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Enzymology of L-Tyrosine Biosynthesis in Mung Bean (*Vigna radiata* [L.] Wilczek)[1]

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ABSTRACT

The enzymes of the 4-hydroxyphenylpyruvate (prephenate dehydrogenase and 4-hydroxyphenylpyruvate aminotransferase) and pretyrosine (prephenate aminotransferase and pretyrosine dehydrogenase) pathways of L-tyrosine biosynthesis were partially purified from mung bean (*Vigna radiata* [L.] Wilczek) seedlings. NADP-dependent prephenate dehydrogenase and pretyrosine dehydrogenase activities coeluted from ion exchange, adsorption, and gel-filtration columns, suggesting that a single protein (52,000 daltons) catalyzes both reactions. The ratio of the activities of partially purified prephenate to pretyrosine dehydrogenase was constant during all purification steps as well as after partial inactivation caused by *p*-hydroxymercuribenzoic acid or heat. The activity of prephenate dehydrogenase, but not of pretyrosine dehydrogenase, was inhibited by L-tyrosine at nonsaturating levels of substrate. The K_m values for prephenate and pretyrosine were similar, but the specific activity with prephenate was 2.9 times greater than with pretyrosine.

Two peaks of aromatic aminotransferase activity utilizing L-glutamate or L-aspartate as amino donors and 4-hydroxyphenylpyruvate, phenylpyruvate, and/or prephenate as keto acid substrates were eluted from DEAE-cellulose. Of the three keto acid substrates, 4-hydroxyphenylpyruvate was preferentially utilized by 4-hydroxyphenylpyruvate aminotransferase whereas prephenate was best utilized by prephenate aminotransferase. The identity of a product of prephenate aminotransferase as pretyrosine following reaction with prephenate was established by thin layer chromatography of the dansyl-derivative.

The shikimate pathway of aromatic amino acid biosynthesis begins with the condensation of erythrose-4-P and phosphoenolpyruvate and culminates in the formation of L-tryptophan, L-tyrosine, and L-phenylalanine. Although many of the pathway reactions have been studied in higher plants (for reviews, see 2 and 23), the enzymology of the distal branches of the shikimate pathway, leading from prephenate to L-tyrosine and L-phenylalanine, has neither been fully investigated nor evaluated for the presence of biosynthetic routes to L-phenylalanine or L-tyrosine that proceed via pretyrosine.

Previous studies showed the following. Labeled tyrosine or phenylalanine was formed when young plant shoots were fed radioactive 4-hydroxyphenylpyruvate or phenylpyruvate (12). Cell-free extracts of plants catalyzed the following enzymic conversions: 4-hydroxyphenylpyruvate to tyrosine; phenylpyruvate to phenylalanine; and prephenate to 4-hydroxyphenylpyruvate, tyrosine, and phenylalanine (13). Prephenate dehydrogenase and an aromatic aminotransferase (utilizing tyrosine to form 4-hydroxyphenylpyruvate) were partially purified and studied in mung bean (10, 11, 14). These results were consistent with tyrosine biosynthesis in higher plants via the 4-hydroxyphenylpyruvate pathway (Fig. 1). However, substantial activities of pretyrosine pathway enzymes (Fig. 1), also present in certain bacteria (7, 25, 27), were recently found in cotyledons of *Phaseolus* (18).

In order to clarify the enzymology of L-tyrosine biosynthesis in higher plants, prephenate dehydrogenase, pretyrosine dehydrogenase, and aromatic aminotransferases from mung bean seedlings were partially purified and characterized.

MATERIALS AND METHODS

Plant Material. Seeds of *Vigna radiata* (L.) Wilczek (originally classified as *Phaseolus aureus* [L.] Roxb. [5]) were immersed in aerated water for 24 h and then spread across single layers of cheesecloth that covered 8 cm of moistened horticultural Perlite in Nalgene tubs. The tubs were loosely covered with clear plastic and placed in a 22 C growth chamber, 90 cm from the light source (12 60-w incandescent bulbs and 16 F48T12/CW/HO fluorescent tubes: about 8,000 lux). After 3 days the roots of the seedlings were removed, and the shoots were immediately frozen in liquid N_2. The frozen tissue was ground to a fine powder in liquid N_2 in a Waring Blendor.

Preparation of Crude Extract. Extraction buffer, containing 50 mM K-phosphate (pH 7.0), 30 mM Na-ascorbate, 1 mM EDTA, and 0.04% (v/v) β-mercaptoethanol, was added to freshly prepared frozen mung bean powder (1:1, w/v) and stirred until melted. All further procedures were carried out at 4 C. The slurry was filtered through four layers of cheesecloth, centrifuged for 20 min at 20,000*g*, and the resulting supernatant was filtered through Miracloth (Chicopee Mills, Inc.) to give a crude extract.

Ammonium Sulfate Fractionation. The crude extract was fractionated by 0 to 30%, 30 to 50% and 50 to 65% saturated solution of ammonium sulfate prepared at 0 C. After each addition of solid ammonium sulfate, the pH was adjusted to 7.0 with 1 N KOH. The preparations were stirred for 20 min and then centrifuged for 10 min at 27,000*g*. The pellet from the 50 to 65% fraction (containing most of the prephenate-pretyrosine dehydrogenase activity) was dissolved in a small volume of 10 mM K-phosphate (pH 7.5) containing 1 mM EDTA and 0.02% (v/v) β-mercaptoethanol, and then passed through a Sephadex G-25 column (1.2 × 70 cm) equilibrated with the same buffer. Those fractions containing protein were pooled.

Column Chromatography. The desalted 50 to 65% ammonium sulfate fraction (192 mg protein) was chromatographed on a DEAE-cellulose (Whatman DE52) column (1.5 × 39 cm) equilibrated with 10 mM K-phosphate (pH 7.5) containing 1 mM EDTA and 0.02% (v/v) β-mercaptoethanol. The column was washed with

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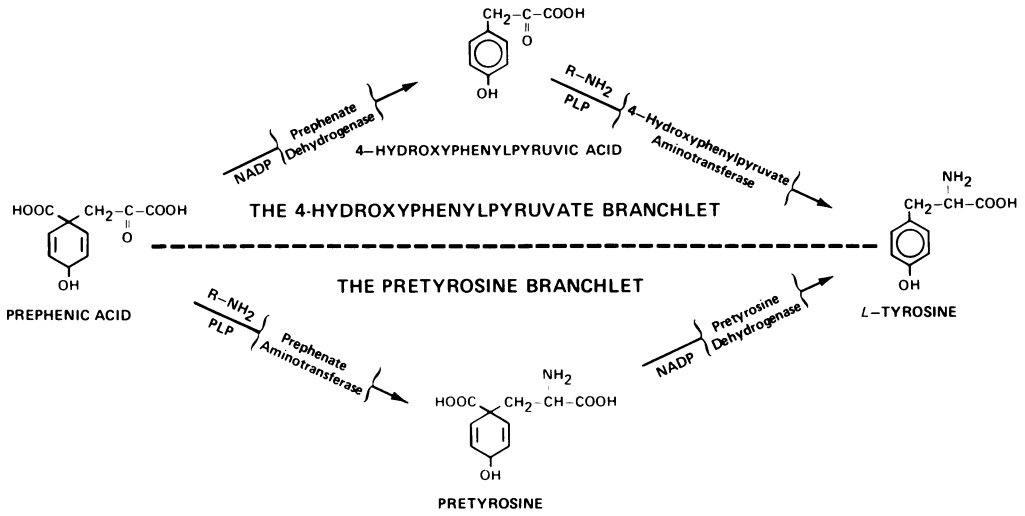

FIG. 1. Enzyme sequences of the 4-hydroxyphenylpyruvate branchlet (top) and the pretyrosine branchlet (bottom) of L-tyrosine biosynthesis. PLP: pyridoxal-5'-P; R-NH_2: amino donor.

the equilibration buffer, and a 400-ml linear gradient (0–0.5 M KCl in the same buffer) was applied: Fractions of 2.8 ml were collected. Fractions containing prephenate-pretyrosine dehydrogenase activity were pooled and concentrated to 10 ml with an Amicon ultrafiltration cell fitted with a PM-10 membrane using 30 psi N_2, and then passed through a Sephadex G-25 column (1.2 × 70 cm) equilibrated with 2.5 mM K-phosphate (pH 7.5) containing 1 mM EDTA. Fractions containing protein were pooled and concentrated as before to 6 ml.

A hydroxylapatite (Bio-Gel HTP) column (1.5 × 14 cm), equilibrated with 2.5 mM K-phosphate (pH 7.5) containing 1 mM EDTA, was loaded with the desalted and concentrated enzyme recovered from the ion exchange step of purification. The column was washed with the equilibration buffer, and proteins were eluted by a 200-ml linear gradient of 2.5 to 200 mM K-phosphate (pH 7.5) containing 1 mM EDTA. Fractions of 2.8 ml were collected. Fractions containing prephenate-pretyrosine dehydrogenase activity were pooled and concentrated as before to 6 ml.

The latter enzyme preparation was chromatographed on an Ultrogel AcA 44 (LKB) column (2.5 × 60 cm, effective fractionation range: 10,000–130,000 daltons) that was equilibrated and eluted with 50 mM K-phosphate (pH 7.5) containing 1 mM EDTA. Fractions of 1.7 ml were collected. Fractions containing prephenate-pretyrosine dehydrogenase activity were pooled and concentrated as before. This was the source of enzyme for all experiments, unless otherwise indicated.

The gel filtration column was calibrated for mol wt determination by the method of Andrews (1) using the following standards: ribonuclease A (13,700 daltons), chymotrypsinogen A (25,000 daltons), ovalbumin (43,000 daltons), and BSA (67,000 daltons). The void volume was determined with blue dextran.

Partial Purification of Aromatic Aminotransferases. Crude extract, prepared as previously described, was passed through a Sephadex G-25 column (1.4 × 49 cm), equilibrated with 10 mM K-phosphate (pH 7.5) containing 1 mM EDTA and 0.02% (v/v) β-mercaptoethanol. Four hundred mg of protein was chromatographed on a DEAE-cellulose (Whatman DE52) column (1.4 × 57 cm) and eluted as described above. Fractions comprising each of two peaks of aromatic aminotransferase activity (HPP-AT[2] and PPA-AT) were separately pooled and concentrated as before in the presence of 1 nmol of pyridoxal-5'-P.

Chromatographic Identification of Enzymically Formed 4-Hydroxyphenylpyruvate and Tyrosine. Reaction mixtures (100 μl) containing prephenate-pretyrosine dehydrogenase (4.0 μg protein recovered from gel filtration column chromatography), 50 mM K-phosphate (pH 7.5), 0.5 mM NADP, and either 0.75 mM prephenate or 0.24 mM pretyrosine were incubated for 2 h at 37 C. Authentic 4-hydroxyphenylpyruvate or L-tyrosine (10 nmol) was added to aliquots of control reaction mixtures lacking NADP to mimic the enzymic formation of product. Fifty-μl aliquots of each sample were spotted on Whatman No. 3MM paper. Chromatograms were developed in the ascending direction for 4 h using a solvent of 70:30 (v/v) *n*-propyl alcohol and water (adjusted to pH 7.9 with pyridine), dried, and sprayed with a solution containing equal volumes of 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride. After several minutes blue spots corresponding to tyrosine, 4-hydroxyphenylpyruvate, prephenate, and NADPH were observed against a yellow background and the chromatogram was dipped in 0.1 N HCl and then distilled H_2O to preserve the colors.

Dansylation and Identification of Enzymically Produced Pretyrosine. Reaction mixtures (100 μl) consisted of enzyme (66.6 μg protein of HPP-AT or 118 μg protein of PPA-AT), 1 mM L-glutamate, L-aspartate, or L-alanine, 0.05 mM pyridoxal-5'-P, 3.5 mM prephenate, and 50 mM K-phosphate (pH 7.5). Controls were prepared that lacked prephenate. After incubation at 37 C for 30 min, 10 μl of 4.0 M K_2CO_3 (pH 9.8), and 50 μl of 1% (w/v) dansyl-chloride in acetone were added, and prephenate was added to the controls. After incubation at 37 C for 30 min, the samples were placed in an ice bath. Ten μl of 6.0 M $HClO_4$ and 100 μl of cold acetone were added with rapid mixing. One-μl aliquots were spotted on micropolyamide plates (5 × 5 cm) (Schleicher and Schuell). The plates were developed in solvent I (concentrated ammonium hydroxide-water [1:2, v/v]) and thoroughly dried. The plates were then placed for 5 min in a covered jar saturated with vapors of 88% formic acid in order to convert dansyl-pretyrosine to dansyl-phenylalanine quantitatively. The plates were thoroughly dried and developed in solvent II (benzene-glacial acetic acid-pyridine [50:5:1, v/v/v]) in a direction perpendicular to that of the first solvent. After drying, the plates were illuminated by shortwave UV light and the acid-converted dansyl-derivative of the enzymically produced pretyrosine, dansyl-phenylalanine, was located by comparison with a plate that had been spotted with authentic dansyl-pretyrosine and developed as described above.

Enzyme Assays. The activities of prephenate dehydrogenase, pretyrosine dehydrogenase, and shikimate dehydrogenase were determined by following the continuous increase in fluorescence

[2] Abbreviations: HPP-AT: 4-hydroxyphenylpyruvate aminotransferase; PPA-AT: prephenate aminotransferase.

of NADPH formed at 37 C using an Aminco-Bowman spectrophotofluorometer (excitation 340 nm, emission 460 nm) (3). Reaction mixtures (200 μl) contained 50 mM K-phosphate (pH 7.5), 0.5 mM NADP, enzyme, and either 0.25 mM prephenate, 0.16 mM pretyrosine, or 1.25 mM shikimate.

The activities of aromatic aminotransferase in crude extracts and partially purified preparations recovered from DEAE-cellulose column chromatography were assayed by determining the amount of keto acid product formed using uniformly labeled radioactive amino acids (24). Reaction mixtures (100 μl) contained 50 mM K-phosphate (pH 7.5), 25 μg BSA, 0.05 mM pyridoxal-5'-P, 2.5 mM prephenate or 4-hydroxyphenylpyruvate or phenylpyruvate, 0.186 μCi of L-[^{14}C]glutamate (0.2 mM unlabeled L-glutamate) or 0.113 μCi of L-[^{14}C]alanine (0.2 mM unlabeled L-alanine) or 0.111 μCi of L-[^{14}C]aspartate (0.2 mM unlabeled L-aspartate) and enzyme. Two controls were prepared for each reaction mixture, one lacking enzyme and the other lacking keto acid. The latter control corrects for enzymes present in crude extracts and partially purified preparations that deaminate L-amino acids and produce keto acid products. After incubation for 20 min at 37 C, the reaction was stopped by the addition of 0.3 ml of 1 N HCl. Enzyme or keto acid was added to control mixtures. The keto acid product was then extracted in 1 ml of ethyl acetate-toluene (4:1, v/v), with extraction efficiencies of 19.7, 21.1, and 30.6% for oxaloacetate, α-ketoglutarate, and pyruvate (determined using α-[^{14}C]ketoglutarate, [^{14}C]pyruvate, and a modification of the colorimetric procedure of Friedemann and Haugen (9) for the assay of oxaloacetate). A 200-μl aliquot of the organic phase was transferred to 10 ml of Liquifluor-toluene (42:1000, v/v) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Determination of Protein Concentrations. Aliquots of enzyme preparations were dialyzed overnight at 4 C against 1,000 volumes of 50 mM K-phosphate (pH 7.5), and protein concentrations were determined by the method of Lowry *et al.* (22) using BSA for the calibration of standard curves.

Chemicals. Uniformly labeled L-[^{14}C]glutamate (290 mCi/mmol), L-[^{14}C]alanine (171 mCi mmol), and L-[^{14}C]aspartate (218 mCi mmol) were obtained from Amersham, and were acidified to 1 N with concentrated HCl and extracted five times with 3 volumes of ethyl acetate before use. Liquifluor was obtained from New England Nuclear.

Ammonium pretyrosine was prepared as described by Jensen *et al.* (20) and was quantified as follows. Samples of ammonium pretyrosine were dried under vacuum using NaOH pellets as desiccant. One hundred μl of 0.5 M $NaHCO_3$, adjusted to pH 9.8 with 2.5 N NaOH, and 50 μl of 1.0% (w/v) dansyl-chloride in acetone were added. After incubation at 37 C for 30 min, 50 μl of cold ethanol and 100 μl of cold acetone were added. Samples were clarified by centrifugation in a clinical centrifuge. Five-μl aliquots of the supernatant were spotted on micropolyamide plates (5 × 5 cm) (Schleicher and Schuell). The plates were developed in solvent I (0.5% [v/v] formic acid, adjusted to pH 5.6 with pyridine, and then to pH 8.0 with *N*-ethylmorpholine), and then in solvent II (glacial acetic acid-*N*-ethylmorpholine-benzene [1:10:40, v/v/v]) in a direction perpendicular to that of the first solvent. The plates were illuminated by shortwave UV light and dansyl-pretyrosine was located by comparison with a plate that had been spotted with authentic dansyl-pretyrosine and developed as described above. The dansly-pretyrosine spot was cut out and placed in 0.5 ml of elution solvent (chloroform-methanol-glacial acetic acid [7:2:2, v/v/v]) for 30 min in order to elute dansyl-pretyrosine from the plate and acid-convert it quantitatively to dansyl-phenylalanine. Two hundred-μl aliquots of the elution solvent were read in an Aminco-Bowman spectrophotofluorometer (excitation 365 nm, emission 510 nm). The amount of dansyl-phenylalanine present was determined by comparison to a standard curve prepared from authentic dansyl-phenylalanine.

Barium prephenate was prepared from culture supernatants of a mutant of *Salmonella typhimurium* (6) and was converted to the K salt with excess K_2SO_4 prior to use. The concentration of prephenate was estimated using a molar extinction coefficient of 17,500 at 320 nm for phenylpyruvate in 1 N NaOH following the acid conversion of prephenate to phenylpyruvate (15).

Protein standards for mol wt calibration were obtained from Pharmacia Fine Chemicals. Acids and bases were obtained from J. T. Baker Chemical Company and organic solvents were obtained from Fisher Scientific Company. L-Aspartate and *p*-hydroxymercuribenzoic acid were obtained from Calbiochem and Mann Research Laboratories, respectively. All other biochemicals were obtained from Sigma Chemical Company and were of the highest grade available.

RESULTS

Prephenate-Pretyrosine Dehydrogenase. As summarized in Table I, prephenate and pretyrosine dehydrogenase activities were partially purified from mung bean seedlings. Both prephenate and pretyrosine dehydrogenase activities precipitated in the 50 to 65% ammonium sulfate fraction. Column chromatographic techniques using DEAE-cellulose, hydroxylapatite, and Ultrogel AcA 44 (Fig. 2) did not separate the two activities. The mol wt of prephenate-pretyrosine dehydrogenase was estimated to be 52,000 daltons by gel filtration column chromatography.

NADP-dependent shikimate dehydrogenase is an extremely active enzyme in mung bean seedlings. This enzyme complicates the assay of pretyrosine dehydrogenase since small amounts of shikimate often contaminate preparations of ammonium pretyrosine. With each successive step of purification of prephenate-pretyrosine dehydrogenase, the level of shikimate dehydrogenase decreased (data not shown). In the enzyme preparation recovered from the gel filtration step of purification (which was used for most experiments), shikimate dehydrogenase activity was not detected.

Enzymic conversion of prephenate to 4-hydroxyphenylpyruvate

Table I. *Partial Purification of Prephenate Dehydrogenase and Pretyrosine Dehydrogenase Activities from Mung Bean Seedlings*

The data in parentheses represent pretyrosine dehydrogenase activities. All other data represent prephenate dehydrogenase activities. The activity of pretyrosine dehydrogenase could not be accurately determined in the crude extract and 50 to 65% $(NH_4)_2SO_4$ fraction due to the presence of shikimate dehydrogenase in these preparations and because of contamination of pretyrosine preparations with small amounts of shikimate.

Enzyme Preparation	Volume	Protein	Total Activity	Specific Activity	Purification	Recovery
[a]	*ml*	*mg*	*nmol NADPH/min at 37 C*	*10^{-9} units/mg*	*fold*	*%*
Crude extract	93	5022	4771	0.95	1.0	100
50–65% $(NH_4)_2SO_4$	10	187	1316	7.04	7.4	27.6
DEAE-cellulose	98	26	627 (260)	24.1 (10.0)	25.3	13.1
Hydroxylapatite	78	4.1	496 (223)	121 (54.5)	127	10.4
Ultrogel AcA 44	44	0.62	199 (68)	321 (110)	338	4.2

[a] Crude extract was dialyzed overnight at 4 C against 1,000 volumes of 10 mM K-phosphate (pH 7.5) with 1 mM EDTA and 0.02% (v/v) β-mercaptoethanol prior to determination of enzyme activity.

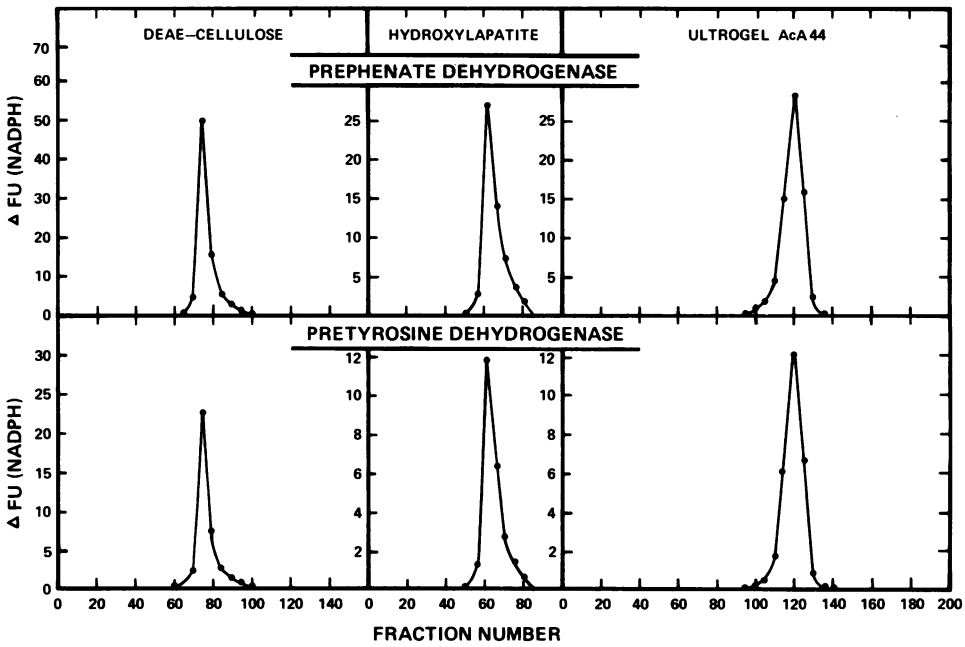

FIG. 2. Co-purification of prephenate dehydrogenase and pretyrosine dehydrogenase activities. Column chromatography was conducted as described under "Materials and Methods." Activities are expressed on the ordinate scale as increase in fluorescence units (ΔFU) per 50 μl of fraction/min. One nmol of NADPH produced corresponds to 19 fluorescence units.

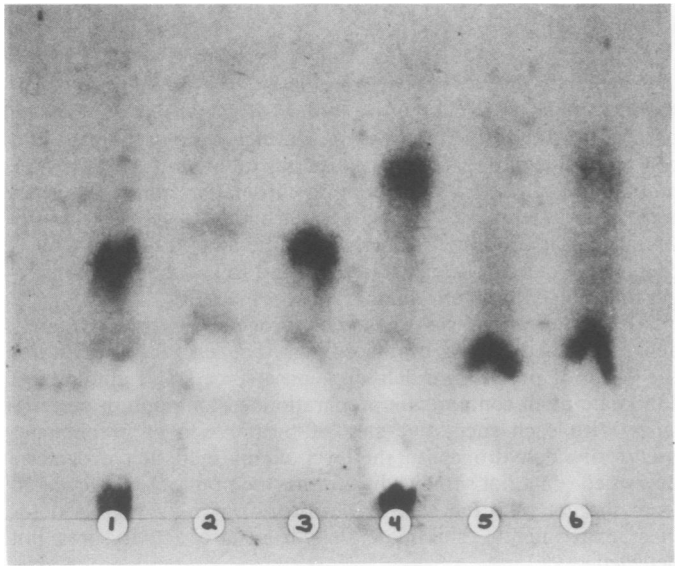

FIG. 3. Paper chromatogram illustrating enzymic formation of tyrosine and 4-hydroxyphenylpyruvate from pretyrosine and prephenate, respectively. Reaction mixtures and controls (which lacked NADP) were prepared, incubated and chromatographed. (1): Reaction mixture for pretyrosine dehydrogenase; (2) control for pretyrosine dehydrogenase; (3) control for pretyrosine dehydrogenase plus 10 nmol authentic L-tyrosine; (4) reaction mixture for prephenate dehydrogenase; (5) control for prephenate dehydrogenase; and (6) control for prephenate dehydrogenase plus 10 nmol authentic 4-hydroxyphenylpyruvate.

and pretyrosine to tyrosine was established qualitatively by paper chromatography. The 4-hydroxyphenylpyruvate and tyrosine products formed were identified by comparison to authentic standards that were added to control mixtures lacking NADP. The control reactions did not form detectable products. The dark V-shaped spot appearing above the origins of sample numbers 5 and 6 of Figure 3 was identified as prephenate since authentic prephenate and this spot exhibited the same mobility. A spot corresponding to prephenate does not appear above the origin of sample number 4 due to the enzymic conversion of prephenate to 4-hydroxyphenylpyruvate. The dark spot near the origin of sample numbers 1 and 4 was identified as NADPH since authentic NADPH and this spot exhibited the same mobility.

Partially purified preparations recovered from gel filtration column chromatography were further studied in order to discern any differential properties that might distinguish the two dehydrogenase activities. The activities of prephenate dehydrogenase and pretyrosine dehydrogenase utilized only NADP as cofactor; NAD was totally inactive. The K_m values for NADP with respect to either dehydrogenase reaction were similar: 14 μM when prephenate was the substrate, and 12 μM when pretyrosine was the substrate. The K_m values of prephenate dehydrogenase for prephenate and pretyrosine dehydrogenase for pretyrosine were 77 μM and 67 μM, respectively. Although the affinities for the two substrates and NADP were very similar, the maximum velocity of prephenate dehydrogenase exceeded that of pretyrosine dehydrogenase (about 2- to 3-fold) throughout each of the purification steps. The specific activity (defined as nmol NADPH formed/min · mg protein at 37 C) of prephenate dehydrogenase in the enzyme recovered from the final step of purification was 2.9 times greater than the specific activity of pretyrosine dehydrogenase. Both activities were maximal at pH 7.5 and were a proportional function of protein concentration in the range of concentrations used (up to 2.4 μg protein recovered from the gel filtration step of purification).

Preincubation of the enzyme preparation recovered from hydroxylapatite column chromatography for 5 min at 37 C in the presence of 1 to 5 μM *p*-hydroxymercuribenzoic acid inhibited the activities of prephenate dehydrogenase and pretyrosine dehydrogenase in parallel. Fifty per cent inhibition of both activities was caused by 1.75 μM *p*-hydroxymercuribenzoic acid, and the addition of excess cysteine reversed the inhibition. These results imply that one or more sulfhydryl groups is essential for the activities of both prephenate dehydrogenase and pretyrosine dehydrogenase. Preincubation of the enzyme preparation recovered from the gel filtration step of purification at 55 C for up to 10 min inactivated

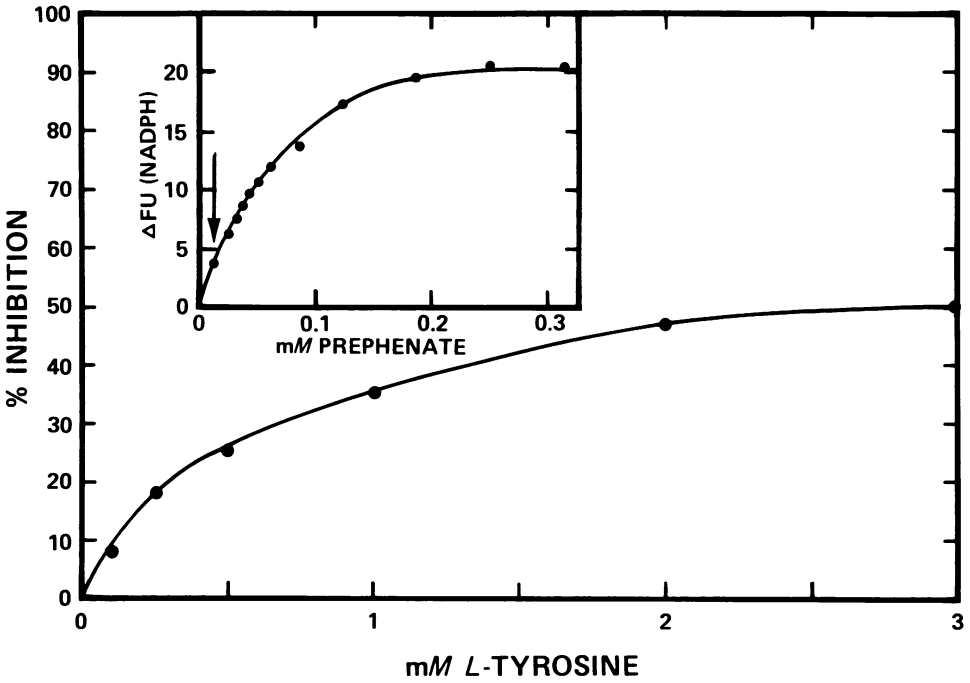

FIG. 4. Inhibition of prephenate dehydrogenase activity by L-tyrosine at nonsaturating levels of prephenate. Concentration of prephenate used was 12.5 μM; relationship of this value to substrate saturation curve for prephenate dehydrogenase is indicated by arrow in inset.

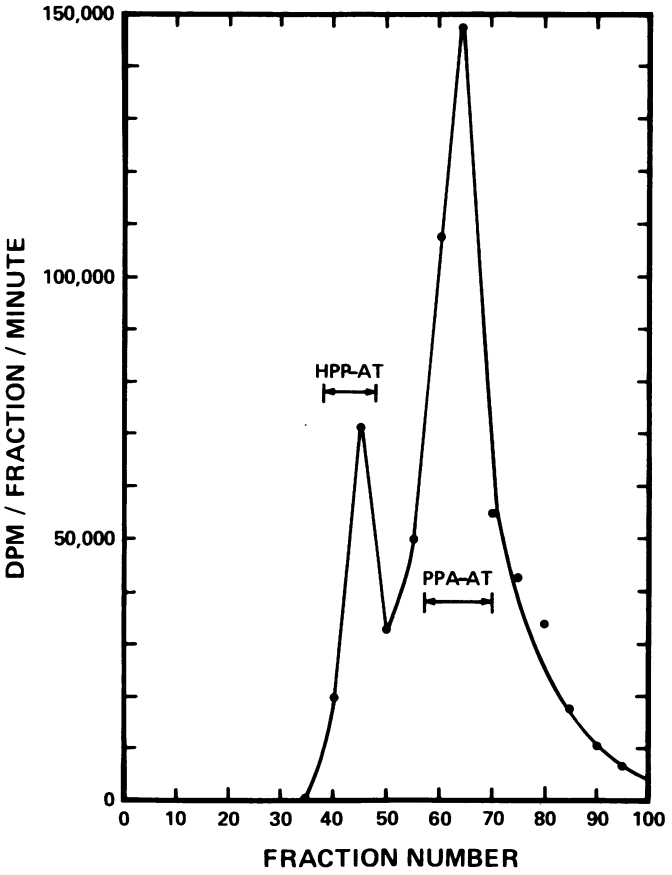

FIG. 5. Isolation of two aromatic aminotransferase activities by DEAE-cellulose column chromatography. Aminotransferase activities were assayed as described under "Materials and Methods" using a mixture of keto acid substrates (prephenate, 4-hydroxyphenylpyruvate, and phenylpyruvate, each at 0.833 mM) and a mixture of radioactive amino acids (L-glutamate, L-aspartate, and L-alanine, each at 66 μM unlabeled L-amino acid and 0.033 μCi L-^{14}C-amino acid). Arrows designate those fractions that were pooled and concentrated.

prephenate dehydrogenase and pretyrosine dehydrogenase activities to the same extent and 1.3 min of incubation caused 50% inhibition of both activities.

The 20 common L-amino acids at 1 mM concentrations did not affect the activities of prephenate dehydrogenase or pretyrosine dehydrogenase (data not shown) under routine assay conditions. In order to detect competitive inhibition by L-tyrosine (the most logical L-amino acid effector of these dehydrogenase activities), the concentrations of prephenate or pretyrosine were decreased 20-fold, and assays for inhibition were repeated. L-Tyrosine inhibited the activity of prephenate dehydrogenase but not of pretyrosine dehydrogenase at nonsaturating levels of substrate (Fig. 4).

Aromatic Aminotransferase Enzymes. The characteristics of aromatic aminotransferases were examined in crude extract and in partially purified preparations recovered from DEAE-cellulose column chromatography. Of the 20 common L-amino acids, only alanine, glutamate, and aspartate were reactive in crude extract in combination with three keto acid substrates (prephenate, 4-hydroxyphenylpyruvate, and phenylpyruvate; data not shown). Eluate fractions were assayed for aromatic aminotransferases by using a mixture of these keto acids and a mixture of radioactive amino acids (L-glutamate, L-aspartate, and L-alanine) in order to detect all aminotransferases reactive with any combination of these keto acid and amino acid substrates. Two peaks of aromatic aminotransferase activity were separated (Fig. 5) and designated HPP-AT and PPA-AT. Both aminotransferase activities were cleanly separated from prephenate-pretyrosine dehydrogenase during partial purification of the latter enzyme (data not shown).

The aminotransferase activities of the crude extract and partially purified enzymes in the presence of the three keto acid substrates and three L-amino acid donors are given in Table II. These data indicate that HPP-AT best utilizes 4-hydroxyphenylpyruvate as substrate, whereas PPA-AT is most active with prephenate. The specific activity (defined as pmol keto acid formed/min·mg protein at 37 C) of PPA-AT (assayed as prephenate aminotransferase) is 2- to 4-fold greater than that of HPP-AT (assayed as 4-hydroxyphenylpyruvate aminotransferase). Both enzymes utilize L-glutamate or L-aspartate, but not L-alanine, as amino donors. Although aromatic aminotransferase activity that utilized L-alanine

as an amino donor was detected in crude extracts, no L-alanine-reactive aminotransferase activity was recovered from DEAE-cellulose by gradient elution.

The results given in Table II indicate the presence of a previously unreported PPA-AT activity in mung bean seedlings. To confirm this, enzymically formed pretyrosine was identified by TLC of the dansyl-derivative. It should be noted that acid conversion of dansyl-pretyrosine to dansyl-phenylalanine was necessary since dansyl-glutamate, dansyl-aspartate, and dansyl-pretyrosine exhibit similar mobilities in both solvents. Dansyl-phenylalanine, but not dansyl-pretyrosine, can be readily separated from dansyl-glutamate and dansyl-aspartate in the second solvent.

Table II. *Comparison of Aromatic Aminotransferase Activities with Various Substrate Combinations in Crude Extract and in Partially Purified Preparations*

Enzyme Preparation	Keto Acid Substrate	Amino-Donor Substrate		
		L-Glutamate	L-Alanine	L-Asparate
		pmol product formed/mg protein · min at 37 C		
Crude extract	Prephenate	702	117	373
	4-Hydroxyphenyl	441	90	91
	Phenylpyruvate	250	123	56
HPP-AT	Prephenate	0	0	283
	4-Hydroxyphenylpyruvate	1167	0	1197
	Phenylpyruvate	0	0	614
PPA-AT	Prephenate	3859	0	2817
	4-Hydroxyphenylpyruvate	572	0	383
	Phenylpyruvate	170	0	192

Figure 6 qualitatively confirms the enzymic formation of pretyrosine when PPA-AT was incubated with prephenate, pyridoxal-5'-P, and either L-glutamate or L-aspartate. No pretyrosine formation was detected by this method using HPP-AT and L-glutamate, L-alanine, or L-aspartate as amino donors, nor with PPA-AT and L-alanine. The small amount of pretyrosine formed by HPP-AT in the presence of prephenate and L-aspartate, as indicated by the data in Table II, is probably below the detection limit of this TLC method. In any event, results indicate the poor ability of aminotransferase HPP-AT to react with prephenate in contrast with the excellent reactivity of aminotransferase PPA-AT with prephenate.

DISCUSSION

Dual Pathways to a Common End Product. It has been shown above that mung bean seedlings have the potential to utilize both the 4-hydroxyphenylpyruvate and pretyrosine pathways of L-tyrosine biosynthesis (Fig. 1). Two separate aromatic aminotransferases were partially purified, one preferentially utilizing 4-hydroxyphenylpyruvate and the other utilizing prephenate to form pretyrosine. The dehydrogenase reactions of each pathway are catalyzed by prephenate-pretyrosine dehydrogenase, which ap-

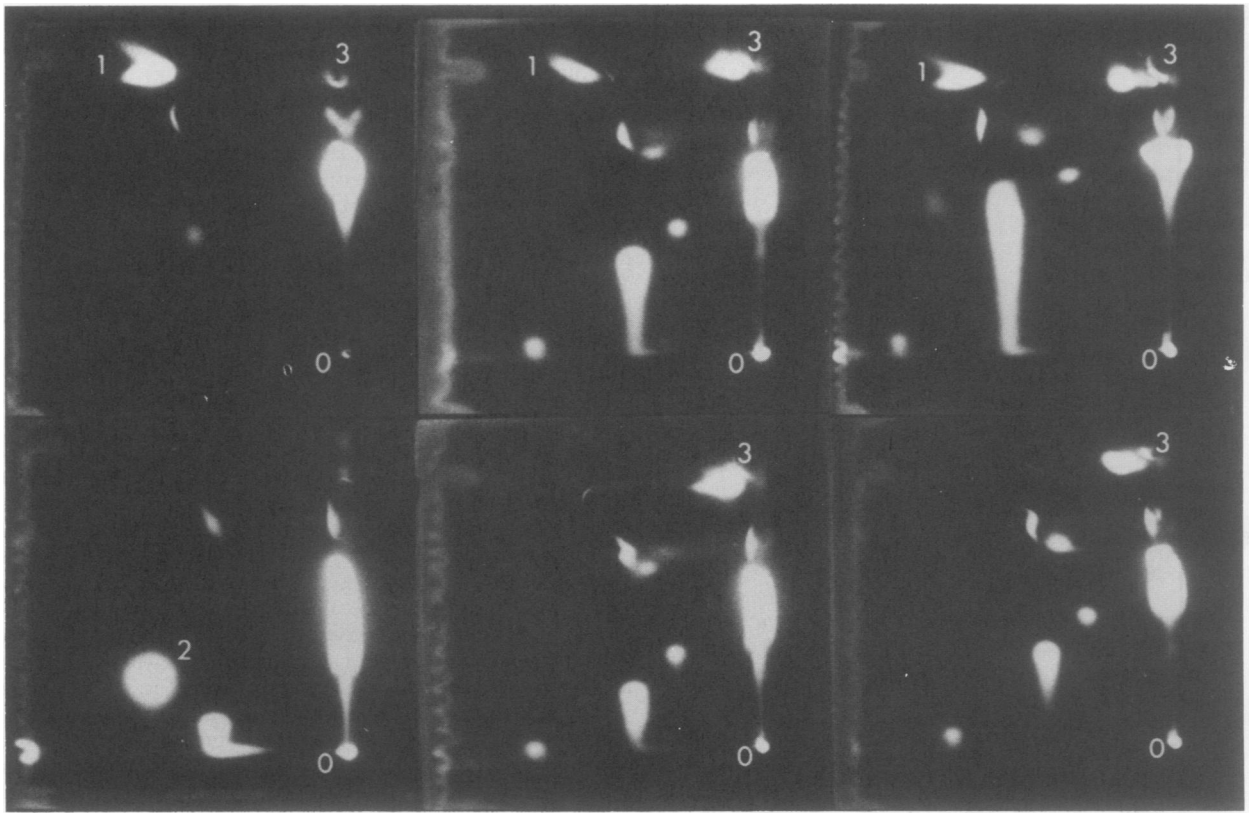

FIG. 6. Identification of pretyrosine formed enzymically by prephenate aminotransferase. Reaction mixtures were prepared, incubated, and dansylated as described under "Materials and Methods." One-μl aliquots were spotted at the origin (0) and the plates were developed in solvent I (bottom to top), exposed to formic acid vapors (to convert dansyl-pretyrosine to dansyl-phenylalanine), and developed in solvent II (right to left). The plates were illuminated by shortwave UV light. Spots corresponding to dansyl-phenylalanine (1) (acid-converted dansyl-pretyrosine) can be seen in the three plates of the top row. The plate in the upper left was spotted with authentic dansyl-pretyrosine. The other two plates in the top row were spotted with reaction mixtures that contained prephenate aminotransferase, prephenate, pyridoxal-5'-P, and either L-aspartate (middle) or L-glutamate (right). The plates in the lower middle and right were spotted with controls for these two reaction mixtures. Spots corresponding to dansyl-phenylalanine are absent in these controls (which were incubated without prephenate). The bottom left plate was spotted with authentic dansyl-phenylalanine and shows that authentic dansyl-phenylalanine (2) and acid-converted dansyl-pretyrosine migrate to the same position in solvent II. The symbol (3) indicates the position of dansyl-aspartate and dansyl-glutamate. All other spots are by-products of the dansylation reaction.

pears to be a single protein. The contribution of each pathway to L-tyrosine biosynthesis *in vivo* is unknown. Perhaps differential carbon flow through the two pathways is a function of tissue differentiation or tissue age.

Another example is known in plants of dual biosynthetic routes to an amino acid involving both dehydrogenase and aminotransferase reactions (2). In the phosphorylated pathway of L-serine biosynthesis, a dehydrogenase, an aminotransferase, and a phosphatase convert 3-P-glycerate to L-serine. In the nonphosphorylated pathway, the loss of the phosphate group precedes the dehydrogenase and aminotranferase reactions. It has been suggested that the phosphorylated pathway is important in rapidly proliferating tissue, whereas the nonphosphorylated pathway is more active in leaf tissue (4).

Cofactor Specificity of Prephenate-Pretyrosine Dehydrogenase. The specificity for NADP of prephenate-pretyrosine dehydrogenase of mung bean seedlings appears to be unique among organisms thus far studied. All prephenate dehydrogenases described in those microorganisms that exclusively employ the 4-hydroxyphenylpyruvate pathway of L-tyrosine biosynthesis have been characterized as NAD-dependent enzymes (19), although it is probable that NADP has not been tested in all cases. The prephenate-pretyrosine dehydrogenase of *Pseudomonas aeruginosa* also utilizes only NAD (25). Recent work by Byng and Jensen (unpublished) reveals other patterns of cofactor specificity among pseudomonad species. In one group (*e.g. P. alkanolytica*) both prephenate dehydrogenase and pretyrosine dehydrogenase utilize only NADP. In a second subgroup (*e.g. P. testosteroni*) prephenate dehydrogenase will use either cofactor while pretyrosine dehydrogenase is NADP-dependent. Yet another subgroup (*e.g. P. cepacia*) contains both NAD-reactive and NADP-reactive species of the two dehydrogenases. L-Tyrosine biosynthesis proceeds exclusively via pretyrosine in blue-green bacteria (19, 27) and coryneform bacteria (7). In blue-green bacteria (DeFuria and Jensen, unpublished) and in coryneform bacteria (7), pretyrosine dehydrogenase can utilize either NAD or NADP, although NADP appears to be the preferred cofactor. In general, NADP specificity is usually associated with pretyrosine dehydrogenase whereas NAD specificity is usually associated with prephenate dehydrogenase.

Is Prephenate-Pretyrosine Dehydrogenase a Multifunctional Protein? If prephenate dehydrogenase and pretyrosine dehydrogenase reactions are catalyzed by a single multifunctional protein, then it would be expected that the two activities not only share common characteristics but also are influenced to the same degree by various inactivating treatments, especially if a single catalytic site accommodates both substrates. It has been shown that both activities of prephenate-pretyrosine dehydrogenase exhibit strict specificity for NADP, and the affinities for this cofactor were similar. The K_m values of prephenate-pretyrosine dehydrogenase for prephenate and pretyrosine were also similar, although the specific activity with prephenate was 2.9 times greater than with pretyrosine at saturating concentrations of substrate. Furthermore, the activities were reduced to the same degree by *p*-hydroxymercuribenzoic acid or heat treatment. These results suggest that a single multifunctional protein catalyzes both dehydrogenase reactions.

One differential characteristic of prephenate dehydrogenase and pretyrosine dehydrogenase activities was noted. In the presence of nonsaturating levels of substrate, L-tyrosine inhibited the activity of prephenate dehydrogenase, but not of pretyrosine dehydrogenase. Prephenate dehydrogenase, but not pretyrosine dehydrogenase, is positioned at a metabolic branch point, and thus the feedback inhibition by L-tyrosine of prephenate dehydrogenase activity, but not of pretyrosine dehydrogenase activity, is consistent with the generalized rules of allosteric regulation. This differential inhibition of prephenate dehydrogenase but not of pretyrosine dehydrogenase activity suggests that prephenate-pretyrosine dehydrogenase may be two different enzyme species having similar chromatographic properties, perhaps as the result of evolutionary derivation from a common ancestral dehydrogenase protein. The possibility of one protein bearing two different catalytic centers cannot be ruled out. In *P. aeruginosa* the two activities of NAD-dependent prephenate-pretyrosine dehydrogenase were not separated (25) using chromatographic procedures similar to those used in the partial purification of prephenate-pretyrosine dehydrogenase from mung bean. However, the failure to obtain *P. aeruginosa* mutants with strict requirements for L-phenylalanine or L-tyrosine suggests the presence of two dehydrogenase genes (Whitaker and Jensen, unpublished).

Spatial Aspects of Enzyme Organization. The localization of the enzymes of the two pathways of L-tyrosine biosynthesis within the plant cell may be particularly interesting. It is known that chloroplasts contain aromatic compounds such as the chloroplastic isoprenoid quinones (16) and flavonoids (28), compounds that are believed to be derived from the shikimate pathway. The enzymes of the entire shikimate pathway may be present within this organelle, and the following evidence supports this possibility. Shikimate dehydrogenase was found to be associated with plastids (8), and all of the enzymes involved in the synthesis of L-tryptophan from chorismate were detected in etioplasts of pea (17). Furthermore, the enzymes of the reductive (21) and oxidative pentose-P cycles (26) have been found in the chloroplast, and thus this organelle generates an internal supply of erythrose-4-P, an early precursor of the shikimate pathway.

Blue-green bacteria synthesize L-tyrosine exclusively by the pretyrosine branchlet (19, 27), which has been hypothesized to be the most ancient pathway of L-tyrosine biosynthesis (18). It has been suggested that the dual enzymic routes to L-tyrosine found in the pseudomonads (25) evolved when pretyrosine dehydrogenase gained reactivity with prephenate and prephenate aminotransferase gained reactivity with 4-hydroxyphenylpyruvate (18). Perhaps genes encoding the enzymes of the 4-hydroxyphenylpyruvate pathway in mung bean likewise evolved from genes of the pretyrosine pathway following the endosymbiotic association of ancient blue-green bacteria with protoeukaryotic cells. If the enzymes of the pretyrosine pathway are found to be localized within the chloroplast, it would support the endosymbiotic theory that chloroplasts arose from endosymbionts similar to modern blue-green bacteria.

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