Tryptophan: a Feedback Activator for Chorismate Mutase from *Neurospora**

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ABSTRACT: Tryptophan, phenylalanine, and tyrosine are synthesized through a multibranched pathway in microorganisms. In *Neurospora crassa* and *Aerobacter aerogenes* tryptophan is the feedback inhibitor of anthranilate synthetase, the first enzyme specific for its own synthesis. In this report initial velocity studies on an enzyme preparation from *Neurospora* show that tryptophan is an activator of an early enzyme in phenylalanine and tyrosine synthesis. The enzyme, chorismate mutase, is also feedback inhibited by phenylalanine or tyrosine and the activity of either inhibitor is antagonized by the presence of tryptophan. This observation establishes a bifunctional role for tryptophan as a feedback effector of two enzymes at the major branch position in the aromatic amino acid pathway in *Neurospora*.

Metabolic pathways can be regulated by specific small molecules or effectors capable of modulating the kinetic behavior of an early enzyme in the reaction sequence. The effector does not participate directly in the reaction but may have either a negative (inhibitory) or positive (activating) effect on the enzyme. Of current interest is the means by which end products of divergent pathways regulate their synthesis. The aromatic amino acids, phenylalanine, tyrosine, and tryptophan, are synthesized by microorganisms through a branched pathway. The major branch position is occupied by a recently discovered intermediate, chorismic acid (Gibson and Gibson, 1964). This compound is substrate for both anthranilate synthetase, the first enzyme specific for tryptophan biosynthesis, and chorismate mutase, the first enzyme on the phenylalanine and tyrosine pathway. Chorismate may also be a precursor of three other aromatic components, 4-hydroxybenzoic acid, 4-aminobenzoic acid, and vitamin K (Cox and Gibson, 1964).

The available information on feedback control of aromatic amino acid synthesis comes principally from bacterial studies. Reports from two laboratories (Smith *et al.*, 1962; Brown and Doy, 1963) have shown that multiple enzymes catalyze the first reaction in aromatic biosynthesis in *Escherichia coli*, the synthesis of D-arabinoheptulosonic acid 7-phosphate. One enzyme is inhibited by tyrosine and another by phenylalanine; a third enzyme exists in small quantities but this enzyme does not appear to be inhibited by tryptophan. Recent work by Jensen and Nester (1966) indicates that only one such enzyme is present in *Bacillus subtilis* and this enzyme is inhibited by chorismate and prephenate rather than the amino acid end products (Jensen and Nester, 1965). Cotton and Gibson (1965) report the presence of multiple enzymes in *E. coli* and *Aerobacter aerogenes* for the conversion of chorismate to prephenate. Again one enzyme is inhibited by phenylalanine and the other by tyrosine.

In *A. aerogenes* (Gibson and Gibson, 1964) as well as *Neurospora crassa* (DeMoss, 1965) tryptophan is an effective feedback inhibitor of anthranilate synthetase. In this paper the author will offer evidence that tryptophan is also an activator of chorismate mutase activity in *Neurospora*.

Experimental Section

Reagents. Chorismate was obtained from the culture fluid of *A. aerogenes* 62-1 according to the procedure of Gibson (1964). Free chorismic acid was obtained by recrystallization from an ether–petroleum ether (60–80°) mixture (F. Gibson, personal communication). L-Tryptophan, L-tyrosine, L-phenylalanine, and phenylpyruvate were purchased from Calbiochem.

Preparation of Chorismate Mutase. The enzyme used in these experiments was obtained from *N. crassa* td 48R (Suyama *et al.*, 1964), a tryptophan mutant that can utilize indole for growth. Growth of the organism and preparation of extracts were performed according to the procedure of DeMoss (1965), except that the standard buffer used throughout this preparation was 0.02 M Tris–maleate, pH 7.0, containing 0.1 mM EDTA. All steps in the preparation of the enzyme were carried out at 3°.

Each 100 ml of extract was treated with 14 ml of 1.5% protamine sulfate and the suspension was centrifuged for 20 min at 12,000*g*. Solid ammonium sulfate, 35 g/100 ml, was added to the protamine sulfate supernatant solution. The suspension was stirred for 10 min and centrifuged at 12,000*g* for 20 min. The precipitate was dissolved in a minimal amount of buffer

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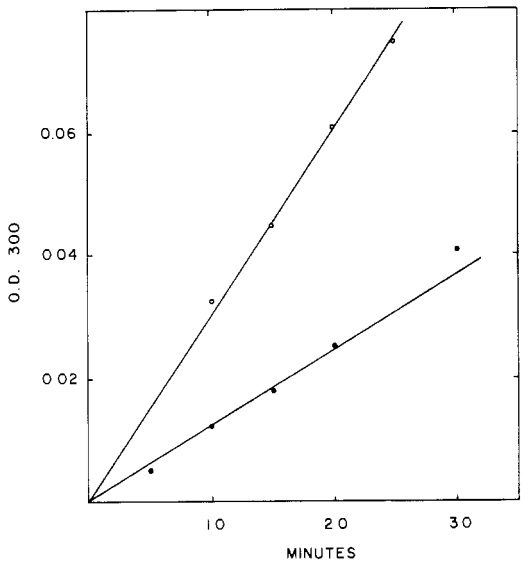

FIGURE 1: Stimulation of chorismate mutase activity by tryptophan. The enzyme preparation was the combined protein fractions from Sephadex G-25 (see *Preparation of chorismate mutase*). Reaction 1, ●, contained 240 μmoles of Tris–maleate, pH 7.0, 0.6 μmole of chorismate, and 0.3 ml of enzyme preparation in a total volume of 3.0 ml. Reaction 2, ○, contained the same plus 0.5 μmole of tryptophan.

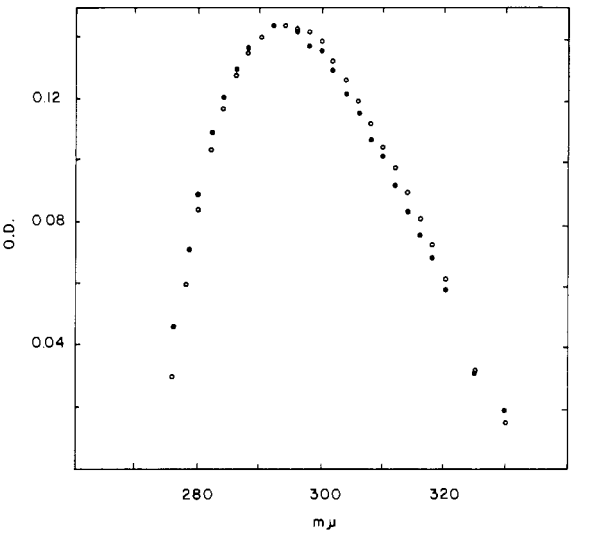

FIGURE 2: Absorption curves of the enzyme reaction product, ●, and authentic phenylpyruvate, ○. The maximal absorption of each curve was set at 0.145 for the purpose of comparison.

and filtered through a Sephadex G-25 column (2.5 × 12 cm). Standard buffer was used for equilibration and elution of the column. All protein fractions were collected and combined. This crude enzyme preparation contained anthranilate synthetase and chorismate mutase activities.

Gel filtration on Sephadex G-200 completely separated these two enzymes. The sephadex, which had been swollen for several days in standard buffer, was packed in a glass column to a bed size of 2.5 × 60 cm. The enzyme solution was layered onto the column and eluted with standard buffer. Fractions with chorismate mutase activity were combined and frozen in 2-ml aliquots to avoid thawing and refreezing. The total procedure gave a threefold purification of chorismate mutase but the preparation was free of anthranilate synthetase activity.

Enzyme Assay. Chorismate mutase activity was assayed by the procedure of Nester and Jensen (1966), a modification of the enol borate–tautomerase method of Lin *et al.* (1958). This assay is based on the enhancement of absorption of the aromatic α-keto acids by borate. The reaction mixture contained Tris–maleate buffer, pH 7.0, 240 μmoles; chorismate, 1.8 μmoles; and enzyme preparation, 0.3 ml (1.68 mg of protein), in a total volume of 3.0 ml. Reactions were initiated by addition of enzyme and stopped by transferring a 0.5-ml aliquot of the reaction mixture to 0.15 ml of 20% trichloracetic acid. The reaction was performed at 37° and aliquots were taken at 30-sec intervals for 2.5 min to determine reaction rates. Samples were kept in trichloracetic acid at 0° for 15 min, sufficient time to convert any prephenate formed during the reaction to phenylpyruvate. Protein was removed by centrifugation and 0.25 ml of the supernatant was transferred to 1.5 ml of 1 M sodium borate in 2 M sodium arsenate, pH 6.5. After 15 min at room temperature the optical density at 300 mμ of the arsenate–borate solution was determined. Phenylpyruvate concentrations were determined using a molar extinction coefficient of 9150 (Lin *et al.*, 1958).

Results

An investigation of the feedback inhibition of anthranilate synthetase activity in *Neurospora* extracts yielded data suggesting that tryptophan clearly inhibited the conversion of chorismate to anthranilate but also increased the rate at which chorismate was removed from the reaction mixture. Further study of this crude enzyme system (See Figure 1) revealed that chorismate mutase activity was present and that this activity was markedly increased in the presence of tryptophan (Figure 1).

A chorismate mutase preparation free from anthranilate synthetase activity was obtained as described above. The enzyme preparation was incubated at 37° for 3 min in the reaction mixture previously described and an aliquot was subjected to the enol borate assay procedure. An absorption curve of the final reaction product was prepared. A second reaction mixture, containing authentic phenylpyruvate but lacking enzyme, was treated in an identical manner. A comparison of the two spectra is presented in Figure 2.

Initial velocities of the chorismate mutase reaction

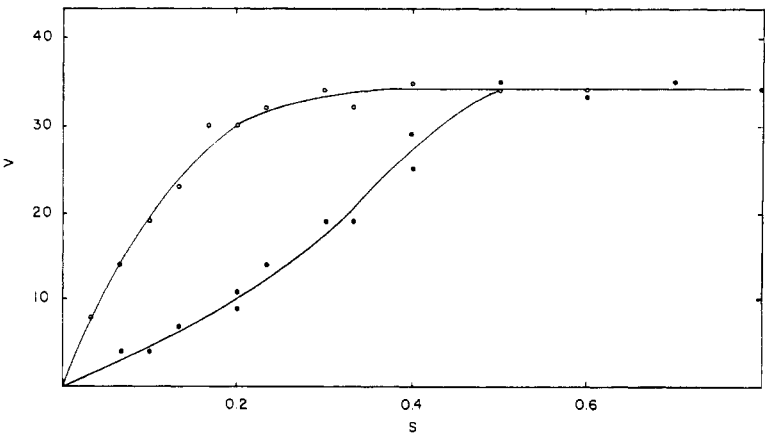

FIGURE 3: Effect of chorismate concentration on chorismate mutase activity in the presence and absence of tryptophan. V is expressed in micromoles of phenylpyruvate per minute. S represents the millimolar concentration of chorismate. Tryptophan concentrations were: ●, none; ○, 0.5 mM.

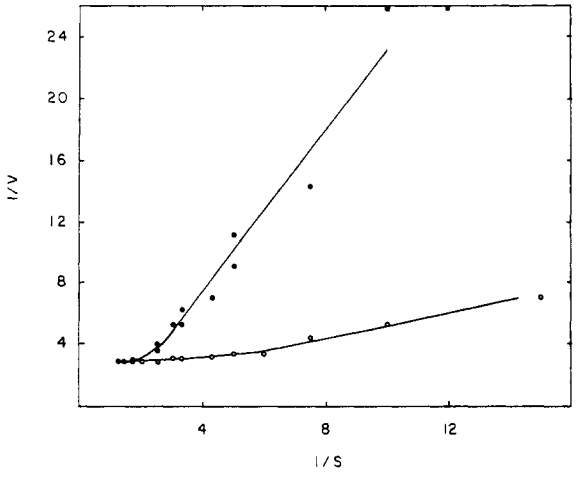

FIGURE 4: Effect of chorismate concentration on chorismate mutase activity, reciprocal plot. V is expressed in mμmoles of phenylpyruvate $\times 10^{-2}$/min. S represents the millimolar concentration of chorismate. Tryptophan concentrations were: ●, none; ○, 0.5 mM.

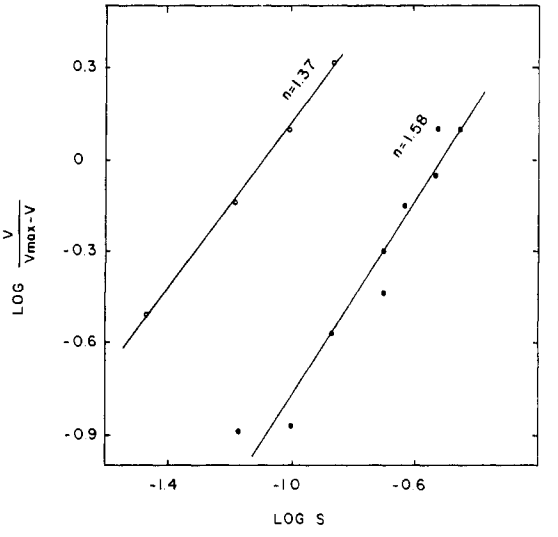

FIGURE 5: Effect of chorismate concentration on chorismate mutase activity in the presence and absence of tryptophan. Data is plotted according to the Hill equation. S represents millimolar concentrations of chorismate. V is expressed in micromoles of phenylpyruvate per minute. Tryptophan concentrations were: ●, none; ○, 0.5 mM.

were measured over a series of substrate concentrations in the presence and absence of tryptophan. The substrate saturation curves are presented in Figure 3. Figure 4 is a double reciprocal plot of the same data.

Table I shows the results of the addition of aromatic amino acids to the reaction mixture. This chorismate mutase preparation is inhibited by phenylalanine and even more strongly by tyrosine. Both inhibitions are reversed by the addition of tryptophan.

Discussion

The substrate saturation curve of chorismate mutase activity (Figure 3) in the absence of tryptophan is strongly sigmoid, *i.e.*, at low concentrations of substrate the slope increases as substrate concentration increases. This is demonstrated again by the curved plot in Figure 4. Such a substrate response curve is typical of many regulatory enzymes belonging to the allosteric K systems (Changeux, 1964). When these data are represented in the Hill plot (Atkinson *et al.*, 1965), a straight line is obtained with slope, n, equal to 1.6 (Figure 5), indicating a strong interaction between substrate binding sites. In the presence of 0.5 mM tryptophan the substrate response curve is shifted toward lower substrate concentrations (Figure 3). Although tryptophan has a marked effect on K_m it does not alter the V_{max} of

TABLE I: Effect of Aromatic Amino Acids on Chorismate Mutase Activity.

Addn to Reaction Mix[a]	Reaction Rate (mμmoles/min)	Inhibn (%)
None	31	—
Tryptophan	34	*b*
Phenylalanine	11	65
Phenylalanine and tryptophan	32	0
Tyrosine	4	87
Tyrosine and tryptophan	34	0

[a] Reaction mixture contained: chorismate, 1.5 μmoles; Tris–maleate buffer, pH 7.0, 240 μmoles; enzyme, 0.3 ml in total volume of 3.0 ml. Amino acid additions were: tryptophan, 1.5 μmoles; phenylalanine 0.9 μmole; and tyrosine, 0.9 μmole. [b] No activation is observed at this substrate level (see Figure 3).

the reaction. Only four experimental points were available for the Hill plot but it is apparent that substrate site interactions, although diminished, still exist at the tryptophan concentration used.

The data in Table I show that 65% inhibition of chorismate mutase activity is obtained in the presence of 0.3 mM phenylalanine. The same concentration of tyrosine gives 87% inhibition. These data do not distinguish between a cumulative system and an isoenzyme system of feedback inhibition, but if isoenzymes are present at least one inhibitor is affecting the activity of both enzymes at these inhibitor concentrations. Regardless of the mechanism, tryptophan will effectively reverse both phenylalanine and tyrosine inhibition.

It appears that in *Neurospora* tryptophan both inhibits anthranilate synthetase activity and stimulates chorismate mutase activity. The importance of this feedback inhibition to the regulation of tryptophan metabolism is obvious. The stimulation of chorismate mutase should result in a more effective discharge of chorismate into the phenylalanine and tyrosine biosynthetic pathways. The coincident inhibition of anthranilate synthetase and removal of substrate may be of special importance to the intricate control of aromatic synthesis. But the significance of the bifunctional role of tryptophan as an effector for two enzymes at the major branch position in this pathway will not be completely understood until more comprehensive studies of the aromatic regulatory systems (enzyme repression as well as feedback control) in *Neurospora* have been made. The presence of two enzymes on different metabolic pathways being modulated by the same effector molecule and sharing a common substrate provides an interesting system for chemical, physical, and genetic studies of allosteric proteins.

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