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Prephenate Dehydrogenase from *Pseudomonas aeruginosa* Is a Regulated Component of the Channel-Shuttle Mechanism Controlling Tyrosine–Phenylalanine Synthesis

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Prephenate dehydrogenase of *Pseudomonas aeruginosa* is unstable in crude extracts or following its partial purification by gel-filtration. However, when ion-exchange chromatography (DEAE-cellulose) is the initial purification step, a stable enzyme preparation is recovered. The enzyme, having a molecular weight of approximately 150,000, has a K_m for prephenate of 0.05 mM, and is inhibited competitively (with respect to prephenate) by L-tyrosine. Feedback inhibition has not been found previously. It is concluded that the entry of prephenate into the tyrosine pathway is regulated largely by L-phenylalanine acting upon a bifunctional enzyme complex bearing activities for chorismate mutase and prephenate dehydratase. Overproduction of L-tyrosine *in vivo* is probably prevented by feedback inhibition of prephenate dehydrogenase.

Within branching biosynthetic pathways, enzymes that compete for common intermediates ordinarily serve as focal points for end-product regulation of metabolite flow through the pathways. Thus, there is ample documentation in the aromatic amino acid pathway of many microorganisms that 3-deoxy-D-*arabino*-heptulosonate 7-P (DAHP) synthetase, anthranilate synthetase, prephenate dehydratase, prephenate dehydrogenase and (often) chorismate mutase function as regulatory proteins. In contrast the prephenate dehydrogenase from *Pseudomonas aeruginosa* has been an apparent exception in this respect. Previous data (1, 4, 6), obtained from unstable enzyme preparations, did not suggest the inhibition of the dehydrogenase activity by L-tyrosine. We now report that stable enzyme preparations do, in fact, exhibit control by L-tyrosine, suggesting the existence of a mechanism for feedback inhibition. Except for prephenate dehydrogenase, the regulatory enzymes cited above all have been partially purified and characterized from *P. aeruginosa* extracts (1, 4, 5, 13). A pattern of regulation that governs aromatic synthesis in *P. aeruginosa* and which is denoted as a channel-shuttle mechanism has been described (4). The enzymological characterization of prephenate dehydrogenase presented here permits a fuller insight into the nature of the pathway-wide control that is achieved by the integrant action of the various regulatory enzymes.

MATERIALS AND METHODS

Microbiological procedures. Pseudomonas aeruginosa strain 1 was obtained originally from B. W. Holloway (12). Cells were grown at 37°C in 1-liter volumes of the minimal salts-glucose medium described by Spizizen (15) modified to contain a fourfold greater concentration of sodium citrate.

Preparation of extracts. Cells were cultured until the late exponential phase of growth and centrifuged at 8500*g* for 15 min. The pellets were resuspended in 4.0 ml of 0.05 M Tris buffer, pH 7.9. The cells were disrupted by a 90-s exposure to ultrasound energy using a Bronwill Biosonik sonifier, and the cell debris was discarded following centrifugation at 30,000*g* for 20 min. The resulting clear supernatant is denoted as crude extract.

Buffers. Columns packed with Sephadex G-200 or DEAE-cellulose were equilibrated with 0.05 M Tris

buffer at pH 7.9. Crude extracts were prepared in the same buffer, as were all reagents for the enzyme assays. Hydroxylapatite columns were equilibrated with 0.01 M potassium phosphate buffer at pH 7.0.

Enzyme assays. (i) Chorismate mutase: a reaction mixture consisting of 0.1 ml of 2 mM chorismate and 0.1 ml of a given fraction of column eluate was incubated 15 min at 37°C. The reaction was terminated by the addition of 0.1 ml of 30% trichloroacetic acid, and the incubation was continued for 10 min. A 0.7-ml volume of 2.7 N NaOH was added just prior to the measurement of the absorbance of phenylpyruvate at 320 nm in a Gilford spectrophotometer.

(ii) Prephenate dehydratase: a 0.1-ml volume of 2 mM prephenate was added to 0.1 ml of column eluate, and such mixtures were incubated for 15 min at 37°C. The reaction was stopped with 0.8 ml of 2.7 N NaOH, and the absorbance of phenylpyruvate at 320 nm was measured.

(iii) Transaminase assays: four transaminase activities were resolved in all, each reactive with L-tyrosine and with L-phenylalanine. Exactly how this multiplicity sorts out *in vivo* is uncertain. For convenience a transaminase is named according to the amino donor used in a given assay, e.g., L-tyrosine transaminase denotes the use of L-tyrosine as the amino donor reactant. It is convenient and accurate to measure formation of phenylpyruvate or 4-hydroxyphenylpyruvate although this does not reflect the biosynthetic direction of transaminase activity in L-tyrosine and L-phenylalanine synthesis. Consequently, it was verified that all the transaminases catalyze the reactions in the biosynthetic direction by measuring phenylpyruvate and 4-hydroxyphenylpyruvate disappearance.

L-Tyrosine transaminase: reaction mixtures of 0.05 ml of 40 mM L-tyrosine, 0.05 ml of 10 mM α-keto glutarate containing 0.1 mM pyridoxal-5'-phosphate, and 0.1 ml of enzyme fraction were incubated at 37°C for 15 min. A 0.8-ml volume of 2.67 N NaOH was added, and the assay mixtures were placed in a 37°C water bath for 10 min. The concentration of 4-hydroxyphenylpyruvate formed during the reaction was measured by reading absorbance at 331 nm in a Gilford spectrophotometer. This procedure is a modification of that of Granner *et al.* (11).

L-Phenylalanine transaminase: the assay procedure is identical to that for the L-tyrosine transaminase except that 0.05 ml of 40 mM L-phenylalanine is used in place of L-tyrosine, and the 10 min incubation following the addition of NaOH is omitted. The absorbance of phenylpyruvate is read at 320 nm.

(iv) Prephenate dehydrogenase: an Aminco-Bowman recording spectrophotofluorometer was used to measure continuously the formation of NADH (7) formed at 37°C in the following reaction mixture: 0.1 ml of 1.0 mM NAD^+, 0.1 ml of partially purified enzyme, and 0.1 ml of 0.3 mM prephenate. The fluorescence of NADH was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm.

DEAE-cellulose chromatography. A 1.5 × 20 cm DE52 (Whatman) column was equilibrated with Tris buffer. Approximately 100 mg of protein were applied to the column. The protein was eluted with a linear gradient of NaCl using one reservoir containing 150 ml Tris and another containing 150 ml Tris plus 0.5 M NaCl. Fractions of 2.2 ml were collected at 4°C.

Hydroxylapatite chromatography. Eluate fractions containing both prephenate dehydrogenase and the aromatic transaminase (following DEAE-cellulose chromatography) were pooled and concentrated to 2 ml by means of an Amicon Diaflow Cell (PM10 filter). This concentrate was applied to a 1.5 × 6 cm Bio-Gel HTP (Bio-Rad) column equilibrated with 0.01 M phosphate buffer (pH 7.0). After application of the protein, the column was washed thoroughly with 0.01 M phosphate buffer prior to the gradient elution of protein. A linear gradient from 0.01 to 0.20 M phosphate buffer (150-ml volume in each reservoir) was used. Eluate fractions of 2.2-ml volume were collected at 4°C.

Sephadex G-200 gel filtration. DEAE-cellulose fractions containing prephenate dehydrogenase were combined and concentrated as described above. The concentrated protein was applied to a 1.5 × 60 cm Sephadex G-200 column. Eluate fractions of 2.2-ml volume were collected at 4°C. The method of Andrews (2) was used to calibrate the column with the following molecular weight markers: γ-globulin (M_r 160,000); bovine serum albumin (M_r 67,000); ovalbumin (M_r 45,000); and chymotrypsinogen (M_r 25,000).

Biochemicals and resins. Chorismic acid was prepared in its free acid form (9) and stored at 4°C under vacuum. Barium prephenate was isolated according to the procedure of Gibson (10) and was converted to the potassium salt just prior to use by the addition of a twofold molar excess of K_2SO_4. The molecular weight standard proteins were purchased from Mann Biochemicals. Amino acids and NAD^+ were obtained from Sigma Chemical Co. All other chemicals were of the highest grade commercially available. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad. Sephadex G-200 and DE52 cellulose were purchased from Sigma and Whatman, respectively.

RESULTS

Preparation of a stable prephenate dehydrogenase. The elution profiles for the five enzyme activities that are uniquely involved in the biosynthesis of L-tyrosine and L-phenylalanine are shown following DEAE-cellulose chromatography in Fig. 1. Two forms of prephenate dehydratase exist

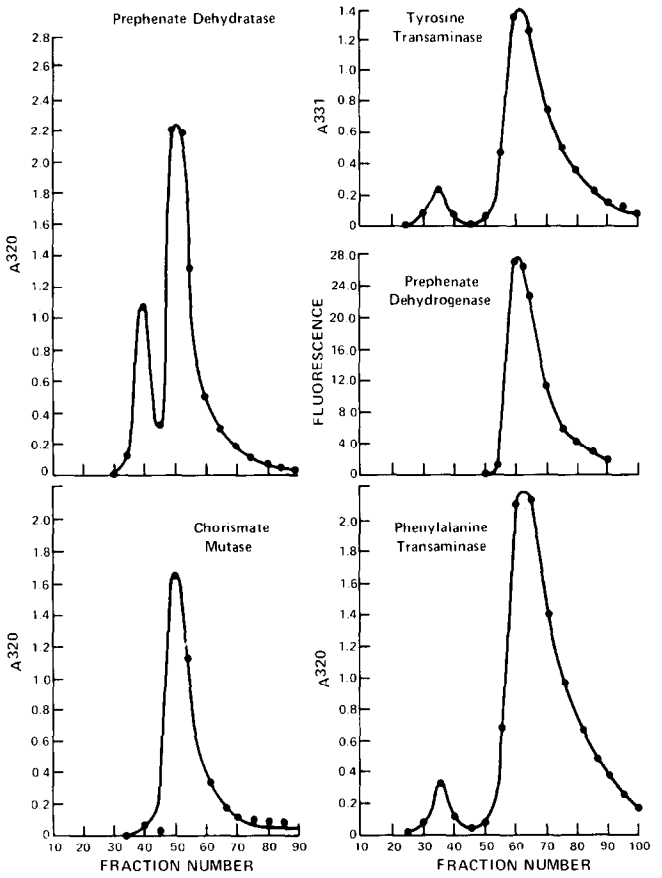

FIG. 1. DEAE chromatography of key aromatic pathway enzymes. Elution profiles are shown for prephenate dehydratase, prephenate dehydrogenase, chorismate mutase, and aromatic transaminase activities. The activities of chorismate mutase, prephenate dehydratase, and L-phenylalanine transaminase are expressed on the ordinate scale as absorbance at 320 nm of phenylpyruvate formed during 15 min of reaction time. An absorbancy value of 1.0 corresponds to 57 nmol of phenylpyruvate per ml at basic pH. The activity of L-tyrosine transaminase is expressed on the ordinate scale as absorbance at 331 nm of 4-hydroxyphenylpyruvate formed during 15 min of reaction time. An absorbancy value of 1.0 corresponds to 50 nmol of 4-hydroxyphenylpyruvate per ml at basic pH. Activity of prephenate dehydrogenase is expressed as fluorescence of NADH formed during five min of reaction time where a value of 1.0 corresponds to 1.6 nmol of NADH per ml. Other enzymological details are given in Methods.

(4) in *P. aeruginosa:* species I (M_r 134,000) and species II (M_r 76,000). These correspond to the right-hand and left-hand peaks (upper left panel) shown in Fig. 1, respectively. As previously shown (4), chorismate mutase and prephenate dehydratase I exist as a bifunctional protein complex that is capable of catalyzing the overall reaction from chorismate to phenylpyruvate. The elution of the enzyme complex at a position peaking in fractions 50–51 is seen by comparison of the two panels shown in the left half of Fig. 1.

Prephenate dehydrogenase is unstable in crude extracts and elutes inconsistently when Sephadex gel-filtration is used as the initial purification step. We have frequently found it to elute from Sephadex G-200 with an irregularly broad profile and to exhibit a progressive decay in activity over a period of 24 h. In contrast, when DEAE-cellulose chromatography is used as

the initial step of purification (middle-right panel of Fig. 1), a sharply defined peak of stable prephenate dehydrogenase activity is recovered. Perhaps a proteolytic activity (or activities) to which prephenate dehydrogenase is susceptible is separated by the ion exchange chromatography step.

Transaminase activities that participate in the biosynthesis of aromatic compounds have not been characterized previously in *P. aeruginosa*. Comparison of the top-right and bottom-right panels of Fig. 1 shows that two transaminase activities were recovered, each exhibiting a similar ratio of reactivity with respect to L-tyrosine or L-phenylalanine (amino donor reactants). Of particular interest in the context of this paper is the major transaminase activity recovered at a peak position of about fraction 62, an elution position coincident with that of prephenate dehydrogenase.

Resolution of prephenate dehydrogenase from transaminase activities. Prephenate dehydrogenase and the co-eluting transaminase recovered from DEAE-cellulose chromatography were chromatographed again, this time over hydroxylapatite (Fig. 2). The transaminase activity then resolved into three fractions, none of which co-eluted with prephenate dehydrogenase. A portion of transaminase activity washed through the column without retardation. Additional major and minor transaminase activities were recovered at peak positions in fractions 75 and 18, respectively. The symmetrical elution of a DEAE-cellulose-purified preparation of prephenate dehydrogenase from a Sephadex G-200 column is shown in Fig. 3. The gel-filtration method led to a molecular weight calculation of 150,000.

Characterization of partially purified prephenate dehydrogenase. The DE52 fractions containing prephenate dehydrogenase were pooled, concentrated by means of passage through an Amicon Diaflow Cell (PM10 filter), and used subsequently to determine substrate affinity and inhibitor specificities of the enzyme. Enzyme activity is feedback inhibited by L-tyrosine, as illustrated in Fig. 4. An inhibition of 50% is achieved in the pres-

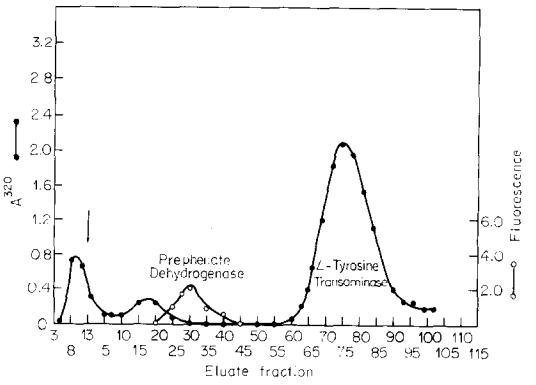

FIG. 2. Purification using hydroxylapatite chromatography. The fractions (55–80) from the experiment shown in Fig. 1 in which the elution of transaminase and prephenate dehydrogenase activities were coincident were pooled, concentrated and applied to the column as described under Methods. The transaminase activities, all heat-stable, were assayed at 45°C in order to increase the detection level. Only about half of the prephenate dehydrogenase activity originally applied to the column is recovered, although the eluted activity remains stable. Symbols: transaminase activities reactive with L-tyrosine, ●——●; prephenate dehydrogenase activity, O——O. The activities given on the ordinate scale are expressed as described in the legend of Fig. 1. The arrow indicates the trailing end of the void volume.

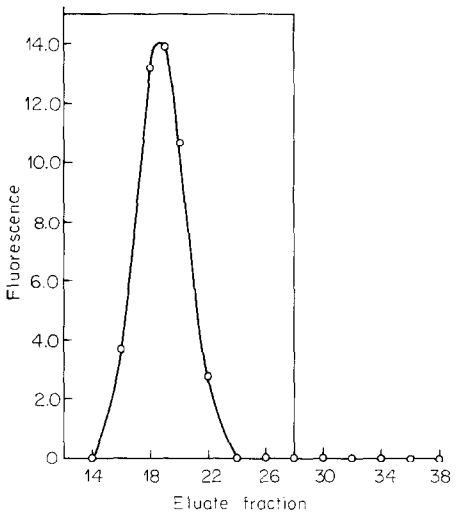

FIG. 3. Elution profile of partially purified prephenate dehydrogenase from Sephadex G-200. The use of enzyme recovered from a first DEAE-cellulose chromatography step, the gel-filtration procedure, and the enzymological procedure are described in Methods. The units of fluorescence given on the ordinate scale are described in the legend of Fig. 1.

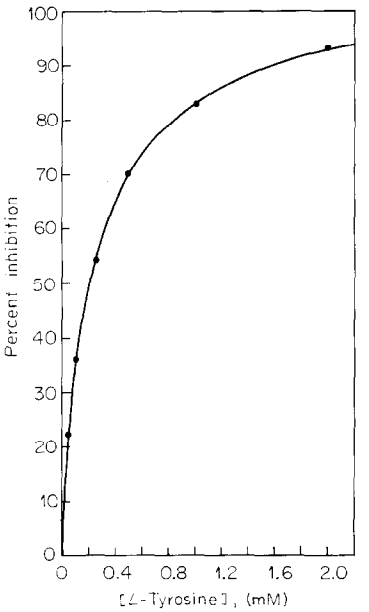

FIG. 4. Feedback inhibition of prephenate dehydrogenase by L-tyrosine. Reaction mixtures containing 0.33 mM NAD^+, 0.1 mM prephenate, 0.1 mg protein (partially purified by DE52 chromatography as in Fig. 1), and the indicated concentration of L-tyrosine in a total volume of 0.3 ml were incubated at 37°C. NADH appearance was measured fluorometrically as described in Methods. The specific activity of the control reaction mixture lacking L-tyrosine (i.e., 0% inhibition) was 18 nmol per min per mg protein.

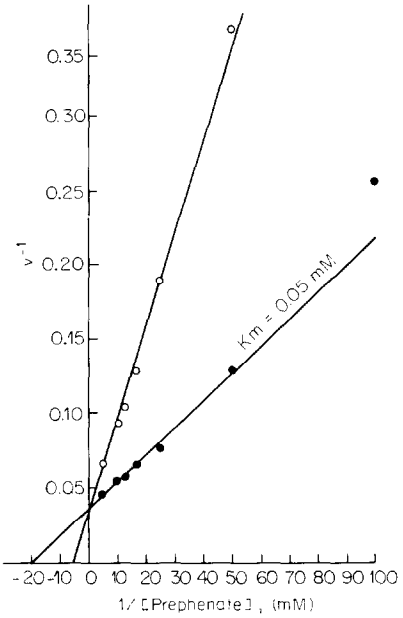

FIG. 5. Double reciprocal plot for kinetic analysis of prephenate dehydrogenase inhibition by L-tyrosine. Velocity was measured as NADH appearance at 37°C as described in Methods. Reaction mixtures having a total volume of 0.3 ml contained 0.3 mM NAD^+, the concentration of prephenate indicated on the abscissa scale, and 0.14 mg of partially purified protein (by DE52 chromatography as shown in Fig. 1). Symbols: no L-tyrosine present, ●——●; L-tyrosine present in reaction mixtures at a fixed concentration of 0.1 mM, O——O.

ence of about 0.2 mM L-tyrosine when the prephenate substrate concentration is 0.1 mM (twice the K_m value, see Fig. 5). Nearly complete inhibition of enzyme activity can be achieved, albeit at rather high concentrations of L-tyrosine. A 1.0 mM concentration of L-phenylalanine or L-trytophan has no effect upon prephenate dehydrogenase activity. The inhibition by L-tyrosine is competitive, as indicated by the kinetic analysis of substrate saturation data. The double reciprocal plot in Fig. 5 shows that L-tyrosine acts by decreasing the apparent affinity of the enzyme for prephenate.

DISCUSSION

Although data have been presented describing prephenate dehydrogenase in *P. aeruginosa* (1, 4, 14), the enzyme has not been characterized under stable conditions of storage and assay. This report shows prephenate dehydrogenase to possess a molecular weight of approximately 150,000 and to have an activity that is controlled by feedback inhibition. Since some allosteric proteins can be desensitized to feedback inhibition with little or no loss of catalytic activity, the apparent lack of feedback inhibition in the unstable enzyme preparations previously used may reflect desensitized enzyme. However, the major factor probably was the previous use of prephenate concentrations that were 10- to 20-fold greater than the K_m value for prephenate (1, 4). Even with stable preparations of feedback-sensitive enzyme, we find very little inhibition by L-tyrosine (up to the limits of its solubility) when substrate concentration is high. The control of the L-tyrosine biosynthetic branch previously appeared (4) to be totally dependent upon

the shuttle mechanism that is under the control of L-phenylalanine. We suggested that L-phenylalanine efficiently inhibits prephenate dehydratase activity, allowing prephenate molecules to become free of the bifunctional chorismate mutase-prephenate dehydratase complex and available to the L-tyrosine pathway. It now appears that while availability of substrate for the L-tyrosine pathway is indeed dependent upon L-phenylalanine concentration, a feedback mechanism responsive to L-tyrosine also exists that can prevent overproduction of L-tyrosine.

In some microbial genera prephenate dehydrogenase is purified in a bifunctional complex with chorismate mutase (8), a second isoenzymic species of chorismate mutase existing as part of another bifunctional complex with prephenate dehydratase. The lack of evidence for two species of chorismate mutase in *P. aeruginosa* contributed to evidence supporting a channel–shuttle mechanism (4) at the aromatic branchpoint in *P. aeruginosa*. The channel–shuttle mechanism affords a regulatory alternative to the channel–channel mechanism utilized by enteric microorganisms. The finding that recovery of stable preparations of prephenate dehydrogenase do not reveal a previously labile and copurifying activity for chorismate mutase reinforces the previous observations (4) that support the channel–shuttle mechanism of regulation.

Prephenate dehydrogenase was separable from a transaminase activity that was coincident with it following elution from DEAE-cellulose. The initial cofractionation may be fortuitous, but it could indicate a weak protein–protein association. Such an association may reflect a spatial organization *in vivo* that selectively channels substrate molecules of 4-hydroxyphenylpyruvate to one species of transaminase. Further experiments providing insight into the gene-enzyme relationships of transaminase function will be important before the control of L-tyrosine and L-phenylalanine synthesis is completely understood.

An additional property of prephenate dehydrogenase was recently described in *P. aeruginosa* (14). The enzyme exhibits a degree of substrate ambiguity that allows it to utilize pretyrosine [β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine] (16) as a substrate reactant. Pretyrosine can be formed in extracts of *P. aeruginosa* following transamination of an appropriate amino donor with prephenate. These observations raise the possibility that the pretyrosine pathway of L-tyrosine biosynthesis may be used *in vivo* in *P. aeruginosa* (14). When the prephenate dehydrogenase is assayed as pretyrosine dehydrogenase, it is subject to product inhibition in the presence of L-tyrosine that is competitive with respect to pretyrosine concentration. It was tentatively concluded that *P. aeruginosa* probably uses the standard 4-hydroxyphenylpyruvate pathway *in vivo* because the dehydrogenase displayed a tenfold greater affinity for prephenate than for pretyrosine (14). The regulation of the prephenate dehydrogenase reaction by L-tyrosine in *P. aeruginosa* also seems to favor the operation of the 4-hydroxyphenylpyruvate pathway *in vivo*, i.e., feedback inhibition of prephenate dehydrogenase at the branch point would be more efficient than product inhibition of pretyrosine dehydrogenase, one step removed from the branch point.

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