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# 3-DEOXY-D-*arabino*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE OF *STREPTOMYCES AUREOFACIENS* TÜ 24

## II. REPRESSION AND INHIBITION BY TRYPTOPHAN AND TRYPTOPHAN ANALOGUES

HELMUT GÖRISCH AND FRANZ LINGENS

*Institut für Mikrobiologie und Molekularbiologie der Universität Hohenheim, Stuttgart 70 (Germany)*

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SUMMARY

1. 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, DAHP synthase) of *Streptomyces aureofaciens* is not repressed in cells grown in minimal medium supplemented with phenylalanine, tyrosine or tryptophan.

2. The three aromatic amino acids, phenylalanine, tyrosine and tryptophan, and metabolites of the aromatic pathway are tested as potential inhibitors of DAHP synthase activity. The activity of enzyme is inhibited only by tryptophan.

3. The inhibition by the allosteric effector, tryptophan, is strongly dependent on the pH. At pH 7 the enzyme activity is inhibited to about 65% at saturating concentrations of tryptophan. The inhibition is noncompetitive for phosphoenolpyruvate and competitive for erythrose 4-phosphate. A cooperative effect of the inhibitor is not found.

4. 25 analogues of tryptophan have been tested as potential inhibitors.

## INTRODUCTION

The regulation of the biosynthesis in branched biosynthetic pathways is complex. Multiple endproduct derivatives of such pathways control the activity of the enzyme catalyzing the initial reaction of the pathway in a number of different ways. The biochemical route of synthesis for aromatic amino acids illustrates such a branching pathway. The first reaction in the biosynthesis of aromatic amino acids, the formation of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) from phosphoenolpyruvate and erythrose-4-*P* is catalyzed by 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-eryth-

Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; erythrose-4-*P*, erythrose 4-phosphate.

rose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, DAHP synthase). Various allosteric patterns of control for DAHP synthase were described by JENSEN *et al.*[1]. In five species of Streptomyces they found that the enzyme is inhibited by tryptophan. Phenylalanine and tyrosine did not influence the activity of the enzyme. This paper describes in some detail the allosteric inhibition of DAHP synthase purified from *Streptomyces aureofaciens* Tü 24, which is also inhibited only by tryptophan.

## MATERIALS AND METHODS

### *Chemicals*

The inorganic chemicals used were of analytical reagent grade. L-Phenylalanine, L-tyrosine, L-tryptophan, L-histidine, anthranilic acid and 2-thiobarbituric acid were obtained from Merck, Darmstadt; dithiothreitol (Cleland's reagent) and D-tryptophan from Calbiochem, Los Angeles, Calif., Phenylpyruvate, *p*-hydroxyphenylpyruvate and 5-hydroxy-DL-tryptophan were obtained from Fluka, Buchs, Switzerland, 5-fluor-DL-tryptophan from Schuchardt, München, 4-Methyl-DL-tryptophan was obtained from Ega-Chemie, Steinheim, and shikimic acid from Hedinger, Stuttgart.

Phosphoenolpyruvate was prepared according to CLARK AND KIRBY[2] and erythrose-4-*P* according to BALLOU[3]. Tryptophan derivatives not obtained commercially were synthesized by LINGENS[4,5].

### *Growth conditions and enzyme preparation*

*S. aureofaciens* Tü 24 was grown in minimal medium on a rotary shaker at 29°. After 48 h the cells were harvested at the exponential growth phase by centrifugation and stored at −25°. For repression the cells were harvested by centrifugation and incubated in minimal medium supplemented with $10^{-2}$ M L-phenylalanine or $10^{-3}$ M L-tyrosine or $2 \cdot 10^{-3}$ M L-tryptophan for 4 h. Combinations of the three aromatic amino acids were also used. The minimal medium contained 10 g glycerol, 1 g $NH_4Cl$, 1 g $K_2HPO_4$, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g NaCl and 1 g $CaCO_3$ per l. DAHP synthase was purified according to procedure B as described previously[6]. Protein was determined according to GROVES *et al.*[7].

### *Enzyme assay*

If not otherwise stated, enzymatic activity was determined in the standard reaction mixture, containing 0.425 $\mu$M phosphoenolpyruvate, 0.3 $\mu$M erythrose-4-*P* and protein in a total volume of 1 ml of Buffer D (0.05 M potassium phosphate (pH 7.0)–$10^{-5}$ M dithiothreitol–0.05 M $(NH_4)_2SO_4$–$2.5 \cdot 10^{-4}$ M $CoCl_2 \cdot 6\ H_2O$). DAHP was determined as described previously[6].

## RESULTS AND DISCUSSION

### *Repression of synthesis of DAHP synthase*

The specific activity of DAHP synthase is nearly identical regardless of whether extracts were prepared from cells grown in a minimal medium or from cells grown in minimal medium with addition of the aromatic amino acids. The addition of the

three aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan, individually or in combination, to minimal medium does not decrease the specific activity of DAHP synthase. The enzyme is either constitutive or is already maximally repressed under conditions of growth in minimal medium.

*Feedback inhibition of enzyme activity*

The three endproducts, L-phenylalanine, L-tyrosine and L-tryptophan, were tested as potential inhibitors of the first enzymatic reaction of the multibranched aromatic biosynthetic pathway. DAHP synthase of *S. aureofaciens* is inhibited only by L-tryptophan; $2 \cdot 10^{-4}$ M L-tryptophan inhibits the enzyme by 65% at pH 7. Some metabolites of the biosynthetic pathway were also tested as potential inhibitors. Shikimic acid, chorismic acid, prephenic acid, anthranilic acid, phenylpyruvic acid

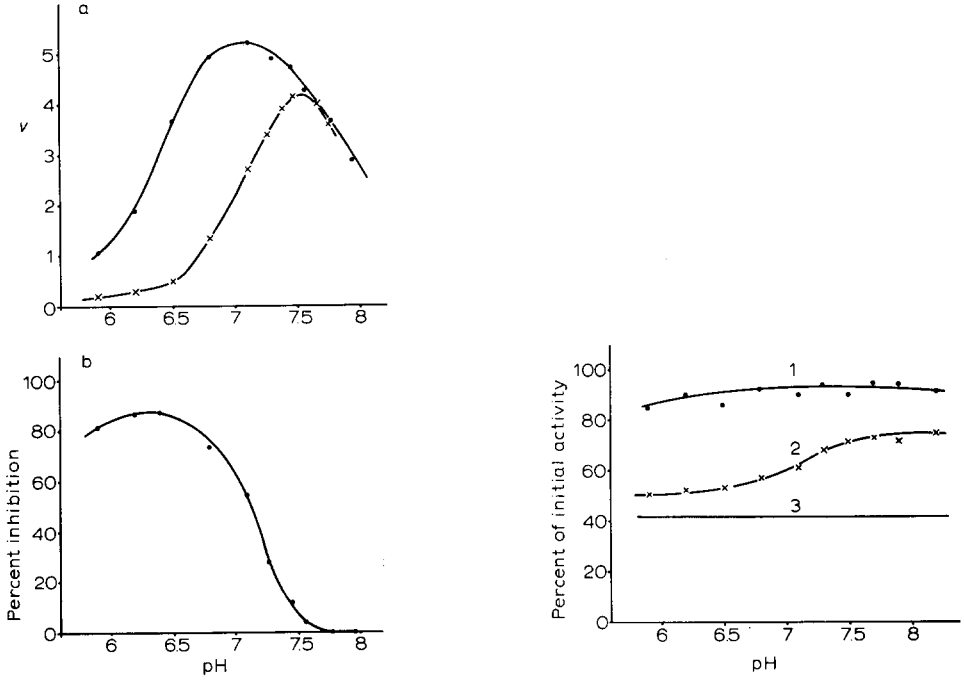


Fig. 1. (a) Effect of pH on inhibition by tryptophan. The reaction mixture contained 60 $\mu$g of protein, 0.425 $\mu$mole phosphoenolpyruvate and 0.3 $\mu$mole erythrose-4-*P* in 1 ml 0.3 M potassium phosphate buffer at various pH values. ●—●, activity of DAHP synthase in the absence of tryptophan; ×—×, activity of DAHP synthase in the presence of $2 \cdot 10^{-4}$ M tryptophan. (b) Inhibition by tryptophan as a function of pH. Data are taken from (a).

Fig. 2. Dependence of enzyme stability and of inhibition of enzyme activity on the pH value. The enzyme preparation was stored at 30° for 2 h in 0.1 M potassium phosphate buffer + $10^{-5}$ M dithiothreitol + $2.5 \cdot 10^{-4}$ M $Co^{2+}$ at the indicated pH. Aliquots of 100 $\mu$l, containing 90 $\mu$g of protein, were removed and the enzymatic activity determined in 0.3 M potassium phosphate buffer (pH 7.0) + $10^{-5}$ M dithiothreitol + $2.5 \cdot 10^{-4}$ M $Co^{2+}$ in the presence and absence of $2 \cdot 10^{-4}$ M tryptophan. The reaction mixture contained 0.425 $\mu$mole phosphoenolpyruvate, 0.3 $\mu$mole erythrose-4-*P* and protein in a total volume of 1 ml. The activity of a sample which had been stored at 0° for 2 h at pH 7.0 was used as a reference. (1) Activity in the absence of tryptophan; (2) activity in the presence of $2 \cdot 10^{-4}$ M tryptophan; (3) activity of the reference in the presence of $2 \cdot 10^{-4}$ M tryptophan.

and *p*-hydroxyphenylpyruvic acid, at concentrations of 0.1 mM, do not influence the enzymatic activity. L-Histidine also does not affect the activity.

The inhibition of DAHP synthase by tryptophan is strongly dependent on the pH. Fig. 1a shows the enzymatic activity as a function of pH in the presence and absence of $2 \cdot 10^{-4}$ M tryptophan. The optimal pH is shifted from pH 7.0 in the absence of tryptophan to pH 7.6 in its presence. Fig. 1b indicates the variation of inhibition of the enzyme with pH. At pH 6.3 the enzyme is inhibited to 90%. Above or below this pH value, the extent of inhibition by tryptophan decreases. With increasing pH the extent of inhibition decreases strongly and at pH 7.7 tryptophan no longer influences the enzymatic activity.

The enzyme activity is relatively stable between pH 6 and 8 (Fig. 2). The sensitivity of the enzyme to tryptophan was determined after storage at various pH values. As indicated in Fig. 2 the enzyme exhibits a remarkable loss of sensitivity to tryptophan after storage above pH 7. Since the stability of the enzyme activity is hardly influenced by pH, whereas the sensitivity to tryptophan is strongly influenced by the pH at which the enzyme was stored, we conclude that the enzyme possesses a distinct binding site for the allosteric inhibitor L-tryptophan, which is not identical to the active centre. Inhibition at pH 7 as a function of tryptophan concentration is demonstrated in Fig. 3a. A plot of these data in terms of the modified Hill equation $\log v - v_{sat}/v_0 - v = m \log [I] - \log K$ according to ATKINSON[8] is shown in Fig. 3b; $v$ and $v_0$ are the initial reaction velocities in the presence and absence of inhibitor,

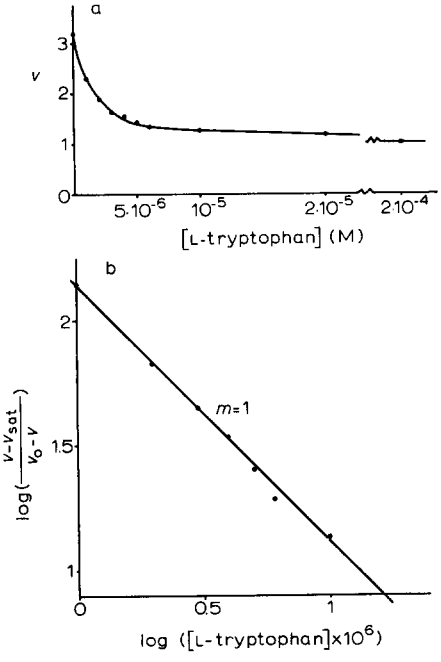


Fig. 3. (a) DAHP synthase activity as a function of tryptophan concentration at pH 7.0. The reaction mixture contained 49 $\mu$g of protein, 0.425 $\mu$mole phosphoenolpyruvate, 0.3 $\mu$mole erythrose-4-*P* and tryptophan concentration as indicated in a total volume of 1 ml of Buffer D. (b) Hill plot of tryptophan inhibition at pH 7.0. Data are taken from (a).

$v_{sat}$ is the initial reaction velocity in the presence of a saturating concentration of inhibitor, $m$ is a function of the number of sites and of the strength of interactions between these sites, $[I]$ is the inhibitor concentration and $K$ is a constant.

The data for inhibition of DAHP synthase by L-tryptophan produce a straight line with a slope of $m = 1$. Thus no cooperativity between tryptophan binding sites is indicated.

The substrate saturation curve for DAHP synthase follows Michaelis–Menten kinetics. Even in the presence of inhibitor, there is no indication of the sigmoid kinetics, which implicates cooperative binding of substrate molecules. The double reciprocal plots of the initial velocity of DAHP synthesis as a function of substrate concentration at several inhibitor concentrations indicate noncompetitive inhibition with respect to phosphoenolpyruvate and competitive inhibition with respect to erythrose-4-*P* (Figs. 4 and 5).

In contrast, the phenylalanine-sensitive DAHP synthase from *E. coli* K 12

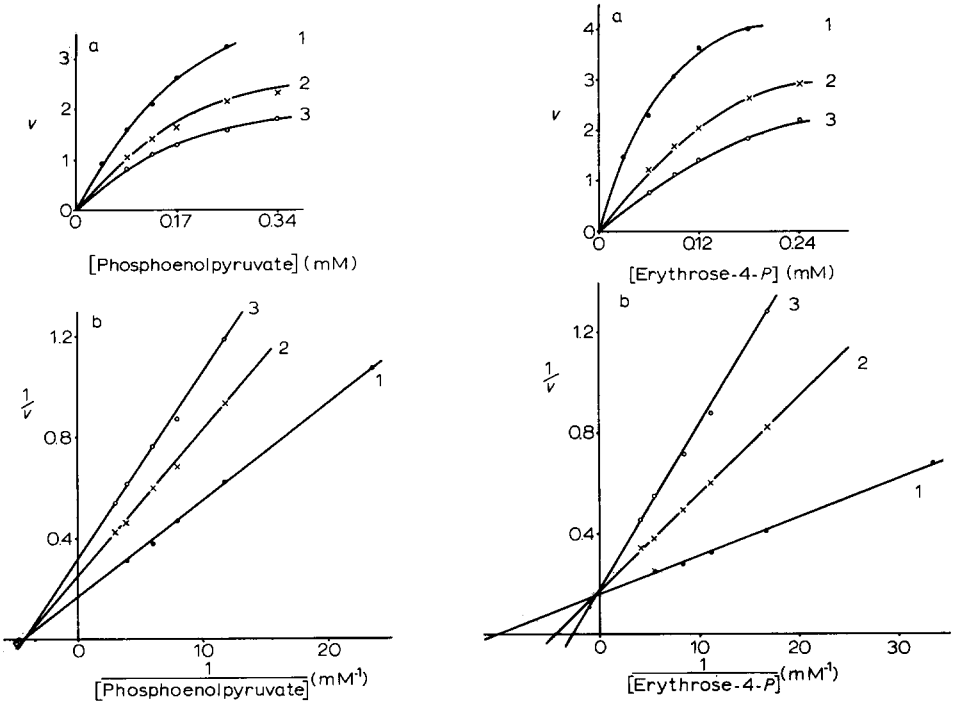


Fig. 4. (a) The initial rate of DAHP synthesis as a function of phosphoenolpyruvate concentration in the presence of various concentrations of L-tryptophan. The reaction mixture contained 66 $\mu$g of protein, 0.3 $\mu$mole erythrose-4-*P*, various concentrations of tryptophan and phosphoenolpyruvate as indicated in a total volume of 1 ml of buffer D. (1) In the absence of tryptophan; (2) in the presence of $2 \cdot 10^{-6}$ M tryptophan; (3) in the presence of $5 \cdot 10^{-6}$ M tryptophan. (b) Lineweaver–Burk plot of the data from (a).

Fig. 5. (a) The initial rate of DAHP formation as a function of erythrose-4-*P* concentration in the presence of various concentrations of tryptophan. The reaction mixture contained 66 $\mu$g of protein, 0.425 $\mu$mole phosphoenolpyruvate, various concentrations of tryptophan and erythrose-4-*P* as indicated in a total volume of 1 ml of Buffer D. (1) In the absence of tryptophan; (2) in the presence of $2 \cdot 10^{-6}$ M tryptophan; (3) in the presence of $5 \cdot 10^{-6}$ M tryptophan. (b) Lineweaver–Burk plot of the data from (a).

examined by STAUB AND DÉNES[9] and the tyrosine-sensitive DAHP synthase from *Salmonella typhimurium* examined by NAGANO AND ZALKIN[10] were inhibited by their effectors noncompetitively with respect to both substrates, phosphoenolpyruvate and erythrose-4-*P*.

*Inhibition of DAHP synthase by tryptophan analogues*

A number of tryptophan analogues were tested as potential inhibitors of DAHP synthase. The results are summarized in Table I.

In contrast to L-tryptophan, D-tryptophan does not inhibit the enzymatic activity at all. The L-configuration at the α-C atom of the alanine side chain is essential for inhibition.

The inhibition of 5-fluoro-DL-tryptophan is similar to that of L-tryptophan. 2-Methyl- and 4-methyl-DL-tryptophan show only weak inhibition. 5-Hydroxy- and 7-methyl-DL-tryptophan have a rather less inhibitory effect. 1-Methyl-, 5-methyl- and 6-methyl-DL-tryptophan do not influence the enzymatic activity.

Compounds with larger substituents such as 5-ethyl-, 7-ethyl-, 4,5-benz- and 6,7-benz-DL-tryptophan show no inhibitory effect. $N^{\alpha}$-Methyl-DL-tryptophan substi-

TABLE I

INHIBITION OF DAHP SYNTHASE OF *S. aureofaciens* BY TRYPTOPHAN ANALOGUES

The activity of 45 μg of protein was determined in the standard reaction mixture with the addition of inhibitors at a concentration of $10^{-5}$ M. Compounds of the DL-form were added at a concentration of $2 \cdot 10^{-5}$ M, assuming that the D-form does not inhibit the enzyme, similarly to D-tryptophan. The specific activities, calculated as units/min per mg of protein were arbitrarily normalized to the specific activity obtained in the standard reaction mixture without any addition.

| *Addition* | *Relative activity* |
|---|---|
| — | 1.00 |
| L-Tryptophan | 0.35 |
| 1-Methyl-DL-tryptophan | 1.01 |
| 2-Methyl-DL-tryptophan | 0.70 |
| 4-Methyl-DL-tryptophan | 0.70 |
| 5-Methyl-DL-tryptophan | 0.96 |
| 6-Methyl-DL-tryptophan | 0.94 |
| 7-Methyl-DL-tryptophan | 0.86 |
| 5-Ethyl-DL-tryptophan | 1.00 |
| 7-Ethyl-DL-tryptophan | 1.01 |
| 5-Fluoro-DL-tryptophan | 0.44 |
| 5-Hydroxy-DL-tryptophan | 0.90 |
| 4,5-Benz-DL-tryptophan | 0.90 |
| 6,7-Benz-DL-tryptophan | 0.94 |
| $N^{\alpha}$-Methyl-DL-tryptophan | 0.40 |
| Indolyl-3-DL-glycine | 0.94 |
| Indol-3-yl acetic acid | 0.95 |
| DL-Homotryptophan | 0.54 |
| DL-Dihomotryptophan | 0.48 |
| 7-Aza-DL-tryptophan | 0.91 |
| DL-Tryptazan | 0.96 |
| β-(Benzothien-3-yl)-DL-alanine | 0.95 |
| β-(Quinol-3-yl)-DL-alanine | 0.95 |
| β-(Indol-2-yl)-DL-alanine | 0.92 |
| DL-*threo*-β-Methyltryptophan | 0.92 |
| DL-*erythro*-β-Methyltryptophan | 0.60 |
| D-Tryptophan | 0.99 |

tuted in the alanine side chain shows an inhibitory effect similar to that of L-tryptophan.

As shown by indolyl-3-DL-glycine (DL-2-amino-2-(indol-3-yl)-acetic acid) and indol-3-yl acetic acid, shortening of the side chain results in a loss of inhibition. A compound elongated by a $CH_2$-group in the side chain, DL-homotryptophan (DL-2-amino-4-(indol-3-yl)-butyric acid), is a potent inhibitor. DL-Dihomotryptophan (DL-2-amino-5-(indol-3-yl)-valeric acid) with a side chain elongated by two $CH_2$-groups, even shows a slightly better inhibitory effect than DL-homotryptophan.

7-Aza-DL-tryptophan and DL-tryptazan (DL-2-amino-3-(indazol-3-yl) propionic acid) are noninhibitory, as are β-(benzothien-3-yl)-DL-alanine, β-(quinol-3-yl)-DL-alanine and β-(indol-2-yl)-DL-alanine.

SNYDER AND MATTESON[11] arbitrarily termed the two isomers of β-methyl-DL-tryptophan, A and B. Isomer B is a strong inhibitor of DAHP synthase of *S. aureofaciens*, whereas Isomer A shows only a slight effect.

H. G. FLOSS (personal communication) elucidated the absolute configuration of the two isomers by treating them with L-amino acid oxidase. Isomer B has the absolute configuration 2'*R*, 3'*S* and 2'*S*, 3'*R* (DL-*erythro*-β-methyltryptophan). Isomer A has the configuration 2'*R*, 3'*R* and 2'*S*, 3'*S* (DL-*threo*-β-methyltryptophan).

These tryptophan analogues were also tested as potential inhibitors of the tryptophan-sensitive DAHP synthase of Claviceps SD 58 by EBERSPÄCHER *et al.*[12]. In most cases both enzymes were inhibited by the same compounds.

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REFERENCES

1 R. A. JENSEN, D. S. NASSER AND E. W. NESTER, *J. Bacteriol.*, 94 (1967) 1582.
2 V. M. CLARK AND A. J. KIRBY, *Biochim. Biophys. Acta*, 78 (1963) 732.
3 C. E. BALLOU, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, p. 482.
4 F. LINGENS, *Z. Physiol. Chem.*, 331 (1963) 56.
5 F. LINGENS, H. KRAUS AND S. LINGENS, *Z. Physiol. Chem.*, 339 (1964) 1.
6 H. GÖRISCH AND F. LINGENS, *Biochim. Biophys. Acta*, 242 (1971) 4.
7 W. E. GROVES, F. C. DAVIS, JR., AND B. H. SELLS *Anal. Biochem.*, 22 (1968) 195.
8 D. E. ATKINSON, *Ann. Rev. Biochem.*, 35 (1966) 85.
9 M. STAUB AND G. DÉNES, *Biochim. Biophys. Acta*, 178 (1969) 599.
10 H. NAGANO AND H. ZALKIN, *Arch. Biochem. Biophys.*, 138 (1970) 58.
11 H. R. SNYDER AND D. S. MATTESON, *J. Am. Chem. Soc.*, 79 (1957) 2217.
12 J. EBERSPÄCHER, H. UESSELER AND F. LINGENS, *Z. Physiol. Chem.*, 351 (1970) 1465.