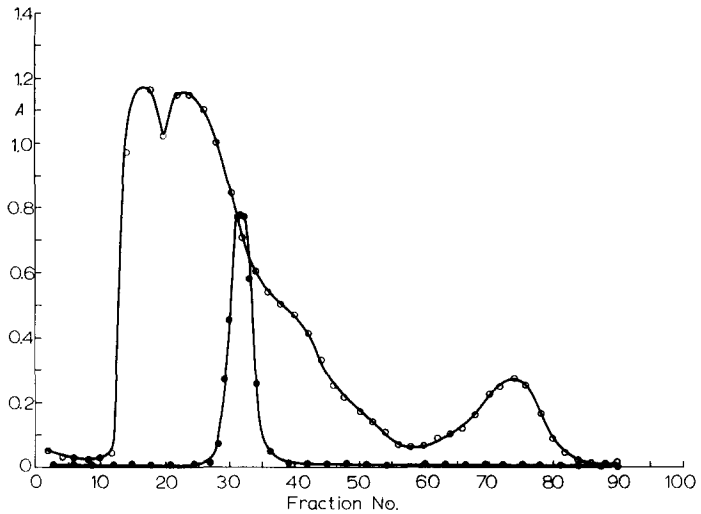

Fig. 1. Elution profile of phospho-2-keto-3-deoxyheptonate aldolase on Sephadex G-100. The column was loaded with 6 ml of a 30–50% $(NH_4)_2SO_4$ fraction. Protein was read at 280 nm; DAHP was measured at 549 nm. ○—○, protein; ●—●, phospho-2-keto-3-deoxyheptonate aldolase activity.

TABLE IV

PARTIAL PURIFICATION OF PHOSPHO-2-KETO-3-DEOXYHEPTONATE ALDOLASE

One enzyme unit equals the amount of enzyme which catalyzes the formation of 1 nmole of DAHP per min.

Fraction	*Vol. (ml)*	*Activity (units/ml)*	*Total activity (units)*	*Protein (mg/ml)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification (-fold)*
Crude extract	80	29.2	2370.5	14.11	2.1	100	1.0
Protamine sulfate supernatant	78	30.2	2361.5	10.44	2.9	99	1.38
30–50% $(NH_4)_2SO_4$	10	167.0	1661.1	15.28	10.5	70.7	5.0
Sephadex G-100	12	45.2	541.5	1.6	28.2	22.8	13.4

with the supposition made on the basis of feedback inhibition data that a single phospho-2-keto-3-deoxyheptonate aldolase exists in *Anacystis*. A purification scheme is presented in Table IV.

Kinetic studies with the purified enzyme

The effect of tyrosine on enzyme kinetics was investigated. The concentration of one substrate was varied in the presence of a fixed saturating concentration of the other at tyrosine concentrations of 5, 10 and 25 μM. Only data for the highest inhibitor concentration is presented, since it is the most informative. Double-reciprocal plots for results of experiments in which PEP was the variable substrate are presented

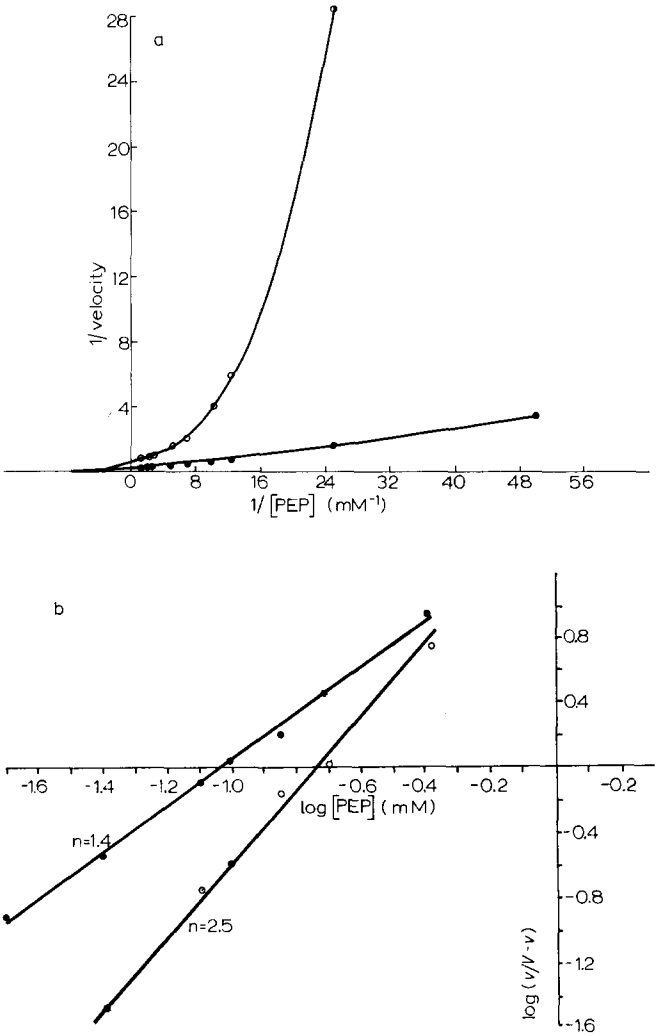

Fig. 2. (a) Lineweaver–Burk plot of reaction velocity as a function of PEP concentration. ●—●, no tyrosine; ○—○, 25 μM tyrosine. An apparent K_m for PEP of $1.5 \cdot 10^{-4}$ M is indicated for the control line under the conditions specified in the text. (b) Hill plot of (a). ●—●, no tyrosine; ○—○, 25 μM tyrosine.

in Fig. 2a. The control line (no tyrosine) is approximately linear. However, in the presence of 25 μM tyrosine an extensive deviation from linearity is observed. At the lower tyrosine concentrations the deviation from linearity was less marked. When the data are replotted according to the Hill equation (Fig. 2b), the line which represents 25 μM tyrosine shows an 80% increase in slope over that of the control (from 1.4 to 2.5).

The corresponding values at lower tyrosine concentrations are 1.3 (control); 1.35 and 1.45 for 5 and 10 μM tyrosine respectively. The preceding results suggest that tyrosine facilitates cooperative interactions in the binding of molecules of PEP.

Lineweaver–Burk plots for data in which D-erythrose 4-phosphate served as

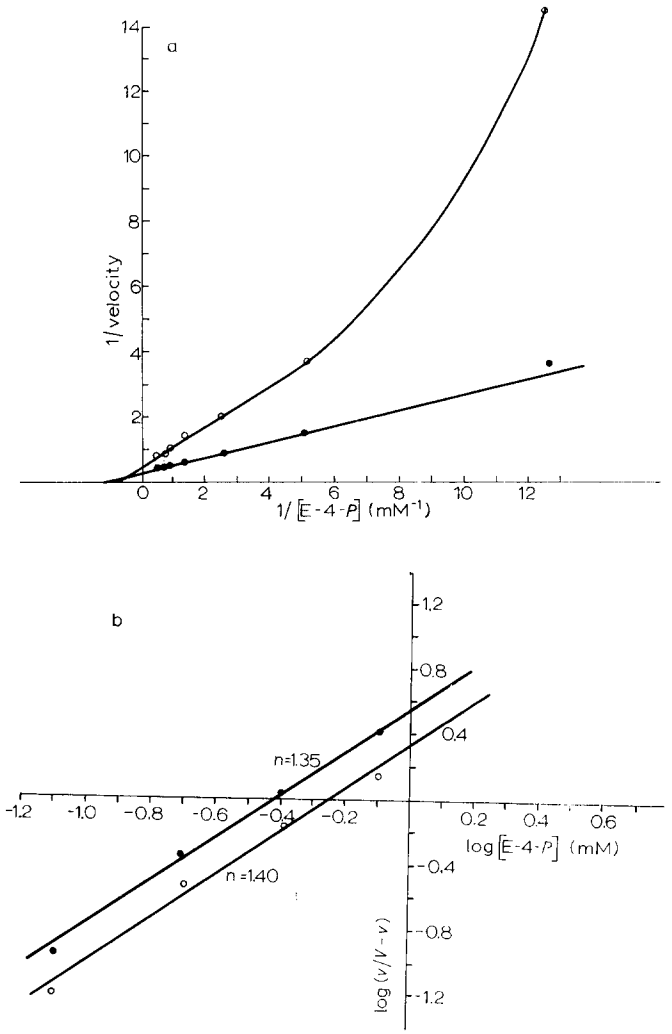

Fig. 3. (a) Lineweaver–Burk plot of reaction velocity as a function of D-erythrose-4-phosphate (E-4-*P*) concentration. ●—●, no tyrosine; ○—○, 25 μM tyrosine. An apparent K_m of $7.1 \cdot 10^{-4}$ M was obtained in the absence of inhibitor. (b) Hill plot of (a). ●—●, no tyrosine; ○—○, 25 μM tyrosine.

the variable substrate are presented in Fig. 3a. Again, the control line is essentially linear. The line representing 25 μM tyrosine exhibits a deviation from linearity only at the point corresponding to the lowest substrate concentration utilized. Hill plots (Fig. 3b) of the data indicate that the presence of tyrosine (25 μM) engenders only a slight increase in slope over that of the control (from 1.35 to 1.4). Similar results were obtained at tyrosine concentrations of 5 and 10 μM. It appears, therefore, that tyrosine affects the binding of D-erythrose 4-phosphate only slightly or not at all.

In both Fig. 2a and Fig. 3a the two lines intersect above the *X*-axis. It can be concluded that the inhibition produced by tyrosine is of the mixed type with regard to both substrates.

DISCUSSION

Several features of phospho-2-keto-3-deoxyheptonate aldolase of *A. nidulans* have been brought to light by the present study. First, the activity exists as a single enzyme which is feedback inhibited primarily by tyrosine. The fact that phenylalanine inhibits activity only weakly and at high concentrations suggests that its effect is non-specific. Since phenylalanine is a structural analogue of tyrosine, weak inhibition by this amino acid is not unexpected. The tyrosine sensitive phospho-2-keto-3-deoxyheptonate aldolase of *E. coli* is also inhibited by high concentrations of phenylalanine[16].

The kinetic properties of the enzyme are a second feature of interest. In the absence of tyrosine the response of reaction velocity to substrate concentration approaches Michaelis kinetics. However, the presence of tyrosine appears to facilitate cooperative interactions between molecules of PEP. On the other hand, tyrosine seems to exert only a minor influence on the binding of D-erythrose 4-phosphate. This differential effect of tyrosine on the binding of the two substrates may be related to the mechanism of the enzymatic reaction. Present evidence suggests that the condensation of PEP and D-erythrose 4-phosphate to give DAHP proceeds *via* a "ping-pong" mechanism[17,18]. First PEP binds to the enzyme and inorganic phosphate is released; then D-erythrose 4-phosphate reacts with the enzyme–PEP complex to give DAHP. Dixon and Webb[19] have indicated that some bisubstrate reactions possibly occur without a definite combination of the second substrate with the enzyme molecule. If the reaction between the phospho-2-keto-3-deoxyheptonate aldolase–PEP complex and D-erythrose 4-phosphate occurs in this manner, then any alterations in enzyme conformation which result from the presence of tyrosine would presumably affect the binding of PEP to a greater extent than the binding of D-erythrose 4-phosphate.

Phospho-2-keto-3-deoxyheptonate aldolases of *E. coli* and *B. subtilis* do not exhibit an altered substrate affinity in the presence of their allosteric inhibitors[3,7]. Weber and Böck[9] reported that this property also characterized the enzyme from *Anacystis*. Our results, however, suggest that the presence of the inhibitor (tyrosine) does affect substrate binding (at least in the case of PEP). The kinetic data which Weber and Böck presented were Lineweaver–Burk plots of reaction velocity as a function of PEP concentration at tyrosine concentrations of 5 and 10 μM. The lowest concentration of PEP which they utilized was about 0.08 mM. In our studies lower PEP concentrations were employed, and deviations from linearity in double-reciprocal plots are more evident at these lower concentrations. The importance of the use of low substrate concentrations in accurately assessing non-linear enzyme kinetics has been well emphasized by Atkinson[19].

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