Tyrosine Biosynthesis in *Sorghum bicolor:* Isolation and Regulatory Properties of Arogenate Dehydrogenase

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Arogenate Dehydrogenase, Prephenate Dehydrogenase, Tyrosine Biosynthesis, Shikimate Pathway, *Sorghum bicolor*

The conversion of prephenic acid to tyrosine can occur by two different routes: (a) oxidative decarboxylation (prephenate dehydrogenase) followed by transamination (aromatic aminotransferase); (b) transamination of prephenate forming the non-aromatic amino acid arogenic acid (prephenate aminotransferase) followed by oxidative decarboxylation (arogenate dehydrogenase).

High activity of arogenate dehydrogenase was found in extracts of etiolated sorghum seedlings, while no evidence of prephenate dehydrogenase was observed. Arogenate dehydrogenase from sorghum eluted, with high recovery of activity (93%), as a single peak on DEAE-cellulose chromatography. The enzyme was strongly inhibited by tyrosine but was unaffected by phenylalanine, prephenate, or tryptophan. Kinetic analysis showed that tyrosine inhibition was competitive with arogenate and that the K_i for tyrosine (61 μM) was much smaller than the K_m for arogenate (350 μM).

The properties of arogenate dehydrogenase indicate that this enzyme is important in the regulation of tyrosine biosynthesis in sorghum. Strong inhibition of the enzyme by tyrosine may indicate that arogenate is a branch point in the shikimate pathway in plants and therefore arogenate may be a precursor to phenylalanine and the numerous phenylpropanoid secondary metabolites derived from phenylalanine.

Introduction

Large quantities of tyrosine are utilized by *Sorghum bicolor* seedlings for the biosynthesis of the cyanogenic glucoside dhurrin [1–3]. In addition, the amino acid is required for protein biosynthesis and the numerous phenylpropanoids known to exist in such seedlings [4]. The level of dhurrin in green seedlings can be as large as 60 μmol per gram of tissue [5] and etiolated seedlings accumulate similar amounts. It can be calculated from the data of Neucere and Sumrell [6], that the total tyrosine (free and protein bound) in sorghum seeds is a small percentage (*e.g.* <5%) of that required for the biosynthesis of dhurrin during the first few days of seedling growth. This fact indicated that the enzymes of tyrosine biosynthesis should be highly active in sorghum seedlings. Although quantitative data are not available, it is probable that most of the carbon flow through the shikimate pathway, in sorghum seedlings, is directed towards dhurrin *via* tyrosine.

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Phenylalanine and tyrosine are important as precursors in the biosynthesis of a wide variety of secondary metabolites in addition to dhurrin, such as flavonoids [7], lignin precursors [8], phenolic acids [9] and coumarins [10]. However, little is known about the regulation of aromatic amino acid biosynthesis or the processes that partition carbon between the branches of the shikimate pathway (for a review see Gilchrist and Kosuge [11]). Indeed the exact sequence of reactions in both the phenylalanine and tyrosine branches of the shikimate pathway in plants has not been established.

Early work demonstrating prephenate dehydrogenase activity in mung bean, waxbean and soybean [12, 13] and the conversion of prephenate to tyrosine and phenylalanine by extracts of mung bean [14], suggested that the pathway in plants was identical to the aromatic keto-acid pathway demonstrated in enteric bacteria and fungi (Fig. 1) [15, 16]. However, it should be noted that Gamborg [13] was unable to detect prephenate dehydrogenase activity in many plants tested.

The generality of the aromatic keto-acid pathway was implicitly accepted for many years until Sten-

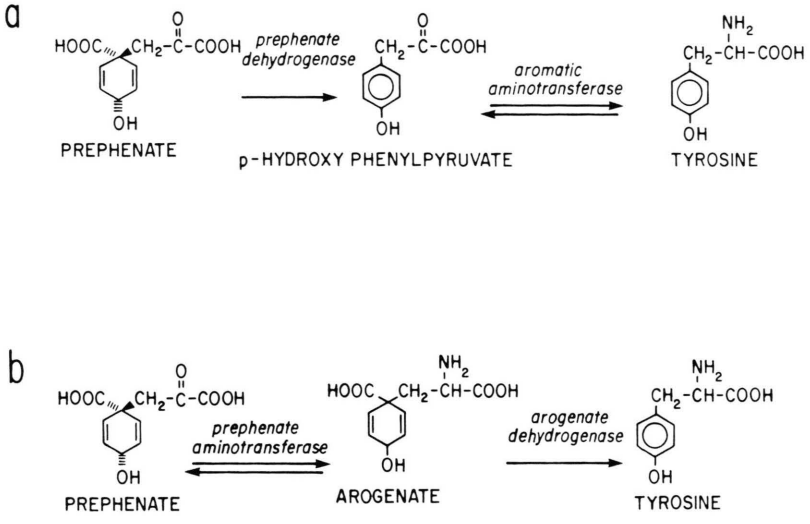

Fig. 1. Routes for the conversion of prephenate of tyrosine. (a) Aromatic keto-acid route. (b) Arogenate route.

mark *et al.* [17] demonstrated an alternate pathway to tyrosine *via* the non-aromatic amino acid, arogenate, initially named pretyrosine (arogenate route, Fig. 1). Rubin and Jensen [18] first described the existence of the arogenate route of tyrosine biosynthesis in higher plants with their study of arogenate dehydrogenase and prephenate aminotransferase from mung bean seedlings (*Vigna radiata,* formerly *Phaseolus aureus*). This work also confirmed the earlier demonstration of prephenate dehydrogenase [12] in this plant. Arogenate dehydrogenase has also been demonstrated in root tissue of *Zea mays* seedlings [19], and suspension cultured cells of *Nicotiana sylvestris* [20]. However, prephenate dehydrogenase was not detected in these tissues. It is apparent that diversity in the routing of tyrosine biosynthesis may occur in higher plants. Such diversity has been well described in microorganisms [21, 22] and indicates the danger of generalizing about metabolic pathways.

The two possible routes for the biosynthesis of either tyrosine or phenylalanine differ only in the sequence of the transamination and aromatization reactions. Although this may seem like a minor difference, it has important implications when considering the regulation and partitioning of carbon flux in the phenylalanine and tyrosine branches of the aromatic pathway. Regulation of tyrosine biosynthesis in sorghum or any other plant cannot be understood without a complete knowledge of the pathway. Therefore, this study was undertaken to determine which, or if both, of the alternatives are operative in sorghum seedlings.

Materials and Methods

Plant material

Sorghum seeds (*Sorghum bicolor* × *Sorghum sudanensis* hybrid, variety WAC Forage 99) were purchased from WAC Seed Inc., Hereford, Texas 79045. Seeds were soaked in aerated tap water for 24 hours, spread into uniform layers between double layers of cheesecloth (grade 10), wetted with tap water and germinated in the dark at 27–30 °C to obtain etiolated seedlings. The cheesecloth wicks were supported by a metal mesh framework in plastic trays. The shoots grew through the cheesecloth and were easily harvested with scissors.

Enzyme extraction

Shoots (54 g) from 3-day etiolated sorghum seedlings were ground with 60 ml buffer (100 mM Tris-HCl pH 8.0, 20% ethylene glycol, 1% β-mercaptoethanol) in a mortar with pestle. The extract was squeezed through two layers of cheesecloth (grade 60) and centrifuged for 60 min at 60,000 × *g* in a Beckman Model L ultracentrifuge. Extraction and all subsequent isolation procedures were performed at 0–4 °C. The crude supernatant (76 ml) was loaded onto a 900-ml Sephadex G-25 column (5 × 45 cm) that had been equilibrated with buffer

(100 mM Tris-HCl pH 8.0, 20% ethylene glycol, 0.1% β-mercaptoethanol). The column was eluted with the equilibration buffer at 10 ml/min. All fractions containing arogenate dehydrogenase activity were pooled.

DEAE cellulose chromatography

Whatman DE52, prepared by the manufacturer's directions, was poured to make a 91 ml bed (2.5 × 18.5 cm) and equilibrated with buffer (100 mM Tris-HCl pH 8.0, 20% ethylene glycol, 0.1% β-mercaptoethanol). The column was loaded with the G-25 pool (192 ml), washed with 500 ml equilibration buffer and eluted with a linear salt gradient (1 liter, 0–0.5 M NaCl in buffer). Fractions of 12 ml were taken and assayed for arogenate dehydrogenase activity. The peak of activity was pooled (242 ml).

Amicon Blue A Gel reverse chromatography

100 ml of the DEAE pool was passed over a 12.4-ml bed (1.5 × 7 cm) of Amicon Blue A gel which had been equilibrated with the same buffer used for the DEAE cellulose step. The flow was 1 ml/min. The arogenate dehydrogenase activity did not bind and was pooled. The enzyme was concentrated to 16 ml in an Amicon ultrafiltration cell (400 ml, Model 8400) fitted with a PM-30 membrane. Nitrogen pressure was 20 PSI. This preparation was used for all characterization studies of arogenate dehydrogenase activity.

Enzyme assay

Arogenate dehydrogenase activity was measured by following the absorbance of the reaction product NADPH at 340 nm ($\varepsilon_{340} = 6220\ M^{-1}cm^{-1}$). Reaction mixtures (200 µl) contained buffer (50 mM Tris-HCl pH 8.0, 10% ethylene glycol, 10 mM β-mercaptoethanol), $NADP^+$ and arogenate. Reactions were started by the addition of enzyme. Absorbance was continuously monitored in a Beckman/Gilford spectrophotometer immediately after mixing in a microcuvette. A unit of enzyme activity was defined as 1 µmol of NADPH produced per minute at 25 °C under the conditions described. Saturating concentrations of arogenate and $NADP^+$, 1.2 mM and 0.1 mM, respectively, were used for quantitative estimation of activity. Similar assay methods, utilizing a wide variety of conditions, were used in the attempts to identify prephenate dehydrogenase activity in crude and partially purified extracts of sorghum.

Preparation of substrates

Arogenate and prephenate were isolated from culture filtrates of an auxotrophic mutant of *Neurospora crassa* 75001/5212/C-167 (ATCC 36373), obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation (stock # 3255). Standard procedures for handling *Neurospora* cultures, as described by Davis and deSerres [23], were employed. Cultures were grown at 25 °C on Trypticase soy agar (BBL) or on solid Vogel's N medium supplemented with 25 µg/ml phenylalanine, tyrosine and tryptophan, in disposable petri dishes. Silica gel was used for long term storage of conidia [23].

Cultures for the accumulation of arogenate and prephenate were grown in carboys containing 15 liters of liquid medium as described by Zamir *et al.* [24], except that sucrose and $CaCl_2$ were added before autoclaving and the final sucrose concentration was 0.5%. The amino acids were dissolved in 1.5 liter of H_2O, filter sterilized and added after the medium had cooled. The carboy was inoculated with spores from several starting cultures which were obtained by placing silica gel grains used for conidial storage on Trypticase soy agar plates and incubating on a lab bench at room temperature for 5 days. The inoculated carboy was aerated at room temperature for 5 days, after which the contents were filtered through glass wool and Whatman # 1 paper. The culture filtrate was used to isolate arogenate by methods similar to Zamir *et al.* [24]. Prephenate was isolated by the method of Metzenberg and Mitchell [25] after separation from arogenate by ion exchange chromatography [24].

Analysis of arogenate solutions

Arogenate was detected during purification by dansylation and micropolyamide thin-layer chromatography as described by Fazel and Jensen [26]. Several qualitative tests for amino compounds were useful for screening cultures and purification fractions for the presence of arogenate. Samples were spotted onto filter paper (10 µl) or placed in small test tubes (50 µl). Ninhydrin reagent [27] or *o*-phthaldehyde reagent (OPA, Fluoraldehyde reagent solution, Pierce Chemical Company) was sprayed on the dried paper or added to the tubes. Ninhy-

drin tests were incubated at 100 °C for 1–2 min; OPA tests were viewed for fluorescence under short-wave UV light.

Quantitation of arogenate by HPLC

Samples containing arogenate were analyzed by either of two procedures. Method 1 involved acid conversion of arogenate to phenylalanine [28] followed by derivitization with OPA and analysis by HPLC. Controls without acid treatment served to detect the presence of phenylalanine. This method, however, could not correct for the possible contamination of arogenate with its gamma-lactam, spiroarogenate [29], as this compound is also converted by acid to phenylalanine. The presence of spiroarogenate would cause overestimation of the arogenate concentration. This method was used for the routine estimation of arogenate during purification because it was technically straightforward and provided quick results.

The estimation of arogenate concentration in solutions to be used for enzyme analysis required more accuracy than available with Method 1. Accurate estimates of arogenate concentration were achieved, in Method 2, by dansylation and analysis by HPLC in a neutral solvent. Spiroarogenate is not subject to derivitization by dansyl chloride and the potential error inherent in Method 1 is avoided. Dansylarogenate could be quantitated directly but it was more convenient to convert it with acid to dansylphenylalanine before analysis. Again, controls lacking the acid treatment served to detect contamination of arogenate solutions with phenylalanine.

Method 1. Samples (10 µl) were placed in a 1.5 ml microfuge tube and treated with 10 µl 2 N HCl for 10 min at room temperature. The acid was neutralized with 30 µl 1 M potassium borate buffer pH 10.4. OPA reagent (50 µl) was mixed with the sample. Two min later, 100 µl H_2O was added and 10 µl of the mixture was injected on a Whatman Partisil PXS ODS-3 HPLC column (4.6 mm × 25 cm, 10 micron particle size). The solvent (acetonitrile/5 mM potassium phosphate buffer, pH 7.2, 25:75) was delivered isocratically at 1.2 ml/min by a Waters pump model 6000A. The absorbance at 336 nm was monitored on a BioRad model 1305 variable wavelength UV monitor. The area of an absorbance peak corresponding to OPA derivitized phenylalanine was compared to a standard curve generated with authentic phenylalanine. Controls without acid treatment were accomplished by reversing the order of addition of the HCl and the borate buffer.

Method 2. Samples (10 µl) were placed in a 1.5 ml microfuge tube and mixed with 10 µl 4 M potassium carbonate buffer pH 9.8 and 25 µl 1% dansyl chloride in acetone. The tube was closed and incubated at 37 °C for 30 min. The reaction was stopped with 6 to 8 µl 6 N H_3ClO_4 and 200 µl HPLC grade acetonitrile. The quantity of H_3ClO_4 needed to neutralize the buffer to pH 7.2 was determined by previous titration of the stock solutions. After mixing, the tube was placed on ice for 20 min and centrifuged for one min in a Beckman microfuge. An aliquot (100 µl) was placed in a 1.5 ml microfuge tube and treated with 20 µl 6 N HCl for 10 min at room temperature to convert dansylarogenate to dansylphenylalanine. Ten µl were injected onto an Alltech econosphere C18 HPLC column (0.5 cm × 15 cm, 5 micron particle size). The solvent (acetonitrile/5 mM potassium phosphate buffer, pH 7.2, 20:80) was delivered at 2 ml/min. Absorbance at 252 nm was monitored and the area of an absorbance peak corresponding to dansylphenylalanine was compared to a standard curve generated with authentic phenylalanine. Substitution of water for HCl served as a control to estimate contamination of arogenate solutions with phenylalanine. An additional control utilizing acid treatment before dansylation served to detect spiroarogenate.

Results

Detection of arogenate dehydrogenase

Arogenate dehydrogenase activity was easily detected in G-25 desalted extracts of etiolated sorghum seedlings. Enzyme assays containing 1 mM arogenate and 0.1 mM $NADP^+$ were linear up to an absorbance change of at least 0.1 absorbance unit. Linearity was more difficult to achieve at lower substrate concentrations; however, all enzyme rate data were taken from the initial linear portion of the assay curve. The continuous spectrophotometric assay allowed detection of as little as 64 pmol NADPH/min, corresponding to an absorbance change of 0.002/min. Under the assay conditions used, linearity with added enzyme was achieved. Controls consisting of either boiled enzyme or lack of substrate (arogenate or $NADP^+$) resulted in only a slight absorbance change.

Several control experiments were carried out in order to rule out the possibility of a side reaction

involving contaminants in the substrate solution. Rubin and Jensen [18] reported that their arogenate preparations were contaminated with shikimic acid which led to overestimation of arogenate dehydrogenase due to the presence of shikimate dehydrogenase in the crude mung bean extracts. The possibility of this problem prompted the following control experiment. Acid treatment is known to convert arogenate to phenylalanine [28] but has no effect on shikimate. Arogenate solutions that were treated with HCl, neutralized and included in reaction mixtures, did not support dehydrogenase activity. Addition of untreated arogenate to such mixtures initiated activity. Therefore the dehydrogenase activity had to be due to the presence of arogenate or other acid labile compounds but not shikimate. In addition, analysis of dansylated reaction mixtures by HPLC indicated that the tyrosine produced by the reaction was stoichiometrically equivalent to the NADPH produced.

Crude enzyme extracts prepared under a wide (but not exhaustive) variety of conditions failed to yield any detectable dehydrogenase activity which utilized prephenate as substrate.

Crude extracts of 3-day etiolated sorghum shoots contained as much as 0.21 units of arogenate dehydrogenase activity per gram of tissue. This activity was relatively stable before and after desalting with G-25. Extracts could be stored at 0 °C or frozen at −20 °C with less than 5% activity loss in 2 weeks. The arogenate dehydrogenase activity had a broad pH optimum, exhibited maximal activity between 6.5 and 9, and was insensitive to the type of buffer used.

A variety of compounds were tested as potential effectors of arogenate dehydrogenase. Tyrosine was found to effectively inhibit the enzyme activity while phenylalanine, anthranilic acid, phenylpyruvate, *p*-hydroxyphenylpyruvate and prephenate were ineffective when tested at a concentration of 1 mM and subsaturating concentration of arogenate (0.5 mM) and/or $NADP^+$ (5 μM). Dhurrin was also tested but the presence of dhurrinase in the enzyme preparation resulted in the rapid production of *p*-hydroxybenzaldehyde (ε_{333nm} = 27,900 $M^{-1}cm^{-1}$) [5] which interfered with the spectrophotometric assay of arogenate dehydrogenase.

Enzyme isolation

Crude extracts were further purified by DEAE cellulose chromatography and reverse chromatography on Amicon Blue A gel. Initial attempts to purify the enzyme on DEAE cellulose resulted in the loss of 75% of the activity. In addition, the activity recovered gave non-linear assays and was lost on storage, dilution, or further purification. This suggested that the enzyme was either separated from stabilizing components of the crude extract or unstable to conditions introduced during chromatography (*i.e.*, high salt or dilution). The addition of KCl to crude extracts had no effect on activity or stability to storage; the same result was obtained in dilution studies. The addition of ethylene glycol (20%) and β-mercaptoethanol (0.1%) stabilized the activity obtained from the DEAE column. Inclusion of these additives to the extraction and DEAE column buffers led to high recovery of activity.

Gradient elution of the DEAE cellulose column resulted in a single peak of activity with 93% recovery (Fig. 2). After pooling, the DEAE purified preparation was passed over an Amicon Blue A column which bound much extraneous protein and gave a 3-fold purification. The isolation results are summarized in Table I. The preparation obtained was concentrated by ultrafiltration and was used for kinetic analysis.

Kinetic and regulatory properties

As mentioned above, prephenate did not support any NAD^+- or $NADP^+$-dependent dehydrogenase activity in crude or DEAE cellulose purified extracts of sorghum shoots. In addition, it was observed that

Table I. Partial purification of arogenate dehydrogenase.

Enzyme preparation	Volume [ml]	Units [μmol/min]	Protein [mg]	Specific activity [Units/mg]	Recovery [%]	Purification [fold]
G-25 pool	192	9.58	233	0.04	–	–
DEAE pool	242	8.89	58	0.15	93	3.7
Blue A pool	91	7.63	18	0.43	80	10.5

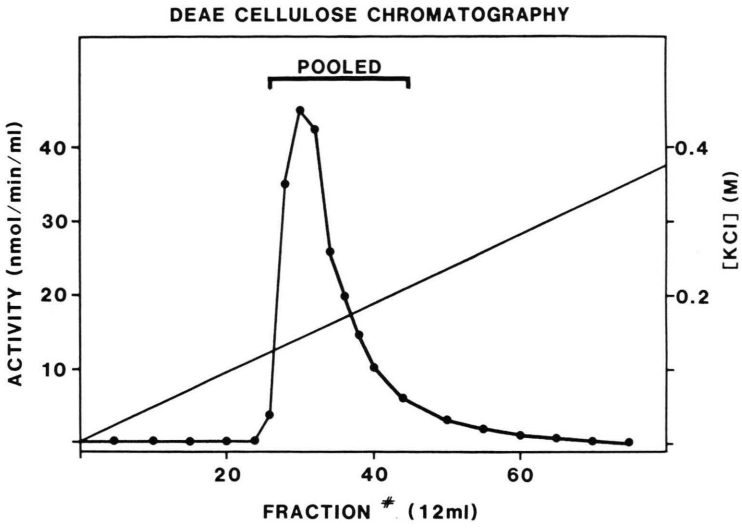

Fig. 2. Elution profile of arogenate dehydrogenase activity on DEAE cellulose.

10 mM prephenate had no inhibitory effect on arogenate dehydrogenase activity when measured at subsaturating as well as saturating concentrations of arogenate and $NADP^+$. The apparent lack of prephenate dehydrogenase activity in extracts of etiolated sorghum seedlings resembles the situation reported for root tissue of *Zea mays* [19] and suspension cultured cells of *Nicotiana sylvestris* [20]. Enzyme extracts from mung bean, on the other hand, contain prephenate and arogenate dehydrogenase activity [18].

The arogenate dehydrogenase of sorghum was specific for $NADP^+$ as cosubstrate. Reactions with NAD^+ provided at concentrations of 50, 100 and 1000 μM resulted in less than 1% of the activity obtained with $NADP^+$ at the same concentration. No further studies of nucleotide cofactor specificity were done. The mung bean [18] and tobacco [20] enzymes were reported to be specific for $NADP^+$; however, the enzyme from maize [19] was specific for NAD^+.

In addition to narrow substrate specificity, arogenate dehydrogenase displayed extreme specificity towards potential effectors. Only tyrosine was found to inhibit enzyme activity. The structurally related compounds phenylalanine, phenylpyruvate and *p*-hydroxyphenylpyruvate had no effect on the enzyme activity at a concentration of 1 mM. Tyrosine has also been reported to effectively inhibit the arogenate dehydrogenase from *Zea mays* [19] and *Nicotiana sylvestris* [20]. The situation in mung bean is, again, different. Tyrosine inhibited prephenate dehydrogenase from mung bean, only at subsaturating concentrations of prephenate, but had no apparent effect on arogenate dehydrogenase activity [18].

Hyperbolic substrate saturation curves were obtained for both arogenate and $NADP^+$. The apparent K_m values were 340 μM for arogenate and 11 μM for $NADP^+$ when measured at saturating concentration of the second substrate (Fig. 3).

The initial observation that 1 mM tyrosine completely inhibited arogenate dehydrogenase activity in the standard assay prompted further investigation of the effect. Reciprocal plots of arogenate saturation curves (Fig. 4a) measured at various tyrosine concentrations demonstrated that tyrosine was competitive with arogenate. Replotting the slopes of the reciprocal plots (Fig. 4b) as a function of tyrosine concentration provided an estimate for the K_i for tyrosine of 60 μM. It should be noted that this value is much lower than the K_m for arogenate.

Discussion

The presence of arogenate dehydrogenase and lack of prephenate dehydrogenase activity in extracts of etiolated sorghum shoots indicates that the biosynthesis of tyrosine, in this species, occurs *via* the arogenate route and not the aromatic α-keto acid route. This conclusion is also supported by the demonstration of prephenate aminotransferase activity in sorghum as described in the accompanying paper [35]. The sole operation of the arogenate route for

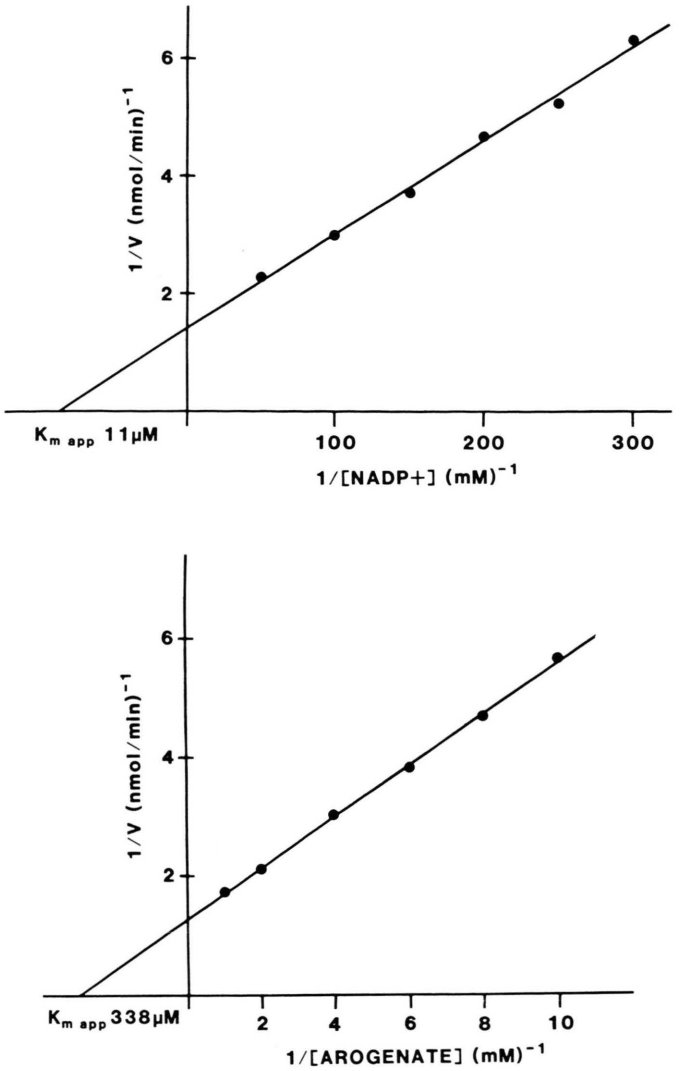

Fig. 3. Substrate saturation of arogenate dehydrogenase. (a) Reciprocal plot for $NADP^+$ saturation at saturating arogenate (1.2 mM). (b) Reciprocal plot for arogenate saturation at saturating $NADP^+$ (0.1 mM).

tyrosine biosynthesis has been suggested for many microorganisms [22, 26] as well as the higher plants, *Zea mays* [19] and *Nicotiana sylvestris* [20]. In the absence of evidence to the contrary, we conclude that this is the only route for tyrosine formation in *Sorghum bicolor.*

It is interesting to note that several authors have reported their inability to detect prephenate dehydrogenase in a number of plant species, including horseradish, rose, *Reseda,* buckwheat, potato, red beet, wheat, pea and rice [12, 13] as well as oak [30] and *Euglena* [31]. It is possible that these plants also utilize the arogenate route exclusively and that the prephenate dehydrogenase activity reported in several closely related bean species [12, 18] is an anomaly among higher plants. It is not clear whether the apparent duality of tyrosine biosynthetic routes observed only in mung bean is due to separate enzymes or to a single enzyme with dual substrate specificity. Regardless of such details, the studies of arogenate dehydrogenase in higher plants indicate a major role for arogenate as a precursor of tyrosine.

The degree of specificity of sorghum arogenate dehydrogenase for the substrates (arogenate and $NADP^+$) and for the inhibitor/product, tyrosine, is remarkable. Compounds that are structurally related

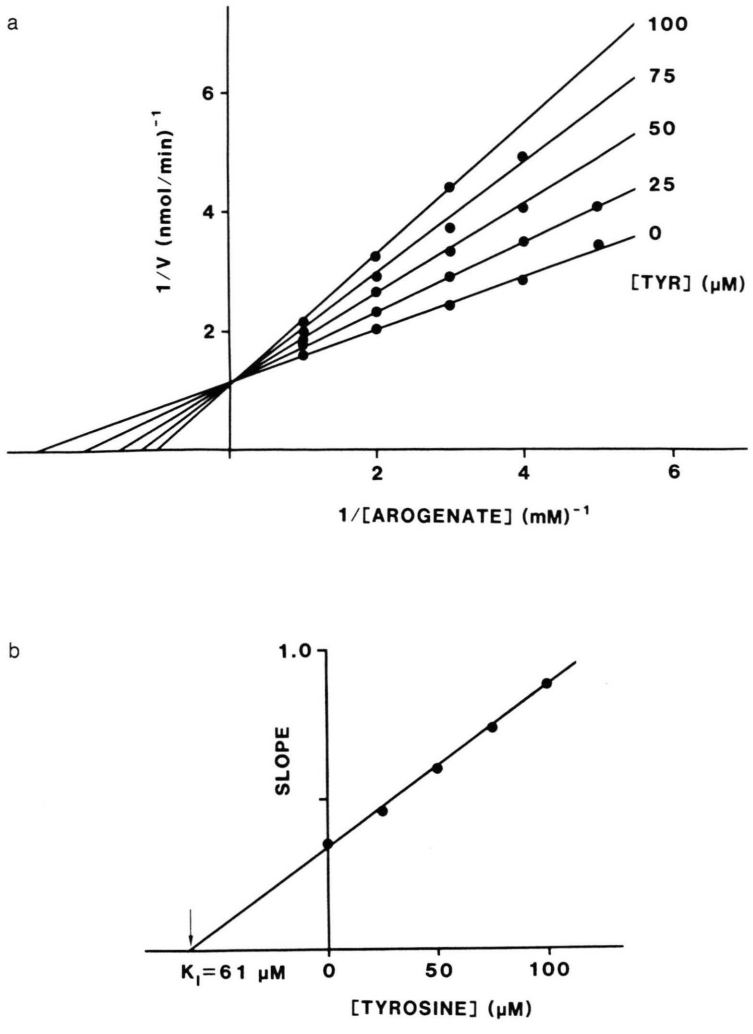

Fig. 4. Inhibition of arogenate dehydrogenase by tyrosine. (a) Reciprocal plot of arogenate dehydrogenase saturation by arogenate at saturating $NADP^+$ (0.1 mM) and various concentrations of tyrosine. (b) Replot of slopes from (a) as a function of tyrosine concentration.

to arogenate and tyrosine, namely prephenate, phenylalanine, phenylpyruvate and *p*-hydroxyphenylpyruvate, had no effect on the activity. Kinetic analysis led to the striking result that sorghum arogenate dehydrogenase has a much greater affinity for tyrosine than the substrate arogenate. This is also the case for the arogenate dehydrogenase isolated from the photosynthetic eukaryote *Euglena gracilis* [22, 32] and the higher plants *Zea mays* [19] and *Nicotiana sylvestris* [20]. For example, the enzyme in *Euglena* [32] was inhibited 50% by 15 μM tyrosine when measured at saturating concentration of substrates. The measured K_i for tyrosine was 4.5 μM and the K_m for arogenate was 200 μM.

It is interesting to note that *Euglena* is the only organism known to utilize arogenate as the sole precursor to both tyrosine and phenylalanine (Fig. 5a). The pathway branches at arogenate in this organism and, therefore, arogenate dehydrogenase is the first committed enzyme for tyrosine biosynthesis. The

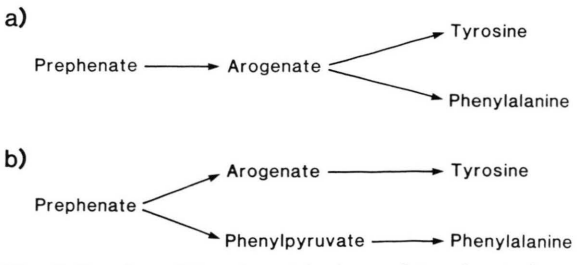

Fig. 5. Routing of the phenylalanine and tyrosine pathway branchlets. (a) Pathway structure found in *Euglena* [22, 32]. (b) Pathway structure found in corynebacteria [26].

Table II. Comparison of shikimate pathway branching in various organisms.

Organism	Pathway structure	Branchpoint	Inhibition of arogenate dehydrogenase by tyrosine	References
Prokaryotes:				
Corynebacterium glutamicum	a	prephenate	−	[26]
Brevibacterium flavum	a	prephenate	−	[26[
Synechocystis	a	prephenate	−	[33]
Eukaryotes:				
Euglena gracilis	b	arogenate	+	[31, 32]
Sorghum bicolor	?	?	+	[this study]
Nicotiana sylvestris	?	?	+	[20]
Zea mays	?	?	+	[19]
Vigna radiata	?	?	−	[18]

regulatory properties of arogenate dehydrogenase in *Euglena* (*i.e.*, strong inhibition by tyrosine) are consistent with the enzyme's position at a metabolic branch point.

In contrast, prokaryotic microorganisms that utilize the arogenate route for tyrosine biosynthesis, such as *Brevibacterium flavum* [26], *Corynebacterium glutamicum* [26] and *Synechocystis* sp. [33], invariably utilize the aromatic keto-acid route for phenylalanine biosynthesis [22]. As illustrated in Fig. 5b, the last common intermediate between the phenylalanine and tyrosine branches, in this class of organism, is prephenate. Arogenate dehydrogenases in prokaryotes have higher affinity for arogenate than for tyrosine and, therefore, are not inhibited by tyrosine. Again, the lack of regulatory properties observed for prokaryotic arogenate dehydrogenases is consistent with the fact that the enzyme is not situated at a metabolic branch point. Table II summarizes the results of various authors concerning the regulation of arogenate hydrogenase by tyrosine and the structure of terminal branches of phenylalanine and tyrosine biosynthesis.

The arogenate dehydrogenases isolated from higher plants (maize [19], tobacco [20] and sorghum [this study]) have been shown to be strongly inhibited by tyrosine. This would indicate that the metabolic branch point in higher plants is at arogenate. The biosynthesis of phenylalanine may then occur by aromatization of arogenate rather than prephenate. No direct evidence for either the arogenate or the phenylpyruvate route has been obtained in plants. The inability to detect phenylalanine branch enzymes in plants may be technical in nature but this fact underscores the lack of knowledge of plant metabolism which is due to, at least in part, overgeneralization of results obtained in microorganisms. It is possible that an, as yet undiscovered, alternate route of phenylalanine biosynthesis exists. Incomplete information about the terminal reactions of phenylalanine biosynthesis and the exact branch point prevents a full understanding of the regulation and partitioning of carbon flux through this important pathway.

Haslam [34] has pointed out that compounds derived from the shikimic acid pathway are second only to carbohydrates in abundance and diversity. Despite the fact that general phenylpropanoid metabolism [7–10] is one of the most important pathways in plants, a clear understanding of the regulatory and structural nature of the phenylalanine and tyrosine branches of the shikimic acid pathway does not exist. Further studies in the enzymology of this pathway in a variety of plants and tissues will either allow the formulation of a general hypothesis for the regulation of aromatic amino acid biosynthesis or reveal a diversity of mechanisms. The piecemeal nature of information pertaining to this subject makes general conclusions difficult.

Acknowledgement

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[1] T. Akazawa, P. Miljanich, and E. E. Conn, Plant Physiol. **35,** 535–538 (1960).
[2] J. Koukol, P. Miljanich, and E. E. Conn, J. Biol. Chem. **237,** 3223–3228 (1962).
[3] J. E. Gander, J. Biol. Chem. **237,** 3229–3233 (1962).
[4] H. A. Stafford, Phytochemistry **3,** 742–752 (1969).
[5] H. J. Gorz, W. L. Haag, J. E. Specht, and F. A. Haskins, Crop Sci. **17,** 578–582 (1977).
[6] N. J. Neucere and G. Sumrell, J. Ag. Food Chem. **27,** 809–812 (1971).
[7] K. Hahlbrock, Flavonoids. In: The biochemistry of plants (E. E. Conn, ed.), **Vol. 7,** Secondary plant products, pp. 425–456, Academic Press, New York 1981.
[8] H. Grisebach, Lignins. In: The biochemistry of plants (E. E. Conn, ed.),**Vol. 7,** Secondary plant products, pp. 457–478, Academic Press, New York 1981.
[9] G. G. Gross, Phenolic acids. In: The biochemistry of plants (E. E. Conn, ed.), **Vol. 7,** Secondary plant products, pp. 301–316, Academic Press, New York 1981.
[10] S. A. Brown, Coumarins, in: The biochemistry of plants (E. E. Conn, ed.), **Vol. 7,** Secondary plant products, pp. 269–300, Academic Press, New York 1981.
[11] D. G. Gilchrist and T. Kosuge, Aromatic amino acid biosynthesis and its regulation. In: The biochemistry of plants (B. J. Miflin, ed.), **Vol. 5,** Amino acids and derivatives, pp. 507–531, Academic Press, New York 1981.
[12] O. L. Gamborg and F. W. Keeley, Biochim. Biophys. Acta **115,** 65–72 (1966).
[13] O. L. Gamborg, Can. J. Biochem. **44,** 791–799 (1966).
[14] O. L. Gamborg and F. J. Simpson, Can. J. Biochem. **42,** 583–591 (1964).
[15] F. Gibson and J. Pittard, Bacteriol. Rev. **32,** 465–492 (1968).
[16] J. Pittard and F. Gibson, Cur. Top. Cell. Reg. **2,** 29–63 (1970).
[17] S. L. Stenmark, D. L. Pierson, and R. A. Jensen, Nature **247,** 290–292 (1974).
[18] J. L. Rubin and R. A. Jensen, Plant Physiol. **64,** 727–734 (1979).
[19] G. S. Byng, R. Whitaker, C. Flick, R. A. Jensen, Phytochemistry **20,** 1289–1292 (1981).
[20] C. G. Gaines, G. S. Byng, R. J. Whitaker, and R. A. Jensen, Planta **156,** 233–240 (1982).
[21] R. A. Jensen and D. L. Pierson, Nature **254,** 667–671 (1975).
[22] G. S. Byng, J. F. Kane, and R. A. Jensen, CRC Crit. Rev. Microbiol. **9,** 227–252 (1982).
[23] R. H. Davis and F. J. deSerres, Meth. in Enzymol. **14a,** 79–143 (1970).
[24] L. O. Zamir, R. A. Jensen, B. H. Arison, and A. W. Douglas, J. Am. Chem. Soc. **102,** 4499–4504 (1980).
[25] R. L. Metzenberg and H. K. Mitchell, Arch. Biochem. Biophys. **64,** 51–56 (1956).
[26] A. M. Fazel and R. A. Jensen, J. Bact. **138,** 805–815 (1979).
[27] I. Smith, Amino acids, amines, and related compounds. In: Chromatographic and electrophoretic techniques (I. Smith, ed.), **Vol. 1,** Chromatography, pp. 104–150, Interscience Publishers, New York 1969.
[28] L. O. Zamir, R. Tibero, and R. A. Jensen, Tetrahedron Letters **24,** 2815–2818 (1983).
[29] L. O. Zamir, E. Jung, and R. A. Jensen, J. Biol. Chem. **258,** 6492–6496 (1983).
[30] P. Gadal and H. Bouyssou, Physiol. Plant. **28,** 7–13 (1973).
[31] H. L. Weber and A. Böck, Arch. Mikrobiol. **66,** 250–258 (1969).
[32] G. S. Byng, R. Whitaker, C. Flick, and R. A. Jensen, Mol. Cell. Biol. **1,** 426–438 (1981).
[33] G. C. Hall and R. A. Jensen, J. Bacteriol. **144,** 1034–1042 (1980).
[34] E. Haslam, The shikimate pathway, J. Wiley and Sons, New York 1974.
[35] D. L. Siehl, J. A. Connelly, and E. E. Conn, Z. Naturforsch. **41c,** 79–86 (1986).