ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 238, No. 1, April, pp. 237-246, 1985

Novel Features of Prephenate Aminotransferase from Cell Cultures of *Nicotiana silvestris*[1]

CAROL A. BONNER AND ROY A. JENSEN[2]

Center for Somatic-Cell Genetics and Biochemistry, Department of Biological Sciences, State University of New York at Binghamton, Binghamton, New York 13901

Received August 13, 1984, and in revised form December 4, 1984

A prephenate aminotransferase enzyme that produces L-arogenate was demonstrated in extracts from cultured-cell populations of *Nicotiana silvestris*. The enzyme was very active with low concentrations of prephenate, but required high concentrations of phenylpyruvate or 4-hydroxyphenylpyruvate to produce activity levels that were detectable. It is the most specific prephenate aminotransferase described to date from any source. Only L-glutamate and L-aspartate were effective amino-donor substrates. Prephenate concentrations greater than 1 mM produced substrate inhibition, an effect antagonized by increasing concentrations of L-glutamate cosubstrate. The enzyme was stable to storage for at least a month in the presence of pyridoxal 5′-phosphate, EDTA, and glycerol, and exhibited an unusually high temperature optimum of 70°C. The identity of L-arogenate formed during catalysis was verified by high-performance liquid chromatography. DEAE-cellulose chromatography revealed two aromatic aminotransferase activities that were distinct from prephenate aminotransferase and which did not require the three protectants for stability. The aromatic aminotransferases were active with phenylpyruvate or 4-hydroxyphenylpyruvate as substrates, but not with prephenate. Both of the latter enzymes were similar in substrate specificity, and each exhibited a temperature optimum of 50°C for catalysis. The primary *in vivo* function of the two aromatic aminotransferases is probably to transaminate between the aspartate/2-ketoglutarate and glutamate/oxaloacetate couples, since activities with the latter substrate combinations were an order of magnitude greater than with aromatic substrates. The demonstrated existence of a specific prephenate aminotransferase in *N. silvestris* meshes with other evidence supporting an important role for L-arogenate in tyrosine and phenylalanine biosynthesis in higher plants.

The cyclohexadienyl amino acid, L-arogenate, is the immediate precursor of L-tyrosine and/or L-phenylalanine in many microorganisms, including those of both procaryotic and eucaryotic lineage (1). Whether L-phenylalanine biosynthesis requires L-arogenate or phenylpyruvate as a precursor in any higher plant has not yet been established. However, L-arogenate does appear to be a major intermediate of L-tyrosine biosynthesis in higher plants, based upon results obtained with mung bean (2), corn (3), tobacco (4), and duckweed (unpublished data). Since L-arogenate is essential at least for L-tyrosine biosynthesis in *Nicotiana silvestris*, the transamination of prephenate (i.e., by prephenate aminotransferase) is an obligatory biosynthetic step.

Figure 1 illustrates the three aminotransferase reactions that are relevant to the biosynthesis of aromatic amino ac-

[1] This research was supported by Department of Energy Contract EP-78-S-024967.

[2] To whom correspondence should be addressed.

0003-9861/85 $3.00

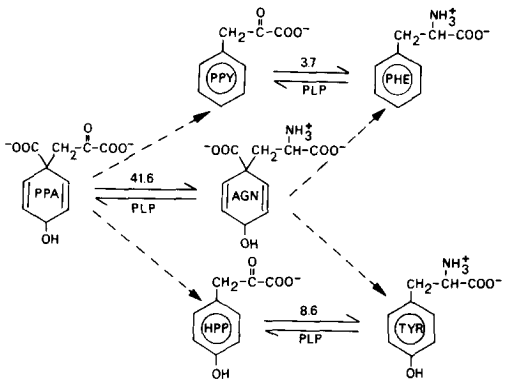

FIG. 1. Aromatic-pathway aminotransferase activities catalyzed by crude extracts prepared from suspension-cultured cells of *N. silvestris*. Specific activities (nmol min^{-1} mg $protein^{-1}$) are shown for prephenate (PPA) aminotransferase which forms L-arogenate (AGN), phenylpyruvate (PPY) aminotransferase which forms L-phenylalanine (PHE), and 4-hydroxyphenylpyruvate (HPP) which forms L-tyrosine (TYR). Specific activities were obtained at 2 mM concentrations of the indicated keto acid and 2 mM L-[^{14}C]glutamate. Dotted arrows indicate dehydratase (upper level) and NAD/NADP dehydrogenase (lower level) enzyme reactions which may function in various combinations in nature (1). PLP indicates pyridoxal 5'-phosphate.

ids in nature. Aromatic-pathway aminotransferases are notorious for their lack of substrate specificity (5). In *Escherichia coli*, genes specifying aspartate aminotransferase (*aspC*), branched-chain aminotransferase (*ilvE*), and aromatic aminotransferase (*tyrB*) exhibit overlapping function between three major metabolic networks (6). It is not uncommon to find that aminotransferases which transaminate only phenylpyruvate and 4-hydroxyphenylpyruvate *in vivo* are additionally able to transaminate prephenate *in vitro*, e.g., as in *Neurospora crassa* (7, 8). On the other hand, aminotransferases that only transaminate prephenate *in vivo* may nevertheless be capable *in vitro* of good catalysis when phenylpyruvate or 4-hydroxyphenylpyruvate are provided as keto acid substrates, e.g., as in *Euglena gracilis* (9). In *Brevibacterium flavum* at least two aminotransferase enzymes that were capable of transaminating 4-hydroxyphenylpyruvate *in vitro* were shown to exist (10), even though 4-hydroxyphenylpyruvate was proven not to be a precursor of L-tyrosine in this organism (11).

In a number of higher plants (2, 12), we have observed excellent aminotransferase activity with prephenate as substrate. As part of an overall objective to characterize the biochemistry and regulation of aromatic biosynthesis in *N. silvestris* (4, 13-16), prephenate aminotransferase is a key enzyme for study. Against the backdrop of information available about microbial aminotransferases, it is of interest to pursue the nature of enzymes in higher plants that can transaminate precursors of L-phenylalanine and L-tyrosine with respect to (i) multiplicity of enzymes, (ii) number of substrate reactants that can be accommodated by any given aminotransferase, and (iii) possible overlapping substrate specificities if multiple aminotransferases exist. Prior to this report, no aromatic-pathway aminotransferase has been found to possess the selectivity for prephenate that we describe here for prephenate aminotransferase from *N. silvestris*.

MATERIALS AND METHODS

Organism. Line ANS-1 of *N. silvestris* has been maintained in suspension culture for about 4 years. It originated from haploid tissue, and its isolation has been described in detail (4). Although the latter paper reported an average chromosome number of 64, strain ANS-1 has exhibited a stable, average chromosome number of 42-44 for the past 2 years. The line is maintained by periodic subculture whereby cultured-cell populations are diluted fivefold with fresh medium after 8 days of incubation at room temperature in continuous light. A typical growth curve consists of a 36-h lag phase, a period of exponential growth (doubling time = 36-40 h) that persists for a little more than two generations, and a transition to stationary phase that is complete at about 6 days.

Preparation of crude extracts. Late exponential-phase cells (5 days after subculture) were harvested on Miracloth filters and washed with a 3% (w/v) mannitol osmoticum. The cell pack was frozen in liquid nitrogen, powdered in a Waring blender, and stored at −80°C until extract preparation. The frozen cell powder (70 g) was dispersed in 100 ml of 50 mM K phosphate buffer (pH 7.2) containing 0.1 mM

pyridoxal 5'-phosphate (PLP),[3] 1.0 mM EDTA, and 20% glycerol (v/v). Additives such as polyvinylpolypyrrolidone, XAD-4 adsorbent, dithiothreitol, β-mercaptoethanol, phenylmethylsulfonyl fluoride, and Na ascorbate were tested as protectants and found to have no beneficial effects. The slurry was passed through two layers of cheesecloth surrounding one layer of Miracloth, and the filtrate was centrifuged at 55,000*g* for 10 min at 4°C. The supernatant was desalted by passage through a Sephadex G-25 column previously equilibrated with the protectant-containing buffer described above.

Aminotransferase nomenclature. Any aminotransferase capable of utilizing prephenate as a substrate *in vitro* is referred to as prephenate aminotransferase. In *N. silvestris* only a single prephenate aminotransferase has been detected. This is denoted aminotransferase AT-C (for cyclohexadienyl). Any aminotransferase capable of utilizing the aromatic keto acids (phenylpyruvate and 4-hydroxyphenylpyruvate) *in vitro* is referred to as aromatic aminotransferase, although this term does not necessarily imply major (or even minor) function *in vivo* with aromatic substrates. In *N. silvestris* the two aromatic aminotransferases are denoted AT-A_1 and AT-A_2 (A for aromatic).

DEAE-cellulose chromatography. A bed of DEAE-cellulose (2.5 × 21 cm) was equilibrated with 50 mM K-phosphate buffer (pH 7.2) containing 0.1 mM PLP, 1.0 mM EDTA, and 20% glycerol. A 142-mg amount of protein was applied to the column, which was then washed at 4°C with 280 ml of equilibration buffer. A 0 to 0.6 M KCl gradient (600 ml), prepared in equilibration buffer, was applied to the column. Fractions of 4.0-ml volume were collected at 4°C.

Aminotransferase assay procedures. Reaction mixtures (100 μl) contained 25 μl of enzyme and the indicated concentrations of keto acid and amino acid substrates in 50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (Epps) buffer, pH 8.6. Reactions were run under conditions where linear rates were maintained. Incubation times varied from 10 to 30 min, depending upon the activity of the preparation used, substrate concentrations, and the assay temperature. At the lowest concentration of prephenate used (1 mM), linear rates were observed up to 20% conversion of prephenate to L-arogenate. Prephenate aminotransferase could be assayed between 37 and 70°C, while aromatic aminotransferase activities (i.e., with phenylpyruvate or 4-hydroxyphenylpyruvate) could be assayed between 37 and 50°C.

Radiolabeled assay: 2 mM concentrations of 0.1 μCi ^{14}C-labeled-amino acids were used. Reactions were stopped by bringing the temperature to 0°C followed by addition of 300 μl of 1 N HCl. One milliliter of ethylacetate:toluene (4:1) was added to the acidified reaction mixture. To ensure consistent extraction efficiencies, each sample was vortexed for 10 s, and the phases were allowed to partition completely before removal of 400 μl from the organic layer into ScintVerse I scintillation fluid for ^{14}C isotope counting. Extraction efficiencies of 21 and 19% were determined for commercially obtained samples of 2-ketoglutarate and oxaloacetate, values in agreement with those previously found (2).

HPLC assay: Reactions were stopped by bringing temperature to 0°C followed by addition of 10 μl of 1 N HCl. Amino acid products were measured by high-performance liquid chromatography. When L-arogenate was the product, it was measured as L-phenylalanine since the acid-labile L-arogenate is quantitatively converted to L-phenylalanine (17). To ensure total conversion, the acid-treated reaction samples were incubated at 37°C for 10 min prior to addition of 5 μl of 2 N NaOH. A 20-μl aliquot was derivatized by addition of 100 μl of *o*-phthalaldehyde (OPA) reagent. After 90 s, the sample was injected and peak heights were determined. Quantitation was carried out by relating peak heights to standard curves constructed for each run using authentic samples of amino acids. A C-18 ODS reverse-phase HPLC column from Altech Applied Science was used. OPA reagent consisted of 200 μl 2-mercaptoethanol added to a mixture of 54 mg of OPA dissolved in 1 ml methanol and 9 ml of 0.4 M Na borate. The OPA derivatives of amino acids were detected with a Gilson Model FL-1B fluorometer (excitation filter, 360 nm; emission filter, 455 nm). Dicarboxylic amino acids eluted with short retention times. L-Arogenate was successfully separated from L-glutamate by use of a 20–60% methanol gradient made with 20 mM Na phosphate buffer, pH 6.9. L-Aspartate and L-glutamate were separated by use of a 10–60% methanol gradient. When L-phenylalanine, L-tyrosine, or L-alanine were measured as reaction products, methanol solvents of 60%, 45% and 45%, respectively, were used.

Determination of protein concentration. The Bradford assay (18) was used to estimate protein concentrations, with bovine serum albumin as reference standard.

Biochemicals. L-Arogenate (90% pure) and prephenate (83% pure) were isolated from a multiple auxotroph of *N. crassa* (ATCC 36373) as described in Ref. (8). The purities were uncorrected for water of solvation, and negligible amounts of organic impurities were present. The barium salt of prephenate was converted to the potassium salt with a twofold excess of K_2SO_4 prior to use as substrate. Bradford

[3] Abbreviations used: PLP, pyridoxal 5'-phosphate; Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; OPA, *O*-phthalaldehyde; Pipes, piperazine-*N*,*N*'-bis[2-ethane sulfonic acid].

reagent was obtained from Bio-Rad (Rockville Center, N. Y.). ^{14}C-Labeled amino acids were obtained from New England Nuclear (Boston, Mass.). ScintVerse I was obtained from Fisher Scientific (Rochester, N. Y.). All other biochemicals were obtained from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Three keto acid intermediates of aromatic amino acid biosynthesis may potentially serve as aminotransferase substrates (Fig. 1). These include a cyclohexadienyl compound (prephenate) and the two aromatic compounds (phenylpyruvate and 4-hydroxyphenylpyruvate). Aminotransferase activities with all three compounds were detected in crude extracts prepared from suspension-cultured cells of *N. silvestris*. However, the specific activity of prephenate aminotransferase exceeded that of either phenylpyruvate aminotransferase or 4-hydroxyphenylpyruvate aminotransferase strikingly. These three activities *a priori* could be the consequence of one aminotransferase of broad specificity, of multiple aminotransferases having narrow specificities, or of multiple aminotransferases having overlapping substrate specificities.

That prephenate aminotransferase activity might be the property of an enzyme distinct from one or more enzymes carrying out reactions with phenylpyruvate and/or 4-hydroxyphenylpyruvate was first suggested by studies evaluating aminotransferase stability in crude extracts. Figure 2 shows that prephenate aminotransferase activity decayed progressively at 4°C with a halflife of 3–6 days, depending on the buffer. After 2 weeks, most or all prephenate aminotransferase activity was lost. This instability was totally prevented by inclusion of PLP, EDTA, and glycerol (see Materials and Methods) in the storage buffer. The latter protectants were only partially effective when present individually. In contrast to prephenate aminotransferase, 4-hydroxyphenylpyruvate aminotransferase (inset of Fig. 2) and phenylpyruvate aminotransferase activities retained stability for at least 2 weeks in the absence of protective agents. These results implicate the existence of

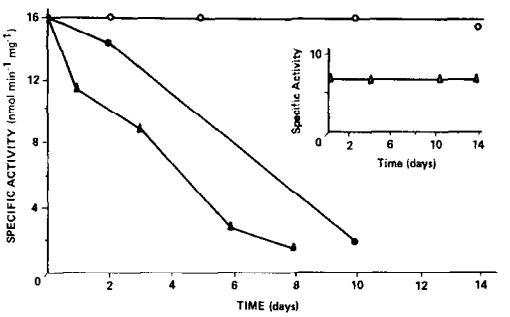

FIG. 2. Stability of aromatic-pathway aminotransferase activities after indicated times of storage at pH 7.0 and 4°C. Crude extracts were prepared as described under Materials and Methods in the following buffers: (▲), 50 mM Pipes buffer; (●), 50 mM K-phosphate buffer; and (○), 50 mM K-phosphate buffer containing 1 mM EDTA, 0.1 mM PLP, and 20% glycerol. Prephenate aminotransferase (prephenate/L-glutamate) specific activities were determined at the indicated elapsed times of storage. The inset (△) shows the retention of stability when aromatic aminotransferase activity with 4-hydroxyphenylpyruvate and L-[^{14}C]glutamate was followed in an extract prepared in 50 mM Pipes buffer. Data shown were obtained at 37°C by radiolabeled assay using 2 mM concentrations of all substrates.

at least two catalytic species, presumably different proteins. This possibility was further supported by results demonstrating differential thermostability of prephenate aminotransferase compared to aromatic aminotransferase. When crude extracts were heated for 25 min at 40, 50, 60, 70, and 80°C prior to assay of aminotransferase activities at 37°C by HPLC assay using substrates at 2 mM, prephenate aminotransferase activity was unchanged up to 70°C of heat treatment, while aromatic aminotransferase activities (with either phenylpyruvate or 4-hydroxyphenylpyruvate) were inactivated 80 and 100% when heated at 60 and 70°C, respectively. This differential thermostability was paralleled by results obtained from comparisons of temperature optima for transamination of prephenate and transamination of phenylpyruvate/4-hydroxyphenylpyruvate. Figure 3 shows that when prephenate aminotransferase activity was followed at progressively higher reaction temperatures, an unusually high temperature optimum of about 70°C was

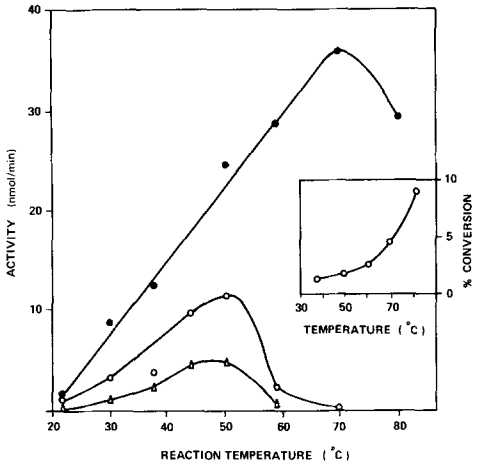

FIG. 3. Differing temperature optima of aromatic-pathway aminotransferase activities depending upon whether prephenate (●), 4-hydroxyphenylpyruvate (○), or phenylpyruvate (△) were used as substrate (2 mM) in combination with L-[^{14}C]glutamate (2 mM). Crude extracts were incubated with substrate mixtures for 30 min at the indicated temperatures. The inset shows the nonenzymatic conversion of prephenate to phenylpyruvate at pH 8.6 in Epps buffer at the indicated temperatures.

observed. On the other hand, both phenylpyruvate and 4-hydroxyphenylpyruvate aminotransferase activities exhibited temperature optima that peaked around 50°C. At 70°C, where prephenate aminotransferase was most active, activities with phenylpyruvate and 4-hydroxyphenylpyruvate were selectively lost. Figure 3 (inset) shows that nonenzymatic formation of phenylpyruvate from prephenate increases with temperature, a potential problem with the radiolabeled assay. However, at temperatures where 5% conversion was approached, aromatic aminotransferase activity was completely inactivated. Hence, the possibility that transamination of phenylpyruvate that was formed nonenzymatically might be mistaken for prephenate transamination at higher temperature can be ruled out.

These results showed that a thermostable prephenate aminotransferase must exist that fails to utilize the two aromatic substrates under the assay conditions employed. However, it was still possible that one or more phenylpyruvate/4-hydroxyphenylpyruvate aminotransferases of lesser thermostability might also utilize prephenate. The latter possibility was diminished, however, by the isolation from DEAE-cellulose of an aminotransferase (AT-A_1) that was able to utilize the aromatic substrates, but not prephenate. Figure 4 shows elution profiles of three aminotransferase activities. Aminotransferase AT-A_1 failed to bind to DEAE-cellulose and eluted in the wash fractions, widely separated from the single, large peak of prephenate aminotransferase activity (AT-C) which eluted in the salt gradient at about 0.13 M KCl. A second aromatic-utilizing aminotransferase peak

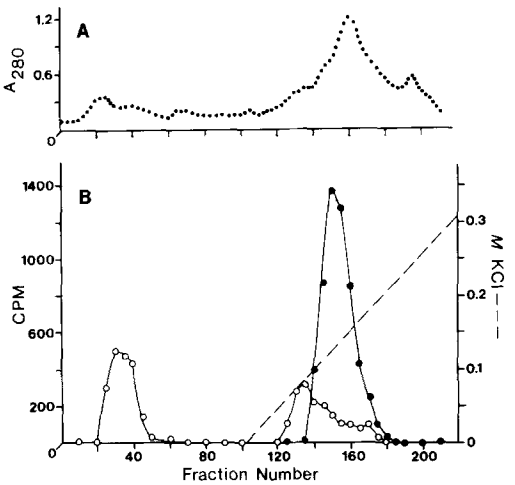

FIG. 4. Separation of aromatic-pathway aminotransferases by DEAE-cellulose chromatography. Procedural details are given under Materials and Methods. (A) The A_{280} profile corresponding to the eluate fractions indicated on the abscissa scale. Fractions were assayed for prephenate aminotransferase (●) with 2 mM prephenate and 2 mM L-[^{14}C]glutamate and for aromatic aminotransferase (○) with 2 mM 4-hydroxyphenylpyruvate and 2 mM L-[^{14}C]glutamate. For clarity data points are not shown for the phenylpyruvate/glutamate couple, but activities were completely parallel (but lower) to those shown for the 4-hydroxyphenylpyruvate/glutamate couple. Activities given on the ordinate scale are cpm of extracted [^{14}C]-2-ketoglutarate. The leftward and rightward peaks of aromatic aminotransferase are denoted AT-A_1 and AT-A_2, respectively. Peak positions were initially located by assay at 37°C. The data shown were obtained by assay of prephenate aminotransferase at 70°C and of aromatic aminotransferase at 50°C.

was located in the salt gradient at about 0.09 M KCl.

Fractions 120-135, containing the leading eluate portion of aminotransferase AT-A_2, did not overlap with aminotransferase AT-C, and these fractions were pooled for preliminary characterization. No significant differences were found between the properties of aminotransferase AT-A_1 and AT-A_2. Both aminotransferase AT-A_1 and AT-A_2 could be temperature inactivated at 70°C prior to assay of aminotransferase AT-C at 37-50°C, with no inactivation of the AT-C enzyme under the same conditions. For example, partially purified aminotransferase AT-A_1 (sp act: = 74.3 nmol min^{-1} mg^{-1} with 4-hydroxyphenylpyruvate and 39.4 with phenylpyruvate at 50°C) was totally inactivated even after several minutes at 70°C prior to assay at 50°C. On the other hand, aminotransferase AT-C can be selectively inactivated over a period of 2 weeks if protectants are removed (as specified under Fig. 2), with no inactivation of aminotransferases AT-A_1 or AT-A_2. The data given in Table I show that thermal inactivation of aminotransferase AT-A_2 (i.e., loss of activity with phenylpyruvate or with 4-hydroxyphenylpyruvate) at 70°C was exceedingly rapid during the assay of aminotransferase AT-C at 70°C. In fact, aromatic aminotransferases can be selectively inactivated in mixtures containing aminotransferase AT-C during assay at 70°C without the necessity of preassay inactivation.

TABLE I

DIFFERENTIAL THERMAL INACTIVATION OF AROMATIC-PATHWAY AMINOTRANSFERASE ACTIVITIES IN A MIXTURE OF AT-C AND AT-A_2 ENZYMES[a]

	Activity (nmol min^{-1} mg^{-1})			
	Assay at 50°C		Assay at 70°C	
Keto acid substrate	Not heated	Heated[b]	Not heated	Heated[b]
Prephenate	64.2	63.1	108	111
4-Hydroxyphenylpyruvate	10.7	-0-	-0-	-0-
Phenylpyruvate	6.1	-0-	-0-	-0-

[a] Fractions 135-180 (Fig. 4) were pooled.

[b] The enzyme preparation was maintained at 70°C for 17 min prior to assay at the indicated temperature in the presence of L-[^{14}C]glutamate (2 mM) and the indicated keto acid substrate (2 mM).

We considered the possibility that transamination of prephenate as determined by the radioactive assay might be only apparent, e.g., owing to contamination of prephenate with some other keto acid. Hence, HPLC was carried out to confirm directly that the product of the reaction was indeed L-arogenate. The results given in Fig. 5 clearly show that L-glutamate decreased in parallel with the formation of L-arogenate. The identity of L-arogenate was established both by coincidence of retention time with that of authentic L-arogenate, and by facile aromatization to L-phenylalanine at acidic pH.

pH-activity profiles were followed separately for preparations of aminotransferase AT-A_1 and AT-C (heat-treated at 70°C) that were recovered from DEAE-cellulose. The two aminotransferases exhibited nearly identical pH optima, with activity rising sharply between pH 6 and pH 8 and presenting optimal activity around pH 8.25.

The cyclohexadienyl-utilizing (AT-C) and aromatic-utilizing (AT-A_1) aminotransferase preparations recovered from DEAE-cellulose were examined in more detail for substrate specificities. The three aromatic-pathway keto acids were tested in combination with all of the common amino acids. Only L-glutamate and L-aspartate were efficient cosubstrates. These amino-donor reactants were then tested in combination with an expanded group of possible keto acid substrates as shown in Table II. Aminotransferase AT-C used prephenate, but not phenylpyruvate or 4-hydroxyphenylpyruvate, in combination with either L-glutamate or L-aspartate. It also utilized oxaloacetate or pyruvate in combination with L-glutamate; 2-ketoglutarate, but not pyruvate, was utilized in combination with L-aspartate. Aminotransferase AT-A_1 differed markedly from aminotransferase AT-C. It failed to utilize prephenate, but was re-

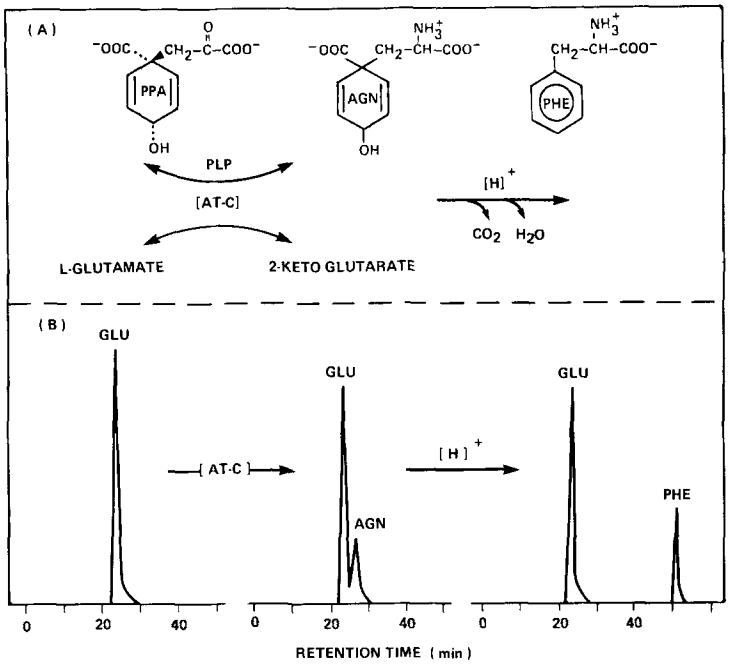

FIG. 5. Confirmation by HPLC of the identity of the prephenate aminotransferase reaction product as L-arogenate. (A) Prephenate and L-glutamate are converted to L-arogenate and 2-ketoglutarate by prephenate aminotransferase (AT-C) recovered from DEAE-cellulose and freed of contaminating AT-A_2 by heat inactivation (see Table I). If the L-arogenate product is acidified, quantitative conversion to L-phenylalanine occurs. (B) HPLC detection of amino acid molecules as described under Materials and Methods. From left to right are shown the results of HPLC injections before initiation of the reaction, after 30 min of reaction time, and after acidification of the latter sample. Authentic samples of L-glutamate (GLU), L-arogenate (AGN), and L-phenylalanine (PHE) yielded peaks identical to those shown with respect to retention times (23, 26, and 50 min, respectively).

active with 4-hydroxyphenylpyruvate and phenylpyruvate in combination with either L-glutamate or L-aspartate. However, the latter activities were modest compared to activities generated with combinations of L-glutamate and oxaloacetate, or of L-aspartate and 2-ketoglutarate. The results obtained with aminotransferase AT-A_2 (not shown) were entirely comparable to the results shown in Table II for aminotransferase AT-A_1. The data indicate that aminotransferases AT-A_1 and AT-A_2 may in fact be aspartate/2-ketoglutarate aminotransferases that happen to possess sufficient breadth of specificity to function weakly with aromatic substrates.

The specificity of aminotransferase AT-C was examined in more detail using the HPLC assay and varying substrate concentrations between 1 and 20 mM as shown in Table III. At progressively higher prephenate concentrations, substrate inhibition was observed. This was overcome partially at high (20 mM) concentrations of L-glutamate. None of the other keto acid substrates showed substrate inhibition. At 20 mM concentrations of 4-hydroxyphenylpyruvate or phenylpyruvate, aminotransferase activity was detected. These activities, however, were more than an order of magnitude lower than those obtained with prephenate at 20-fold lower concentrations. Maximal activity for the prephenate/L-glutamate couple appears to occur between 0.5 and 1.0 mM prephenate.

DISCUSSION

In vivo function of aromatic-pathway aminotransferases. Figure 1 illustrates

TABLE II

SUBSTRATE SPECIFICITIES OF AROMATIC-PATHWAY AMINOTRANSFERASES FROM *N. silvestris*

Keto acid substrates (2 mM)	^{14}C-Labeled amino acid substrates (2 mM)	Aminotransferase[a] (nmol min^{-1} mg^{-1})	
		AT-C	AT-A_1
Prephenate	L-Glutamate	157	0
4-Hydroxyphenylpyruvate		0	85
Phenylpyruvate		0	41
2-Ketoglutarate		251	739
Oxaloacetate		125	592
Pyruvate		73	6
Indolepyruvate		0	0
Prephenate	L-Aspartate	77	0
4-Hydroxyphenylpyruvate		0	9
Phenylpyruvate		0	3
2-Ketoglutarate		156	262
Oxaloacetate		118	428
Pyruvate		0	0

[a] Enzyme recovered from DEAE-cellulose was used. Aminotransferase AT-C was assayed at 70°C while aminotransferase AT-A_1 was assayed at 50°C.

that endogenous formation of phenylpyruvate and 4-hydroxyphenylpyruvate depends upon the existence of prephenate dehydratase and prephenate dehydrogenase. If the latter enzymes are absent, then *in vitro* capability for transamination of phenylpyruvate and 4-hydroxyphenylpyruvate is superfluous. In higher plants prephenate dehydratase activity has never been found. Prephenate dehydrogenase has only been observed in mung bean (2), and it seems possible that this activity might be specialized to the developmental stage of seedling germination. If transamination of prephenate observed *in vitro* is physiologically significant, then arogenate dehydrogenase and arogenate dehydratase enzymes would be obligatory for conversion of L-arogenate to L-tyrosine and L-phenylalanine, respectively. In *N. silvestris* both an NADP-linked arogenate dehydrogenase (4) and arogenate dehydratase (unpublished data) have indeed been found.

N. silvestris possesses three separable species of aminotransferase that are capable of utilizing phenylpyruvate, 4-hydroxyphenylpyruvate, or prephenate as keto acid substrate. Phenylpyruvate and 4-hydroxyphenylpyruvate are poor substrates for all three aminotransferases. Species AT-A_1 and AT-A_2 both exhibited high preference for aspartate/2-ketoglutarate, while species AT-C showed preference for prephenate. Aminotransferases AT-A_1 and AT-A_2 probably play no role in biosynthesis of aromatic amino acids. Most likely, they are general aspartate/2-ketoglutarate aminotransferases. Since 4-hydroxyphenylpyruvate has been shown to be a precursor of tocopherol and plastoquinone (19), aminotransferases AT-A_1 and/or AT-A_2 might function to transaminate L-tyrosine to 4-hydroxyphenylpyruvate.

In extracts containing mixtures of all three aminotransferase species, conditions have been found for selective inactivation of prephenate aminotransferase (i.e., AT-C) or for selective loss of phenylpyruvate or 4-hydroxyphenylpyruvate transamination (i.e., AT-A_1 and AT-A_2). These selective inactivations were complete at 2 mM substrate concentrations, concentrations chosen for order-of-magnitude approximation of intracellular conditions.

TABLE III

EFFECT OF SUBSTRATE CONCENTRATIONS UPON APPARENT SPECIFICITY OF AMINOTRANSFERASE AT-C

Keto acid substrate	Rate (nmol/min) with L-Glutamate	
	2 mM	20 mM
1 mM		
Prephenate	2.22	4.37
4-Hydroxyphenylpyruvate	-0-	0.09
Phenylpyruvate	-0-	0.06
Oxaloacetate	1.93	2.87
Pyruvate	0.23	0.55
2 mM		
Prephenate	1.78	3.86
4-Hydroxyphenylpyruvate	-0-	0.12
Phenylpyruvate	-0-	0.11
Oxaloacetate	2.27	4.63
Pyruvate	0.21	0.75
20 m*M*		
Prephenate	0.86	3.05
4-Hydroxyphenylpyruvate	0.17	0.57
Phenylpyruvate	0.06	0.40
Oxaloacetate	2.50	5.72
Pyruvate	0.23	0.87

Note. Crude extracts of *N. silvestris* were assayed by the HPLC method as described under Materials and Methods. Reactions were run for 10 min at 70°C. Similar relative numbers were obtained when extract was thermally treated at 70°C for 20 min prior to assay at 37°C. Each reaction vessel contained 37 μg of extract protein.

Prephenate aminotransferase. The narrow substrate specificity of aminotransferase AT-C strongly supports a specialized role *in vivo* for transamination of prephenate. Transamination of phenylpyruvate or 4-hydroxyphenylpyruvate by aminotransferase AT-C was only detectable at substrate concentrations that seem unreasonably high within the intracellular milieu. The specificity for prephenate is quite striking against the background of existing data in the microbial literature, where prephenate aminotransferases thus far described utilize phenylpyruvate or 4-hydroxyphenylpyruvate *in vitro* about as well as prephenate. The specificity for prephenate seen in *N. silvestris* may apply to higher plants in general since aminotransferase activity with prephenate has consistently exceeded that found with phenylpyruvate or 4-hydroxyphenylpyruvate in all other higher plants examined so far, e.g., mung bean (2) and other plant organisms (corn, sorghum, and duckweed; unpublished data).

Aminotransferase AT-C showed substrate inhibition with prephenate, but not with oxaloacetate or pyruvate. Increasing concentrations of L-glutamate partially antagonized the extent of substrate inhibition. This is consistent with expectations for a ping-pong mechanism in which prephenate is the second substrate to bind to the enzyme. With such a mechanism, the substrate with the highest affinity (prephenate) can show relatively low activities at high substrate (prephenate) concentrations. As the preferred substrate for aminotransferase AT-C, prephenate utilization is most apparent at concentrations of 1 mM and below. Although detailed kinetic characterization remains to be carried out, it is apparent that aminotransferase AT-C has greater affinity for prephenate than for other substrates such as oxaloacetate. High affinity for prephenate would seem to be a physiologically significant feature since intracellular concentrations of a compound like prephenate are expected to be low.

The temperature optimum for aminotransferase AT-C of *N. silvestris* is strikingly high. The physiological significance of this characteristic is unknown, and it remains to be seen whether other prephenate aminotransferases from higher plants will also exhibit resistance to denaturation at elevated temperature. Practical advantages of the thermal stability of aminotransferase AT-C include (i) inactivation and denaturation of other proteins at 70°C in purification routines, (ii) ability to monitor prephenate aminotransferase free from possible interference by other enzymes present in crude extracts, and (iii) good prospects for preparation of L-arogenate through use of the tobacco enzyme immobilized on a column matrix operated at high temperature. A precedent for successful immobilization of prephenate aminotransferase exists

whereby a cyanobacterial prephenate aminotransferase was immobilized for operation at 37°C (20).

ACKNOWLEDGMENT

We thank Robert Tiberio for advice and assistance with HPLC techniques.

REFERENCES

1. BYNG, G. S., KANE, J. F., AND JENSEN, R. A. (1982) *Crit. Rev. Microbiol.* **9,** 227-252.
2. RUBIN, J. L., AND JENSEN, R. A. (1979) *Plant Physiol.* **64,** 727-734.
3. BYNG, G. S., WHITAKER, R. J., FLICK, C., AND JENSEN, R. A. (1981) *Phytochemistry* **20,** 1289-1292.
4. GAINES, C. G., BYNG, G. S., WHITAKER, R. J., AND JENSEN, R. A. (1982) *Planta* **156,** 233-240.
5. JENSEN, R. A., AND CALHOUN, D. H. (1981) *Crit. Rev. Microbiol.* **8,** 229-266.
6. GELFAND, D. H., AND STEINBERG, R. A. (1977) *J. Bacteriol.* **130,** 429-440.
7. JENSEN, R. A., ZAMIR, L. O., ST. PIERRE, M., PATEL, N., AND PIERSON, D. L. (1977) *J. Bacteriol.* **132,** 896-903.
8. ZAMIR, L. O., JUNG, E., AND JENSEN, R. A. (1983) *J. Biol. Chem.* **258,** 6492-6496.
9. BYNG, G. S., WHITAKER, R. J., SHAPIRO, C. L., AND JENSEN, R. A. (1981) *Mol. Cell Biol.* **1,** 426-438.
10. FAZEL, A. M., AND JENSEN, R. A. (1979) *J. Bacteriol.* **140,** 580-587.
11. FAZEL, A. M., BOWEN, J. R., AND JENSEN, R. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1270-1273.
12. STENMARK, S. L., PIERSON, D. L., GLOVER, G. I., AND JENSEN, R. A. (1974) *Nature (London)* **247,** 290-292.
13. RUBIN, J. L., GAINES, C. G., AND JENSEN, R. A. (1984) *Plant Physiol.* **75,** 839-845.
14. D'AMATO, T. A., GANSON, R. J., GAINES, C. G., AND JENSEN, R. A. (1984) *Planta* **162,** 104-108.
15. GOERS, S. K., AND JENSEN, R. A. (1984) *Planta* **162,** 109-116.
16. GOERS, S. K., AND JENSEN, R. A. (1984) *Planta* **162,** 117-124.
17. ZAMIR, L. O., TIBERIO, R., AND JENSEN, R. A. (1983) *Tetrahedron Lett.* **24,** 2815-2818.
18. BRADFORD, M. M. (1976) *Anal. Biochem.* **72,** 248-254.
19. FIEDLER, E., SOLL, J., AND SCHULTZ, G. (1982) *Planta* **155,** 511-515.
20. ZAMIR, L. O., JUNG, E. D., AND TIBERIO, R. D. (1982) *Bioorg. Chem.* **11,** 32-42.