Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7231–7235, October 1986
Biochemistry

Chloroplasts of higher plants synthesize L-phenylalanine via L-arogenate

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Communicated by Anton Lang, June 23, 1986

ABSTRACT **The specific enzymological route of L-phenylalanine biosynthesis has not been established in any higher plant system. The possible pathway routes that have been identified in microorganisms utilize either phenylpyruvate or L-arogenate as a unique intermediate. We now report the presence of arogenate dehydratase (which converts L-arogenate to L-phenylalanine) in cultured-cell populations of *Nicotiana silvestris*. Prephenate dehydratase (which converts prephenate to phenylpyruvate) was not detected. Arogenate dehydratase was also found in washed spinach chloroplasts, and these data add to emerging evidence in support of the existence in the plastidial compartment of a complete assembly of enzymes comprising aromatic amino acid biosynthesis. Arogenate dehydratase from tobacco and spinach were both specific for L-arogenate, inhibited by L-phenylalanine, and activated by L-tyrosine. Apparent K_m values for L-arogenate (0.3×10^{-3} M), pH optima (pH 8.5–9.5), and temperature optima for catalysis (32–34°C) were also similar.**

The current understanding of the post-prephenate pathways of phenylalanine and tyrosine biosynthesis and of their regulation has depended almost entirely upon a large base of comparative data from both prokaryotic and eukaryotic microorganisms (1). Until recently it had been assumed that higher plants utilize phenylpyruvate and 4-hydroxyphenylpyruvate as biosynthetic precursors of L-phenylalanine and L-tyrosine, respectively. A similar assumption prevailed for microorganisms until 1974, when enzymatic formation of L-arogenate from prephenate and enzymatic conversion of L-arogenate to L-tyrosine was first recognized in cyanobacteria (2). Since then, the arogenate pathway to L-tyrosine has been demonstrated in mung bean (3), corn (4), sorghum (5), tobacco (6), spinach (7), and buckwheat (J. L. Rubin and R.A.J., unpublished data). Prephenate dehydrogenase activity has not been found in any plant system, except in the developmental stage of seed germination in mung bean. Hence, the arogenate pathway appears to be the major, if not exclusive, mode of L-tyrosine biosynthesis in higher plants.

Progress with the phenylalanine pathway has been slower, largely because of the technical difficulty of the enzyme assay (8, 9). Fig. 1 illustrates the two alternative biosynthetic paths to L-phenylalanine that exist in nature. In some microorganisms both pathways are present simultaneously (1). The arogenate pathway utilizes transamination of prephenate to form L-arogenate, followed by decarboxylation, aromatization, and dehydration to yield L-phenylalanine. The phenylpyruvate pathway carries out an initial decarboxylation, aromatization, and dehydration of prephenate to yield phenylpyruvate, which is then transaminated to form L-phenylalanine. In spinach leaves and *Nicotiana silvestris* cultures, we have demonstrated the existence in the plastidial compartment of the arogenate route for phenylalanine biosynthesis.

MATERIALS AND METHODS

Biochemicals. Potassium L-arogenate was prepared from *Neurospora crassa* by a modification of the method of Zamir *et al.* (11). After elution from the third anion-exchange column, the lyophilized L-arogenate was desalted on a Sephadex G-10 column equilibrated with 10 mM potassium phosphate (pH 7.6). For K_m determinations, aliquots of the desalted L-arogenate (≈90% pure) were concentrated by lyophilization. The concentrated solution was diluted, when necessary, with 50 mM potassium Epps [4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid] buffer (pH 9.0). Prephenate (≈75% pure) was prepared as described by Dayan and Sprinson (12). Epps and Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffers were purchased from Research Organics (Cleveland, OH). All other biochemicals were obtained from Sigma.

Plant Materials. Spinach leaves were Waldorf cultivar, obtained from Grand Union (Binghamton, NY). The background and characteristics of the cell line ANS-1 of *N. silvestris* have been described (6). Suspension cultures of these cells were maintained by 1:4 dilution every 8 days into fresh medium. In this subculture routine, the cells undergo a lag phase of ≈36 hr followed by a period of exponential growth that lasts until about day 6, when the cells enter a stationary phase.

Cells were harvested in late-exponential phase between days 4 and 5 on Miracloth filters and were washed three times with 3% (wt/vol) mannitol. Excess wash solution was removed with the aid of a suction filter. The cell pack was frozen in liquid nitrogen and stored at −80°C or was ground in liquid nitrogen using a Waring blender and stored as a frozen powder at −80°C.

Preparation of Partially Purified Extract from *N. silvestris* Cells. Frozen powdered cells (100 g) were combined with 100 ml of extraction buffer (0.1 M potassium Epps, pH 8.0/10% glycerol), thawed in a 37°C bath, and maintained on ice. Cell debris was removed by centrifugation for 10 min at 18,000 × *g*. The supernatant was treated with sufficient ammonium sulfate, added slowly as a chilled saturated solution at pH 8.0, to give a 40%-of-saturation concentration. After a 30-min equilibration with gentle mixing at 4°C, the suspension was centrifuged for 15 min at 18,000 × *g*. The pellet was suspended in 9.5 ml of chilled 50 mM potassium Epps buffer, pH 8.0/10% glycerol (standard buffer). This preparation was further clarified by centrifugation at 18,000 × *g* for 10 min. The partially purified extract, largely free of interfering proteases (determined by detection of free phenylalanine by HPLC after incubation of the extract at 32°C), was desalted on a 34 × 1.5 cm Sephadex G-25 column equilibrated in standard buffer. Protein concentration was determined by the method of Bradford (13).

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Abbreviation: OPA, *o*-phthalaldehyde.

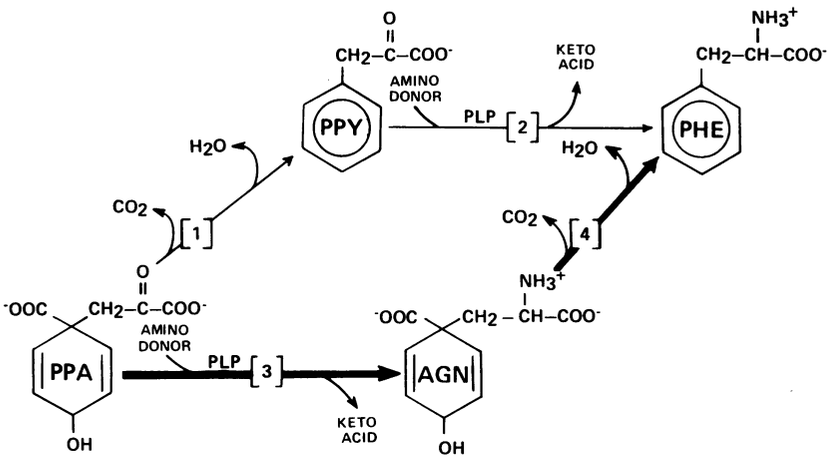

FIG. 1. Alternative pathway routes to L-phenylalanine (PHE) in nature. The flow route through L-arogenate (AGN) is shown with bold arrows and consists of enzymes prephenate aminotransferase [3] and arogenate dehydratase [4]. The flow route through phenylpyruvate (PPY), not presently known to exist in higher plants, is shown with light arrows and consists of enzymes prephenate dehydratase [1] and phenylpyruvate aminotransferase [2]. PPA, prephenate; PLP, pyridoxal 5'-phosphate.

Isolation of Spinach Chloroplasts and Preparation of Extract. Chloroplasts from 100 g of spinach leaves, mid-ribs removed, were isolated by the method of Mills and Joy (14) with the following modifications of the protocol: (*i*) substitution of 50 mM Hepes buffer (pH 7.9) for Tricine buffer, (*ii*) grinding by means of three 1-sec bursts at full speed in a Waring blender, and (*iii*) sedimentation of chloroplasts through the Percoll medium by centrifugation for 1 min at 2000 × *g*. The chloroplast pellet was washed with 20 ml of extraction medium and again centrifuged for 1 min at 2000 × *g*.

Chloroplasts were ruptured osmotically by suspending the pellet in 5–6 ml of cold (4°C) 25 mM potassium phosphate buffer, pH 7.5/1 mM dithiothreitol. The suspension was maintained on ice for 15 min before clarification by a 10-min centrifugation at 12,000 × *g*. The crude extract was desalted on a Sephadex PD-10 column (Sephadex G-25 M, Pharmacia Fine Chemicals) which had been equilibrated in 50 mM potassium Epps buffer, pH 8.2/15% glycerol. All buffers were degassed prior to use.

Assay Method. Reaction mixtures for enzyme assay (100-μl volume) contained 2.5 mM L-arogenate and 0.25 mM L-tyrosine in 0.1 M Epps buffer (pH 9.0). In extract preparations in which protease activity was found to produce undesirable background levels of phenylalanine, 1 μM leupeptin and 1 μM pepstatin were also included. Reactions were terminated after 20 min at 32°C by bringing the mixtures to ice temperature. Enzyme concentrations used were within a range producing proportional activities as discussed below. L-Phenylalanine formation was detected and quantitated as the *o*-phthalaldehyde (OPA) derivative. OPA-phenylalanine was separated from other OPA derivatives by reversed-phase HPLC, and peak-area fluorescence was measured and quantitated by comparison with an OPA-phenylalanine standard curve. The OPA-phenylalanine derivative was prepared by mixing 30 μl of reaction mixture or L-phenylalanine standard with 150 μl of the OPA reagent (200 μl of 2-mercaptoethanol added to a mixture of 54 mg of OPA dissolved in 1 ml of methanol and diluted with 9 ml of 0.4 M sodium borate buffer, pH 9.4). After 90 sec the reaction was complete, and the sample was injected into a 20-μl sample loop. The sample was then loaded onto an Ultrasphere 3-μm ODS column (7.5 × 4.6 mm; Altex, Berkeley, CA) at a rate of 1 ml per min. The OPA derivatives were separated and eluted with methanol/20 mM potassium phosphate buffer, pH 6.9, 55:45 (vol/vol). OPA-phenylalanine, having a retention time of 2.8 min, was detected with a Model FL-1B fluorometer (Gilson) equipped with a 360-nm (cutoff) excitation filter and 455-nm (cutoff) emission filter. Peak areas were measured with a Hewlett–Packard model 3390 A integrator. Activity is reported as nmol of L-phenylalanine formed per min per mg of protein.

RESULTS

Extract Preparation. Crude extracts from *N. silvestris* cells initially failed to yield activity for arogenate dehydratase or for prephenate dehydratase, even after routine testing of many combinations of possible stabilizing or activating conditions of enzyme preparation, storage, and assay. Once conditions for the assay of arogenate dehydratase were worked out in partially purified preparations of enzyme, it became possible to detect enzyme activity in crude extracts qualitatively, but quantitative results were still unsatisfactory. The major problem appeared to be the high protease activities that generated L-phenylalanine. Since dehydratase activity was low (about 1 nmol/min per mg of crude-extract protein) and the assay depends upon measurement of L-phenylalanine, the background phenylalanine causes large errors. We recently have found that protease activity is very high in stationary-phase cultures, and a substantial amount is carried over into populations that have been in exponential phase for only two generations (i.e., 4–5 days after subculture, when cultures were usually harvested for extract preparation). Routine detection of arogenate dehydratase was improved by inclusion of the protease inhibitors leupeptin and pepstatin at 1 μg per ml.

In attempts at separating most of the protease activity from arogenate dehydratase, it was found that arogenate dehydratase salted out in the 25–40% cut during ammonium sulfate fractionation, leaving the bulk of protease activity in solution. This step resulted in a 2-fold increase in specific activity (to 2.5 nmol/min per mg of protein), with recovery of total starting activity estimated to be 60%. The presence of glycerol, dithiothreitol, EDTA, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and L-tyrosine during extract preparation from *N. silvestris* did not result in a greater yield of arogenate dehydratase in the 25–40% ammonium sulfate fraction. Residual protease activity was inhibited by a combination of leupeptin and pepstatin. All further characterizations of the *N. silvestris* enzyme in this report were carried out with desalted preparations of this partially purified enzyme.

In crude extracts prepared from leaf tissue of spinach, low specific activities of about 0.09 nmol/min per mg of protein were obtained, and background protease activities were again a problem. When extracts were prepared from washed chloroplasts, in contrast, protease activity was not a hindrance. Specific activity in the best preparations was increased about 1 order of magnitude. Specific activities in extracts prepared from different chloroplast preparations varied, giving values from 0.8 to 3.4 nmol/min per mg of protein, presumably because of variation in different batches of market spinach.

Stability. Desalted extracts (after the ammonium sulfate step) obtained from *N. silvestris* cells or from spinach chloroplast extracts were stable at least 4 weeks during storage at −80°C. A 15-min incubation of enzyme from either tobacco or spinach at 37°C in the absence of substrate resulted in about a 30–40% loss of activity prior to addition of substrate and assay at 32°C (Fig. 2). Glycerol, dithiothreitol, or L-tyrosine (allosteric activator) failed to protect arogenate dehydratase from thermal inactivation at 37°C. Each dehydratase enzyme was substantially inactivated (85–95%) at 45°C for 15 min.

Catalytic Properties. The optimal temperature for arogenate dehydratase catalysis was similar in both tobacco and spinach, about 32°C and 34°C, respectively. Fig. 3 shows the temperature profile obtained with the spinach chloroplast enzyme. In the presence of L-tyrosine, the temperature-optimum profile was skewed to the right, indicating the ability of L-tyrosine to protect against thermal denaturation. This was surprising because the thermal inactivation shown in Fig. 2 in the absence of substrate was not altered by the presence of L-tyrosine (data not shown). Hence, the combined presence of L-arogenate and L-tyrosine is evidently required for protection. Assays were usually carried out at 32°C, where activities were a proportional function of time for at least 25 min (Fig. 4) over a range of enzyme concentrations up to that present in 100 μg of extract protein per reaction vessel (Fig. 5).

The pH profile for catalysis was almost identical for both tobacco and spinach arogenate dehydratase (Fig. 6). Because of the difficulty in finding buffers having a $pK_a > 9.5$ that did not either inhibit the enzyme (as did potassium carbonate buffer) or interfere with the HPLC detection of L-phenylalanine, Epps buffer ($pK_a = 8.0$) was used at higher pH values. A final concentration of 0.2 M Epps buffer satisfactorily stabilized the pH during the assay period, even at pH 11. The facile nonenzymatic conversion of L-arogenate to L-phenylalanine at acidic pH (15) complicated the assay at pH values <7.0.

Substrate saturation data indicated a first-order dependence of reaction rate upon substrate concentration. When these data were plotted on double-reciprocal coordinates (Figs. 7 and 8), apparent K_m values of 0.3×10^{-3} M for arogenate dehydratase from both tobacco and spinach were obtained.

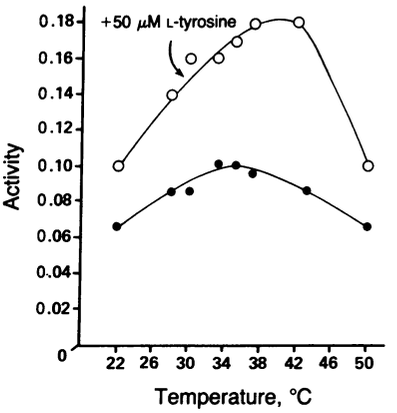

FIG. 3. Temperature optimum for catalytic function of arogenate dehydratase from spinach chloroplasts. Reaction mixtures contained 0.10 μmol of potassium L-arogenate, 94 μg of protein, 60 μl of 0.4 M potassium Epps buffer (pH 9.0), and L-tyrosine, if indicated, in a final volume of 100 μl. Reaction mixtures were incubated at the specified temperatures for 20 min. Activity on the ordinate scale is in nmol of L-phenylalanine formed per min.

Up to 2.5 mM Mg^{2+} and Mn^{2+} did not affect arogenate dehydratase activity from tobacco. Zn^{2+} or Cu^{2+} at 2.5 mM abolished the activity completely, while 2.5 mM Co^{2+} reduced activity about 50%. Dithiothreitol, reduced glutathione, or ATP (in the presence of 1 mM Mg^{2+}) did not affect enzyme activity when tested at 0.25 mM.

Allosteric Regulation. L-Tyrosine activated arogenate dehydratase from both tobacco and spinach. Maximal activation was obtained at about 0.25 mM L-tyrosine (data not shown). The double-reciprocal plots shown in Figs. 7 and 8 indicate that L-tyrosine increases the apparent maximum velocity, V_{max}, and does not affect affinity for L-arogenate.

Background levels of L-phenylalanine, added as inhibitor,

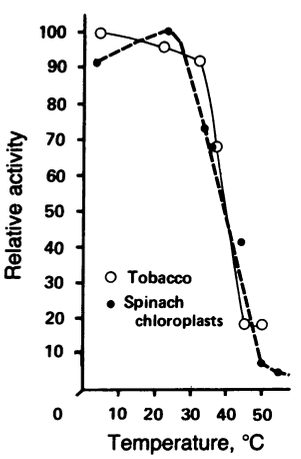

FIG. 2. Thermal stability of arogenate dehydratase. For *N. silvestris* (tobacco) extracts, 87 μg of protein in 0.26 M potassium Epps buffer (pH 9.0) was incubated 15 min at the specified temperature. Activity was then determined by incubating at 32°C for 20 min with 2.4 mM L-arogenate. For spinach chloroplast extracts, 100 μg of protein in 0.13 M potassium Epps buffer (pH 9.5) was incubated 15 min at the specified temperature. Activity was then determined by incubating at 33°C for 30 min with 1.4 mM L-arogenate. Relative activities of 100 correspond to specific activities of 2.5 and 2.2 nmol/min per mg of protein for tobacco and for spinach chloroplast extracts, respectively.

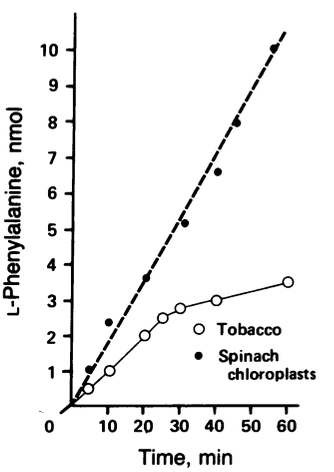

FIG. 4. Proportionality of reaction rate as a function of time. For *N. silvestris* (tobacco) extracts, reaction mixtures contained 0.24 μmol of potassium L-arogenate (added as a 9.4 mM solution in 20 mM potassium phosphate buffer, pH 8.5), 89 μg of protein, and 50 μl of 0.4 M potassium Epps buffer (pH 9.0) in a total volume of 100 μl. Samples were incubated at 32°C for the specified elapsed times. For spinach chloroplast extracts, a total volume of 100 μl of reaction mixture contained 0.14 μmol of potassium L-arogenate (added as a 5.6 mM solution in 20 mM K phosphate, pH 8.5), 100 μg of protein, and 50 μl of 0.2 M potassium Epps buffer (pH 9.5). Samples were incubated at 32°C for the specified elapsed times.

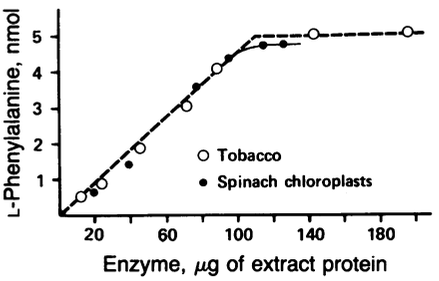

FIG. 5. Proportionality of reaction rate as a function of enzyme concentration. For *N. silvestris* (tobacco) each reaction mixture contained 0.24 μmol of potassium L-arogenate, the indicated amount of extract protein, and 50 μl of 0.4 M potassium Epps buffer (pH 9.0) in a final volume of 100 μl. Samples were incubated at 32°C for 20 min. For spinach chloroplasts, each reaction mixture contained 0.2 μmol of potassium L-arogenate, the indicated amount of protein, and 50 μl of 0.4 M potassium Epps buffer (pH 9.0) in a final volume of 100 μl. Samples were incubated at 33°C for 20 min.

were relatively high compared to the L-phenylalanine product generated enzymatically. Table 1 shows that arogenate dehydratase from spinach chloroplasts was increasingly inhibited as substrate levels were decreased, indicating that inhibition is probably competitive or mixed. We estimated that at K_m levels of L-arogenate, the arogenate dehydratase from spinach chloroplasts was inhibited 50% by about 35 μM L-phenylalanine. Better determinations will be possible when radiolabeled L-arogenate becomes available so that labeled reaction product can be distinguished from unlabeled inhibitor.

Arogenate dehydratase activity was not affected by L-tryptophan, and the presence of L-tryptophan in combination with L-tyrosine or L-phenylalanine did not affect activation or inhibition by these allosteric agents. Caffeic acid, an inhibitor of some aromatic-pathway isoenzymes present in the cytosol of higher plants (7), did not affect the activity of arogenate dehydratase either.

DISCUSSION

Enzymatic Makeup of the Phenylalanine Pathway. Radiolabeled shikimate has been shown to be incorporated into both L-phenylalanine and L-tyrosine in higher plants (16). Two avenues of evidence have been offered in the literature in support of the conclusion that phenylpyruvate is the immediate precursor of L-phenylalanine in higher plants. (*i*) When prephenate and L-glutamate were incubated with extracts prepared from various plants, L-phenylalanine was produced (17). (*ii*) When labeled phenylpyruvate was administered to plant cells, labeled L-phenylalanine was produced (18). The first line of evidence is, however, equally consistent with the operation of the arogenate pathway of phenylalanine

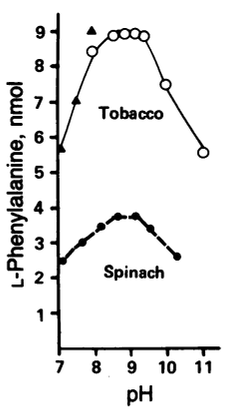

FIG. 6. Arogenate dehydratase activity as a function of pH. For *N. silvestris* (tobacco) the pH of reaction mixtures containing 0.24 μmol of potassium L-arogenate and 87 μg of protein was adjusted with 50 μl of 0.4 M potassium phosphate (▲) or potassium Epps buffer (○) in a total volume of 100 μl. Reactions proceeded for 20 min at 32°C. For spinach chloroplasts, the pH of reaction mixtures containing 0.12 μmol of potassium L-arogenate and 100 μg of protein were adjusted with 50 μl of 0.4 M potassium Epps buffer (●) in a total volume of 125 μl. Reactions were terminated after 30 min at 33°C.

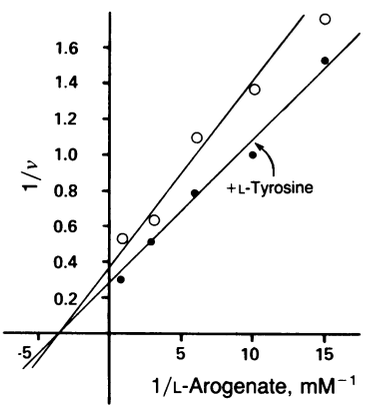

FIG. 7. Double-reciprocal plot of substrate saturation data for arogenate dehydratase from *N. silvestris*. A final volume of 100 μl of reaction mixture contained the specified amount of potassium L-arogenate, 87 μg of extract protein, and a final Epps buffer concentration of 0.1 M at pH 9.0. Elapsed reaction times were 20 min at 32°C. Where specified, L-tyrosine was present at 0.25 mM. *v*, Velocity.

biosynthesis, since either pathway requires only the input of prephenate plus L-glutamate. In fact, the presence of the arogenate pathway probably explains the inability of Gamborg and Simpson (17) to detect prephenate dehydratase, even though the overall conversion was demonstrated.

The second line of evidence is unconvincing because aminotransferases of broad specificity are inevitably present in microbial and plant cells (19) and will function with phenylpyruvate. It has been shown, for example, that a mutant of *Brevibacterium flavum* lacking arogenate dehydrogenase accumulates L-arogenate and is auxotrophic for L-tyrosine, thus proving the role of L-arogenate as the sole precursor of L-tyrosine (20). Nevertheless, the mutant can utilize exogenous 4-hydroxyphenylpyruvate in place of tyrosine. It fails to use the 4-hydroxyphenylpyruvate pathway for endogenous synthesis of L-tyrosine because it cannot synthesize 4-hydroxyphenylpyruvate. By analogy, ability to transaminate exogenous phenylpyruvate is irrelevant to phenylalanine biosynthesis from prephenate if the organism lacks the enzymic capacity to form phenylpyruvate from prephenate.

To the extent that phenylpyruvate plays any important role(s) in plant metabolism [e.g., in formation of ubiquinones (21) or certain alkaloids (16)], it seems likely that phenylpyruvate would be formed by transamination of L-phenylalanine rather than vice versa. An entirely parallel case would

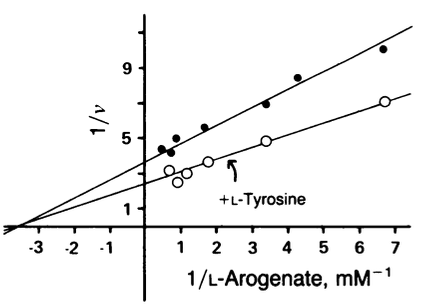

FIG. 8. Double-reciprocal plot of substrate data for arogenate dehydratase from a spinach chloroplast extract. A final volume of 100 μl of reaction mixture contained from 15 to 200 nmol of potassium L-arogenate, 100 μg of protein, and a final Epps buffer concentration of 0.2 M at pH 9.0. Elapsed reaction times were 30 min at 33°C. Where specified, L-tyrosine was present at 0.37 mM concentration. *v*, Velocity.

Table 1. Inhibition of arogenate dehydratase from spinach chloroplasts by L-phenylalanine

L-Arogenate, mM	L-Phenylalanine, μM	% inhibition
2.10	25	18
1.50	25	16
1.00	25	25
0.52	25	39
2.10	50	23
1.50	50	26
1.00	50	34
0.52	50	61

Arogenate dehydratase was assayed in extracts prepared from washed spinach chloroplasts as described in the text. Specific activities corresponding to the four substrate concentrations used in the absence of L-phenylalanine were 2.2, 1.3, 1.0, and 0.8 $nmol \cdot min^{-1} \cdot mg^{-1}$.

be the important role established for 4-hydroxyphenylpyruvate in the biosynthesis of tocopherol and plastoquinone in spinach chloroplasts (22). Since spinach chloroplasts have good activity for arogenate dehydrogenase and tyrosine/2-ketoglutarate aminotransferase but not for prephenate dehydrogenase (7), the metabolite order undoubtedly is:

prephenate → L-arogenate → L-tyrosine → 4-hydroxyphenylpyruvate.

The demonstration of arogenate dehydratase activity in two unrelated plant species indicates that phenylalanine in higher plants may be synthesized exclusively or mainly via L-arogenate. This possibility is reinforced by a number of other findings. Prephenate dehydratase activity has never been reported in even a single plant species. L-Arogenate is already known to be at least a major precursor of L-tyrosine in various higher plant species (3–7). A highly active aminotransferase enzyme having an unusually narrow substrate specificity for prephenate exists in mung bean (3), tobacco (23), and sorghum (C. A. Bonner and R.A.J., unpublished data). L-Arogenate plays a role as a feedback inhibitor of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase-Mn) in the chloroplast (24, 25). This regulation would be maximally attractive (see later section) if L-arogenate accumulates as a consequence of feedback-inhibition of arogenate dehydrogenase and arogenate dehydratase.

Compartmentation and Regulation. Most if not all of the arogenate dehydratase from spinach was estimated to be present in the chloroplast compartment (7). This adds a major element to the emerging picture of the arrangement of the aromatic pathway and its regulation in the plastidial compartment. Arogenate dehydrogenase is sensitive to inhibition by micromolar levels of L-tyrosine (6), while arogenate dehydratase is sensitive to inhibition by micromolar levels of L-phenylalanine. Thus, excess levels of L-phenylalanine and L-tyrosine should increase plastidial levels of L-arogenate, which then would inhibit via feedback inhibition of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase-Mn, the plastidial isoenzyme that catalyzes the initial step of aromatic biosynthesis (24, 25). Anthranilate synthase in spinach chloroplasts (7) is sensitive to feedback inhibition by micromolar levels of L-tryptophan. Thus, the plastid-localized pathway appears to be subject to precise, fine-tuned allosteric control consisting of feedback circuits that operate in a pattern termed "sequential feedback inhibition" (26, 27).

In contrast to prephenate/arogenate dehydratase [e.g., that from *P. aeruginosa* (28)], substrate-specific species of prephenate dehydratase or arogenate dehydratase have so far proven to be highly sensitive to inhibition by L-phenylalanine (1). Although prephenate dehydratase is commonly activated by L-tyrosine, this report cites activation of an arogenate dehydratase by L-tyrosine.

Whether arogenate dehydratase activity might exist in the cytosol as well as in plastids is unknown. However, it has been found that in buckwheat, when phenylalanine-ammonia lyase was specifically inhibited by L-α-aminooxy-β-phenylpropionate *in vivo*, substantial phenylalanine accumulated (29). This indicates that phenylalanine biosynthesis in the cytosol must not be subject to feedback inhibition. Therefore, a so-far-undetected species of arogenate dehydratase (or prephenate dehydratase) may exist in the cytosol that is insensitive to feedback. The established presence of unregulated isoenzymes of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase and chorismate mutase in the cytosol (7), together with suggestive findings in potato of a second, feedback-insensitive isoenzyme of anthranilate synthase (30) and in wax bean of a feedback-insensitive prephenate dehydrogenase (31), lend support to the contention that secondary-metabolite formation in the cytosol is essentially an overflow phenomenon (10).

Euglena gracilis (32) and higher plant chloroplasts now stand as examples of the aromatic-pathway type in which L-arogenate is located at the metabolic branchpoint of phenylalanine and tyrosine biosynthesis, L-arogenate thus being a precursor of both amino acids.

This research was supported by Grant PCM 8309070 from the Metabolic Biology Program of the National Science Foundation.

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