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Control of Aromatic Acid Biosynthesis in *Bacillus subtilis*: Sequential Feedback Inhibition

E. W. NESTER AND R. A. JENSEN

Departments of Microbiology and Genetics, University of Washington School of Medicine, Seattle, Washington

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ABSTRACT

NESTER, E. W. (University of Washington, Seattle), AND R. A. JENSEN. Control of aromatic acid biosynthesis in *Bacillus subtilis*: sequential feedback inhibition. J. Bacteriol. **91**:1594–1598. 1966.—The three major end products of aromatic acid synthesis, tyrosine, phenylalanine, and tryptophan, were tested for their ability to inhibit the first enzymes of the three terminal branches of the pathway as well as the enzyme common to both tyrosine and phenylalanine synthesis. Tyrosine inhibits the activity of prephenate dehydrogenase and also prephenate dehydratase to a limited extent. Phenylalanine inhibits the activity of prephenate dehydratase and, at much higher concentrations, prephenate dehydrogenase. Tryptophan inhibits the activity of anthranilate synthetase and, to some extent, prephenate dehydrogenase and prephenate dehydratase. Chorismate mutase is not inhibited by either 1 mM tyrosine or 1 mM phenylalanine when these are present singly or together in the reaction mixture. The significance of the feedback control of the terminal branches to the feedback control of that part of the pathway common to the synthesis of all three amino acids is discussed.

A variety of physiological mechanisms by which feedback inhibition controls the entry of low molecular weight metabolites into branched biosynthetic pathways have been described. Thus, some pathways have isoenzymes individually controlled by different end products of the pathway (12, 15). In other cases, a single enzyme may be inhibited only if all end products are present simultaneously (13), or the presence of an increasing number of the end products may result in a proportionately greater cumulative inhibition of an enzyme common to the synthesis of all of the end products (16).

In *Bacillus subtilis*, the portion of the biosynthetic pathway common to tyrosine, phenylalanine, and tryptophan is under the feedback control of two intermediates of the pathway, chorismic acid and prephenic acid (6). For effective feedback control, the accumulation of these intermediates should somehow reflect the intracellular concentration of the three major end products. Presumably, these intermediates would accumulate upon supplementation with the aromatic amino acids if each of the individual pathways leading from chorismic and prephenic acids were under feedback control of the specific end product of the pathway. This paper presents evidence that this is indeed the case. Data are also presented which indicate, however, that the control of one terminal branch may in part be controlled by the end products of the other branches.

MATERIALS AND METHODS

Bacterial strains. Two strains of *B. subtilis* were employed: 168, a tryptophan-requiring mutant of the Marburg strain (2), and WB672, a revertant of the threonine-requiring strain 23. For the preparation of extracts active for anthranilate synthetase, a tryptophan-requiring mutant of WB672 (WB2023) was grown on limiting tryptophan (1 μg/ml) in a chemostat. The enzyme activity in the prototroph is very low, presumably because of repression by internal tryptophan. Prephenate dehydratase was determined in extracts prepared from WB746, a prototrophic revertant of strain 168.

Growth and harvesting of bacteria. Cultures were grown at 37 C with vigorous aeration on a rotary shaker in 200 ml of a glucose minimal salts solution (11) in 1-liter flasks. The medium for the 168 culture was supplemented with 5 μg/ml of L-tryptophan. Cells were harvested in the exponential phase of growth for the preparation of enzyme extracts. After centrifugation, the pellets were washed once with the minimal medium and resuspended in an appropriate suspending fluid, depending on the enzyme to be assayed and the method of cell breakage.

Extract preparation and enzyme assays. Extracts of prephenate dehydrogenase were prepared by resus-

pending the washed bacterial pellet in 10^{-2} M tris-(hydroxymethyl)aminomethane (Tris)-HCl, 10^{-3} M potassium phosphate (*p*H 7.5) and treatment in an MSE ultrasonic disintegrator (Instrumentation Associates, New York, N.Y.) for 2 min. The crude extract was centrifuged at 25,000 × *g* for 30 min. The supernatant fluid was used directly or, in some cases, dialyzed against 500 volumes of 10^{-2} M Tris-HCl buffer, 10^{-3} M potassium phosphate (*p*H 7.5) for 16 hr. In the enzyme assay, the reaction mixture contained (per milliliter): barium prephenate, 2.5 μmoles; potassium phosphate (*p*H 8.1), 100 μmoles; nicotinamide adenine dinucleotide, 0.8 μmole; Tris-HCl buffer (*p*H 8.1), 50 μmoles. The reaction vessel (a 13 by 100 mm test tube) was incubated for 20 min at 37 C on a reciprocal shaker, and the *p*-hydroxyphenylpyruvic acid was measured as a Millon positive product by the procedure described by Schwinck and Adams (10). Because tyrosine also reacts in the assay, an appropriate blank was run for each concentration of tyrosine used in the inhibition studies. The amino acid was highly inhibitory at concentrations which gave only a barely detectable color in the assay.

Extracts of prephenate dehydratase were prepared by sonic disintegration as described for prephenate dehydrogenase, except that the cells were resuspended in a buffer (*p*H 8.1) of the following composition: 10^{-2} M Tris-HCl, 10^{-4} M ethylenediaminetetraacetic acid (tetra sodium), 10^{-3} M $MgCl_2$, 6×10^{-3} M mercaptoethanol. After the extract was centrifuged, 3.0 ml of the supernatant fluid was placed on a Sephadex G-25 column (1 by 7 cm) and 1.5 ml was collected. Extracts prepared by lysozyme lysis had much lower activity. The reaction mixture contained (per milliliter): barium prephenate, 1.25 μmoles; potassium chloride, 50 μmoles; Tris-HCl buffer (*p*H 8.1), 50 μmoles. The inclusion of the potassium chloride greatly stimulated enzyme activity. The product of the reaction, phenylpyruvic acid, was measured in 0.8 N NaOH at 320 mμ according to the procedure of Cotton and Gibson (3).

Extracts of chorismate mutase were prepared by lysozyme lysis of the cells in the Tris-phosphate medium described above to which was added 0.1 M NaCl and 6×10^{-3} M mercaptoethanol. Lysozyme (100 μg/ml), 10 μg/ml of deoxyribonuclease and 5×10^{-3} M $MgCl_2$ were added to the cells, and the mixture was incubated for 30 min at 44 C. The extract was processed by the procedure described for prephenate dehydrogenase, except that 6×10^{-3} M mercaptoethanol was always included in the dialyzing fluid. The reaction mixture contained (per milliliter): chorismic acid, 2 μmoles; glycine buffer (*p*H 8.9), 100 μmoles. The reaction was terminated by the addition of trichloroacetic acid (5% final concentration), and the prephenate was converted quantitatively to phenylpyruvate by incubation for 10 min at 0 C. The absorbancy of the enol tautomeric form of the acid was measured at 310 mμ in a borate-arsenate solution as described by Lin et al. (7).

For the preparation of anthranilate synthetase, the cells were suspended in 0.04 M potassium phosphate buffer (*p*H 7.0) containing 10^{-3} M ethylenediaminetetraacetate, 5×10^{-3} M mercaptoethanol, and 0.5 M KCl, and were lysed with lysozyme as previously described. After centrifugation, the supernatant fluid was dialyzed for 16 hr at 4 C in the above solution. The reaction vessel contained (per milliliter): chorismic acid, 4 μmoles; glutamine, 20 μmoles; potassium phosphate (*p*H 7.5), 100 μmoles; $MgCl_2$, 10 μmoles. The reaction was terminated by the addition of 5% trichloroacetic acid (final concentration), and the anthranilate was assayed by a modified Bratton-Marshall reaction (1). With the concentrations of tryptophan employed, there was no inhibition of the assay system for anthranilate.

All studies were performed on extracts which had not lost any significant enzyme activity upon storage. The assays were done at saturating substrate concentrations and at the *p*H optimum for enzyme activity unless otherwise specified. None of the inhibitory amino acids had any effect on the assay system per se.

Preparation of enzyme substrates. Chorismic acid was isolated from the culture supernatant fluid of *Aerobacter aerogenes* mutant 62-1 grown under the conditions described by Gibson (5). After purification and crystallization by the procedures described by Edwards and Jackman (4), the free acid was 100% pure, based upon an extinction coefficient of 2,440 at 274 mμ (Gibson, *personal communication*).

Barium prephenate was prepared by heating chorismic acid at *p*H 9 for 90 min at 70 C, and, after adding excess barium chloride, was isolated by the procedure of Metzenberg and Mitchell (8). The barium prephenate was approximately 85% pure upon converting and measuring the absorption of phenylpyruvate at 320 mμ. The amount of prephenate added in the reaction mixture was corrected for its purity.

All other chemicals were purchased from commercial sources and were of the highest purity obtainable. They were used without further purification.

RESULTS

That part of the pathway of aromatic acid synthesis relevant to the present study is diagrammed in Fig. 1. Chorismate mutase, prephenate dehydrogenase, prephenate dehydratase, and anthanilate synthetase are those enzymes which, if inhibited, would likely result in the accumulation of chorismic and prephenic acids, the feedback inhibitors of 7-phospho-2-oxo-3 deoxy-D-*arabino*-heptonate D - erythrose - 4 - phosphate - lyase (DAHP).

Each enzyme was tested for inhibition by the end product of its part of the pathway, as well as by the other two aromatic amino acids.

Prephenate dehydrogenase. Figure 2 indicates that this enzyme from strain 168 is inhibited by low levels of tyrosine, a concentration of 0.023 mM resulting in 50% inhibition. At concentrations 100-fold higher, phenylalanine and tryptophan also inhibit the enzyme.

Prephenate dehydratase. Both phenylalanine and tryptophan are effective inhibitors of this enzyme, 0.020 mM and 0.027 mM, respectively,

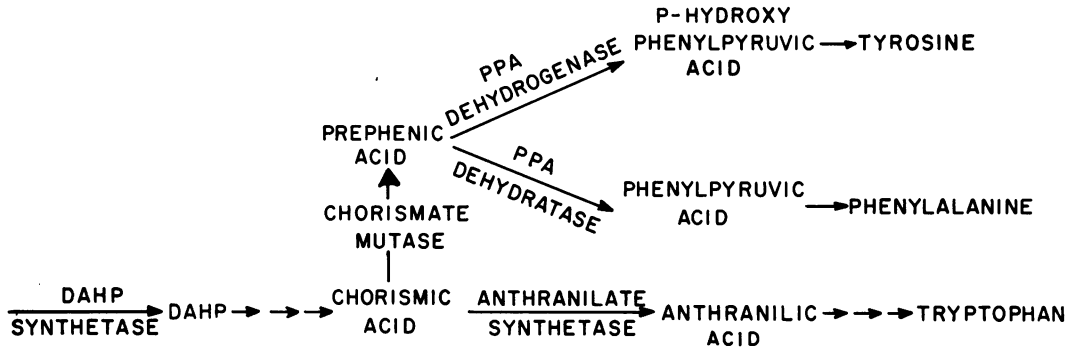

FIG. 1. *Biosynthetic pathway of aromatic acid synthesis. DAHP, 7-phospho-2-oxo-3 deoxy-D-arabino-heptonate D-erythrose-4-phosphate-lyase; PPA, prephenic acid.*

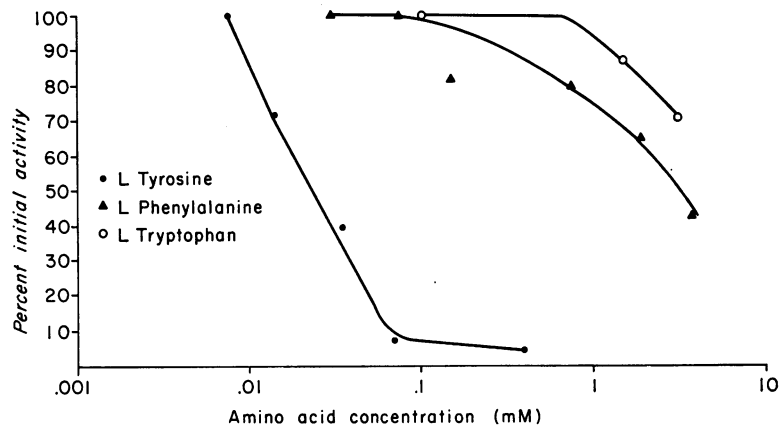

FIG. 2. *Effect of various concentrations of L-tyrosine, L-phenylalanine, and L-tryptophan on the enzyme activity, prephenate dehydrogenase, in strain 168.*

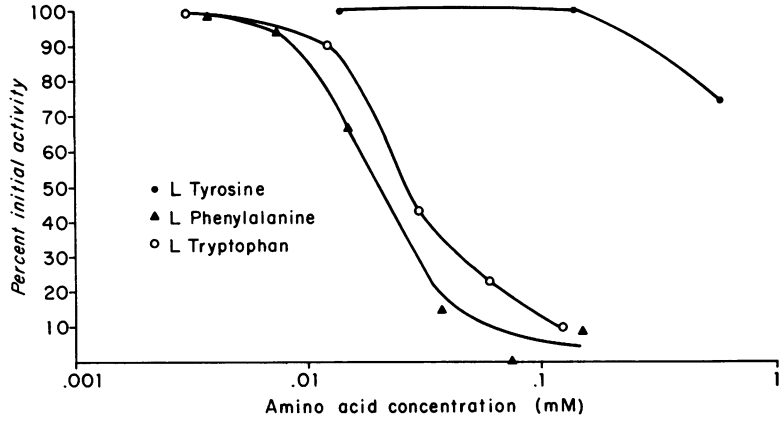

FIG. 3. *Effect of various concentrations of L-phenylalanine, L-tyrosine, and L-tryptophan on the enzyme activity, prephenate dehydratase, in strain 746.*

giving 50% inhibition (Fig. 3). Tyrosine also inhibits, but only at considerably higher concentrations.

Anthranilate synthetase. This enzyme is specifically inhibited by tryptophan (Fig. 4), with 0.07 mM resulting in a 50% inhibition. Tyrosine and phenylalanine at concentrations of 2 mM have no inhibitory effect.

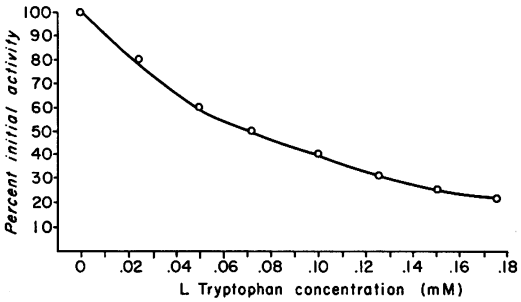

FIG. 4. *Effect of various concentrations of* L*-tryptophan on the enzyme activity, anthranilate synthetase, in strain 168.*

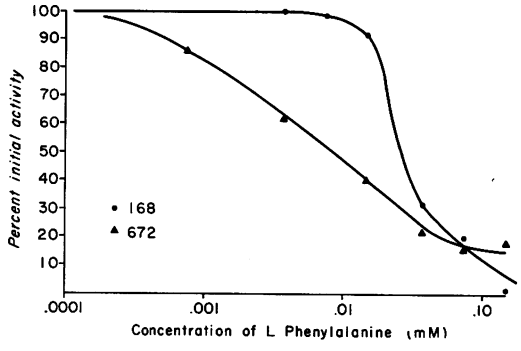

FIG. 5. *Comparison of feedback inhibition of prephenate dehydratase by* L*-phenylalanine in strain 672 and 168.*

Chorismate mutase. This enzyme from either strain 672 or 168 is not significantly inhibited by tyrosine or phenylalanine alone (each at 10^{-3} M), or both together at these concentrations. No inhibition was observed if the assay was run in the range of *p*H 7.0 to 8.9, nor if the substrate concentration was decreased to one-tenth the saturating level.

Comparison of the feedback inhibition of enzymes from strain 23 and 168. These two strains are apparently not from the same stock and differ from one another in a number of characteristics (14; Nester, *unpublished data*). Since a vast number of mutants currently in use are hybrids between the 23 and 168 strains, we determined whether the same enzymes in the two strains were distinguishable by their feedback properties. The data in Fig. 5 and 6, which compare the kinetics of feedback inhibition of prephenate dehydratase and anthranilate synthetase in the two strains, show that these enzymes are distinguishable. Similar studies revealed no significant differences in the prephenate dehydrogenase. Further, the chorismate mutase of strain 23 differs markedly from that of strain 168. Thus, the two enzymes

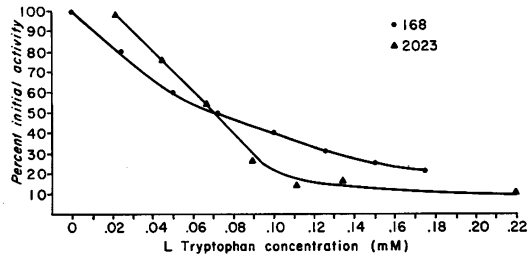

FIG. 6. *Comparison of feedback inhibition of anthranilate synthetase by* L*-tryptophan in strain 2023 and 168.*

have different chromatographic properties and different sensitivities to heat (Lorence and Nester, *unpublished data*). These observations may have relevance to the efficiency of regulation of the overall pathway in a hybrid strain. It is conceivable that an efficiently regulated pathway of aromatic acid synthesis in strain 168 will be severely deranged if loci of strain 23 replace some 168 alleles.

DISCUSSION

These studies not only help to clarify the control of the terminal branches of aromatic acid synthesis by feedback inhibition, but they support the predictions previously made on the control of the initial portion of the pathway in *B. subtilis* (6). Our results indicate that the first enzyme of each terminal branch is under the feedback control of its respective end product, tyrosine, phenylalanine, or tryptophan. These amino acids inhibit the entrance of metabolites into their branch of the pathway which, in turn, would likely result in an increase in the level of the substrates of the branch point enzymes, either chorismic acid in the case of tryptophan, or prephenic acid in the case of phenylalanine and tyrosine. These intermediates in turn regulate the activity of the first enzyme common to all three amino acids, DAHP synthetase. That this type of "sequential feedback" control actually does operate in vivo can be inferred from studies in which DAHP synthetase activity was measured after the addition of the three end products. Feedback inhibition was observed only several hours after the addition of end products (6).

The importance in vivo of the cross inhibitions of tryptophan on prephenate dehydratase and phenylalanine and tryptophan on prephenate dehydrogenase is difficult to assess from our present data. However, we have made several observations which are consistent with the possibility that these cross inhibitions observed in vitro play a role in vivo. Thus, the addition of high

levels of L-tryptophan (100 μg/ml) to a culture results in a lag of several hours before the culture begins to grow. This lag could be the time required for the cell to become derepressed and synthesize sufficient levels of prephenate dehydratase to overcome the tryptophan inhibition. This situation would be analogous to the transient inhibition of 2-thiazolealanine on histidine biosynthesis in *Escherichia coli* (9). Although we have not observed any significant inhibition in growth of wild-type cells by phenylalanine, we have observed that a mutant which has a partial defect in tyrosine synthesis, by virtue of a mutation in the locus controlling prephenate dehydrogenase, is inhibited by phenylalanine. This inhibition is relieved by the addition of tyrosine. A reasonable interpretation consistent with the in vitro data is that the phenylalanine falsely feedback-inhibits the already limited supply of prephenate dehydrogenase, thereby starving the cell for tyrosine.

ACKNOWLEDGMENTS

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