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Biochemistry

Arogenate (pretyrosine) is an obligatory intermediate of L-tyrosine biosynthesis: Confirmation in a microbial mutant

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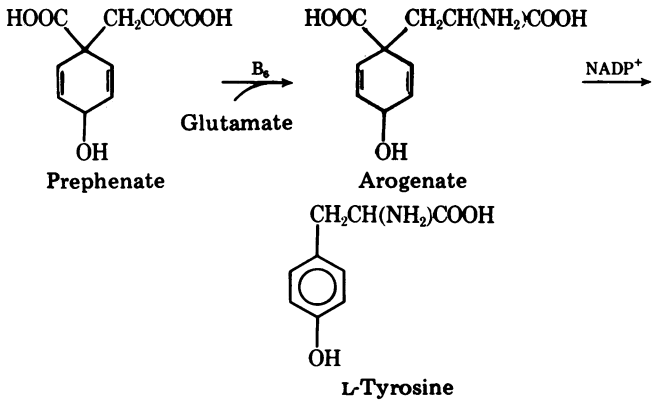
ABSTRACT **Wild-type *Brevibacterium flavum* has been shown to possess arogenate dehydrogenase activity and to lack prephenate dehydrogenase, thereby providing presumptive evidence that arogenate (previously named "pretyrosine") is an obligatory intermediate of L-tyrosine biosynthesis. A similar enzymological pattern has been discerned in extracts made from wild-type cultures of various species of cyanobacteria. Application of rigorous molecular genetic criteria in confirmation of the exclusive role of arogenate in L-tyrosine synthesis was made possible by the isolation of an auxotrophic mutant exhibiting a nutritional requirement for L-tyrosine. The mutant was found to lack activity for arogenate dehydrogenase and to accumulate substantial amounts of arogenate behind the mutant block during starvation for L-tyrosine.**

Two enzymatic sequences occur in nature*, separately or simultaneously, to govern L-tyrosine biosynthesis. The 4-hydroxyphenylpyruvate branchlet has been well known for over 20 years:

$$\text{prephenate} \xrightarrow{NAD^+} \text{4-hydroxyphenylpyruvate} \xrightarrow{B_6} \text{L-tyrosine.}$$

This reaction sequence uses prephenate dehydrogenase and 4-hydroxyphenylpyruvate aminotransferase, respectively. A second sequence, the arogenate branchlet, was reported to exist in at least some species of cyanobacteria in 1974 (1):



Arogenate [β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine] was initially named "pretyrosine."† Since then, a growing list of microorganisms (5–9) and plants (5, 10) is emerging in which arogenate functions as an intermediate of L-tyrosine biosynthesis. We recently reported (11) that species of coryneform bacteria appear to rely exclusively upon the arogenate branchlet for L-tyrosine biosynthesis. Wild-type cells of *Corynebacterium glutamicum*, *Brevibacterium flavum*, and *B. ammoniagenes* lack prephenate dehydrogenase activity and were shown to contain prephenate aminotransferase and arogenate dehydrogenase, the two enzyme activities illustrated above.

More rigorous establishment of conclusions based upon data derived from wild-type cells would be provided by the demonstration that tyrosine auxotrophy corresponds to the mutational loss of arogenate dehydrogenase and that arogenate accumulates behind the mutant block. The latter approach has been fulfilled in *B. flavum*, and this system now offers the most rigorous documentation to date of an exclusive role *in vivo* of the arogenate branchlet for L-tyrosine biosynthesis.

MATERIALS AND METHODS

Microbial Stocks. *B. flavum* (ATCC 14067) was obtained from the American Type Culture Collection. Mutant *tyrB2* was derived from the prototrophic strain after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Cells (10 ml in a 125-ml flask) were cultured at 30° in minimal glucose medium with vigorous shaking in a New Brunswick gyratory shaker to the late exponential phase of growth. Mutagen (1 mg) and 0.5 mg of chloramphenicol (12) were added and incubation was continued for 30 min. The culture was centrifuged, and the pellet was washed several times with prewarmed minimal glucose medium. This pellet was resuspended in 10 ml of fresh medium containing 50 μg of L-tyrosine per ml and incubated with shaking at 30°C for 4 hr to allow segregation of mutant cells. Surviving cells were plated out on solid medium containing 50 μg of L-tyrosine per ml. Colonies failing to replicate in minimal glucose medium lacking L-tyrosine were sought, and mutant *tyrB2* was initially recognized in this way. Mutant *tyrB2* was carried through three rounds of single-colony isolation to ensure microbiological purification from wild-type background.

Microbiological Procedures. Stock cultures were maintained on tryptic soy medium solidified with 1.5% (wt/vol) Difco agar. The composition of basal minimal salts medium has been described (11). Glucose at a final concentration of 3.6% (wt/vol) was the carbon source. In experiments in which arogenate excretion into the culture medium was monitored, the

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Abbreviation: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

* Although L-tyrosine potentially could be derived from L-phenylalanine via phenylalanine hydroxylase, all species of this enzyme thus far described in microbes are inducible catabolic enzymes that probably make no significant contribution to L-tyrosine biosynthesis.

† Zamir *et al.* (2) replaced the name "pretyrosine" with "arogenate" in recognition of the enzymatic capability of some organisms to convert arogenate to phenylalaine—i.e., arogenate dehydratase. It is an interesting analogy that Davis (3) initially named prephenate "prephenylalanine" until its role as a common precursor of both phenylalanine and tyrosine was appreciated (4).

medium contained (per liter): glucose, 100 g; ammonium acetate, 15 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.2 mg; $MnSO_4 \cdot H_2O$, 2 mg; biotin, 30 μg; and thiamine·HCl, 100 μg. To prevent acid-catalyzed conversion of arogenate to phenylalanine by pH changes of medium during accumulation,* the pH of the medium was maintained between 7.2 and 8.0 by periodic addition of 10 M NaOH as necessary.

Growth curves were constructed by the measurement of turbidities of 10-ml cultures contained within 125-ml sidearm flasks. Turbidity measurements were made in a Klett–Summerson colorimeter (filter no. 64). *B. flavum* achieves very high densities through exponential growth and, when necessary, culture aliquots were diluted to allow measurements within a turbidity range of 25–250.

Quantitation of Arogenate. Samples (100 μl) containing arogenate were treated with 10 μl of 4 M $KHCO_3$ (pH 9.8) and 100 μl of 1% dansyl chloride in acetone. After 30 min at 37°C the reaction vessels were cooled and 10 μl of 6 M $HClO_4$ and 100 μl of ice-cold acetone were added with rapid mixing. After brief centrifugation, analysis by high-pressure liquid chromotography was carried out. A 100-μl aliquot of dansyl chloride-treated supernatant was diluted with 100 μl of 0.1 M sodium maleate (pH 5.7), and 20 μl was injected onto a column of LiChrosorb RP-18 (3.2 × 250 mm). The mobile phase was acetonitrile/0.05 M sodium maleate (pH 5.7), 12:88 (vol/vol), with a linear velocity of 2.5 cm/min. A Gilson Filter-Glo fluorometer was used for detection. Dansyl arogenate was identified by peak retention time and quantitated from peak height (which was proportional to dansyl arogenate concentrations).

Thin-Layer Chromatography of Arogenate Dehydrogenase Product. The formation of L-tyrosine from L-arogenate by arogenate dehydrogenase was confirmed by thin-layer chromatography as follows. Reaction mixtures (100 μl) contained 0.25 mM $NADP^+$, 0.25 mM arogenate, and 250 μg of extract protein. After 20 min at 35°C, 10 μl of 4 M $KHCO_3$ (pH 9.8) and 100 μl of 1% dansyl chloride in acetone were added. After 30 min at 37°C the reaction vessels were cooled and 10 μl of 6 M $HClO_4$ and 100 μl of ice-cold acetone were added with rapid mixing. After brief centrifugation, a 1-μl aliquot of sample was applied near one corner of a 5 × 5 cm micropolyamide plate. The plates were developed in solvent I [H_2O/NH_4OH, 2:1 (vol/vol)]. Plates were dried in a cool air stream, exposed for 5 min to the vapor of 88% formic acid, dried again, and developed in a second dimension with solvent II [benzene/glacial acetic acid/pyridine, 50:5:1 (vol/vol)]. Dansyltyrosine was located by its fluorescence under short-wave ultraviolet illumination and recognized by comparison to the mobility of authentic dansyltyrosine chromatographed on the reverse side of the same plate.

Enzymological Procedures. Crude extracts were prepared from cell populations disrupted by sonic oscillation at 4°C. Cell pellets obtained by centrifugation (5000 × *g* for 20 min) at 4°C were washed twice in 0.05 M Tris·HCl at pH 7.5 and resuspended in 4 ml of buffer per g (wet weight) of pellet. Cells were disrupted by sonication at 60 mV (Lab-line Ultratip, Labsonic System) through application of four 30-sec bursts. The crude extract was clarified by centrifugation at 27,000 × *g* for 50 min at 4°C. The supernatant was dialyzed against 1000 vol of Tris·HCl buffer at pH 7.5 overnight.

The reaction mixture for arogenate dehydrogenase (200 μl) contained 0.25 mM $NADP^+$, 0.5 mM arogenate, and enzyme. NADPH formation was followed continuously by using an Aminco–Bowman spectrophotofluorometer (excitation at 340 nm and emission at 460 nm).

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase was assayed according to Srinivasan and Sprinson (13) as modified by Smith *et al.* (14). Reaction mixture (100 μl) contained 50 mM potassium phosphate buffer at pH 7.0, 0.5 mM phospho*enol*pyruvate, 0.5 mM erythrose 4-phosphate, 1 mM $CoCl_2$, 1 mM dithiothreitol, and enzyme. An ϵ_{549} of 45,00 for DAHP (15) was used to calculate specific activities.

The assays for prephenate dehydrogenase and for prephenate aminotransferase were as described (11). Reaction mixtures (100 μl) for prephenate aminotransferase contained 50 mM Tris·HCl at pH 7.5, 2.5 mM prephenate, 0.25 mM pyridoxal 5′-phosphate, 0.125 μCi of 1 mM L-[^{14}C]glutamate, and enzyme (1 Ci = 3.7×10^{10} becquerels).

Biochemicals and Chemicals. Tryptic soy broth and agar were obtained from Difco. Prephenate and arogenate were isolated from a mutant strain of *Neurospora crassa* (16) (an improved isolation procedure for arogenate is described in ref. 2). Prephenate preparations were about 90% pure and were free of detectable arogenate or phenylpyruvate. Arogenate preparations were 90–95% pure and free of prephenate, phenylpyruvate, or phenylalanine. Amino acids, keto acids, cofactors, and buffers were obtained from Sigma. Liquifluor and radioactive amino acids were from New England Nuclear and Amersham, respectively. The method of Bradford (17) as outlined in Bio-Rad Technical Bulletin 1051 with Bio-Rad reagents were used for the analytical estimation of protein concentrations. Micropolyamide plates for thin-layer chromatography were from Schleicher & Schuell.

RESULTS

Characterization of Mutant *tyrB2*. Tyrosine auxotroph *tyrB2* grew in the presence of L-tyrosine at 30°C with a doubling time of about 100 min during exponential growth, a growth rate that is identical to that of the wild-type prototroph in minimal glucose medium (Fig. 1). The mutation imposed an absolute nutritional requirement for L-tyrosine, as illustrated by the absence of leaky growth after resuspension in the absence of L-tyrosine. The tight block in mutant *tyrB2* is also apparent from the abrupt cessation of growth following exhaustion of tyrosine initially supplied at the growth-limiting concentration of 5 μg/ml.

The absence of arogenate dehydrogenase was found to be the distinctive feature of mutant *tyrB2* (Table 1). Extracts prepared from the wild-type parent of the mutant lacked prephenate dehydrogenase but exhibited excellent arogenate dehydrogenase activities. Verification that the product of the arogenate dehydrogenase reaction obtained in the presence of arogenate and $NADP^+$ was indeed tyrosine was achieved by thin-layer chromatography of the dansylated reaction mixture. Extracts from the mutant did not differ significantly from wild-type extracts with respect to specific activities measured for DAHP synthetase and prephenate aminotransferase. The data in Table 1 also illustrate that specific activities of DAHP synthetase, prephenate aminotransferase, and arogenate dehydrogenase were not influenced by the presence of excess tyrosine in the wild type. Additionally, data obtained with mutant *tyrB2* grown under conditions of tyrosine limitation allow the conclusion that neither DAHP synthetase nor prephenate aminotransferase specific activity is influenced by tyrosine starvation.

Accumulation of Arogenate by Mutant *tyrB2*. In contrast to wild type, which does not accumulate detectable levels of arogenate under any conditions, mutant *tyrB2* exhibited substantial capability for accumulation of arogenate in the culture medium (Table 2). The extent of arogenate excretion was exceedingly sensitive to the presence of L-tyrosine. Resuspension of cells under conditions of starvation for L-tyrosine yielded

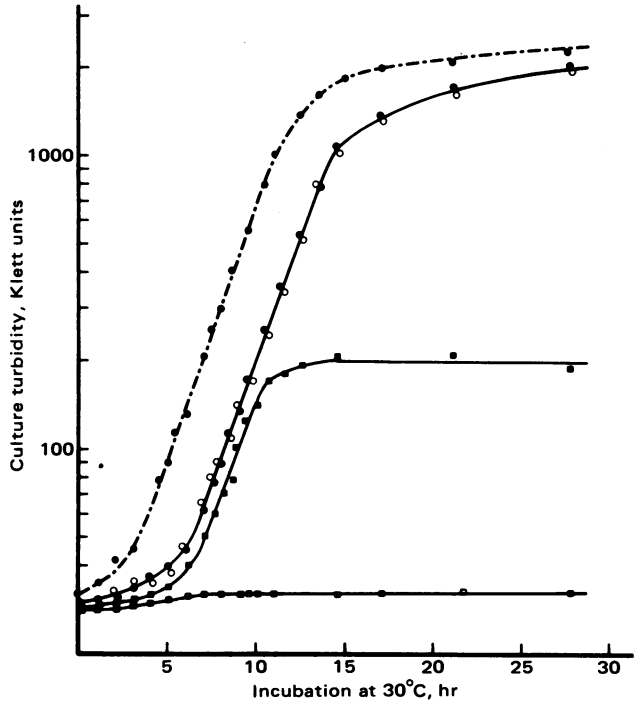

FIG. 1. Nutritional responses of auxotroph *B. flavum tyrB2*. All cultures were contained in 125-ml sidearm flasks holding 10-ml cultures that had been diluted 1:100 from starter cultures of minimal glucose (wild type) or minimal glucose containing 50 μg of L-tyrosine per ml (mutant *tyrB2*) cultured to the late exponential phase of growth. Cultures used for the growth curve shown were incubated at 30°C with vigorous shaking (170 rpm) in a New Brunswick constant-temperature incubator. ●--●, Wild-type *B. flavum* in minimal glucose medium; ●—●, mutant *tyrB2* in minimal glucose medium supplemented with L-tyrosine at 50 μg/ml; ○—○, mutant with L-tyrosine at 25 μg/ml; ■—■, mutant with L-tyrosine at 5 μg/ml; □—□, mutant starved in the absence of L-tyrosine.

0.1–0.2 mM arogenate in the culture medium, an accumulation level that was diminished about 95% when L-tyrosine was present in the accumulation medium. Undoubtedly, regulation of early-pathway enzymes (particularly DAHP synthetase) by L-tyrosine decreased precursor flow to arogenate. However, supplementation with shikimate, potentially able to bypass allosteric effects impinging at the level of DAHP synthetase, did not result in increased arogenate accumulation. Shikimate enters these cells because auxotrophs responsive to shikimate (11) are readily isolated after mutagenesis. Very likely, chorismate mutase (an enzyme that is not by-passed by exogenous shikimate) is a significant control point. Recently, tyrosine has been shown to exert both allosteric and repressive regulatory effects upon chorismate mutase in *B. flavum* (18).

Table 2. Accumulation of arogenate in *B. flavum tyrB2*

Added (500 μg/ml)	Arogenate accumulated, μM: At resuspension	Arogenate accumulated, μM: After 31 hr	SEM
None	<0.6	146	±4
Shikimate	<0.6	149	±9
L-Tyrosine	<0.6	9.3	±0.4
Shikimate + L-tyrosine	<0.6	7.3	±1.5

A culture in the late exponential phase of growth (minimal glucose plus 100 μg of L-tyrosine per ml) was diluted into accumulation medium supplemented as indicated to yield initial turbidities of about 250 Klett units. Cultures were incubated at 30°C with vigorous shaking. In the presence of L-tyrosine, cultures grew to a turbidity of 1300–1400 Klett units.

Fig. 2 illustrates the identification, by thin-layer chromatography, of arogenate accumulated in minimal glucose medium under conditions of starvation for L-tyrosine. The sample showing the presence of arogenate was taken after 31 hr of accumulation (the condition shown on line 1 of Table 2, for comparison with the middle right plate of Fig. 2). The results illustrated in Fig. 2 involved the formation of dansyl arogenate, chromatography, conversion at acidic pH to dansylphenylalanine directly on the chromatogram, and chromatography in a second direction with another solvent. The abolition of arogenate accumulation by exogenous L-tyrosine seen in the data of Table 2 is also apparent in Fig. 2.

DISCUSSION

We previously deduced that arogenate is an obligatory precursor of L-tyrosine in species of cyanobacteria (1, 5) and coryneform bacteria (11) on the basis of enzymological studies of wild-type prototrophs. A similar interpretation was made for the yeast *Hansenula henricii* (9). This conclusion is now established most rigorously in *B. flavum* because tyrosine auxotrophy can be equated with lack of arogenate dehydrogenase and because arogenate (the substrate for arogenate dehydrogenase) accumulates behind the enzyme block.

Tyrosine auxotrophs of *C. glutamicum* (12) and *B. flavum* (19) were reported to excrete phenylalanine. However, it now seems a strong possibility that the phenylalanine observed originated from accumulated arogenate. Because arogenate converts to phenylalanine at even mildly acidic pH (1), it seems likely that the stationary-phase decrease in culture pH, further

Table 1. Enzymological comparison of wild-type and mutant *tyrB2*

B. flavum strain	Initial L-tyrosine in medium, μg/ml	Specific activity, nmol/min per mg: DAHP synthetase	Prephenate dehydrogenase	Arogenate dehydrogenase	Prephenate aminotransferase
Wild-type	0	2.9	0	13.2	0.7
	50	2.1	0	13.6	0.6
Mutant *tyrB2*	5	3.6	0	0	0.6
	10	2.7	0	0	0.6
	50	2.3	0	0	0.6

Cell populations were cultured in minimal glucose medium at 30°C with vigorous shaking. Cultures of wild-type and of mutant *tyrB2* grown with 50 μg of L-tyrosine per ml were harvested in the late exponential phase of growth. The remaining *tyrB2* cultures were growth-limited at the initial concentrations of L-tyrosine supplied and were harvested several hours after exhaustion of L-tyrosine from the growth medium.

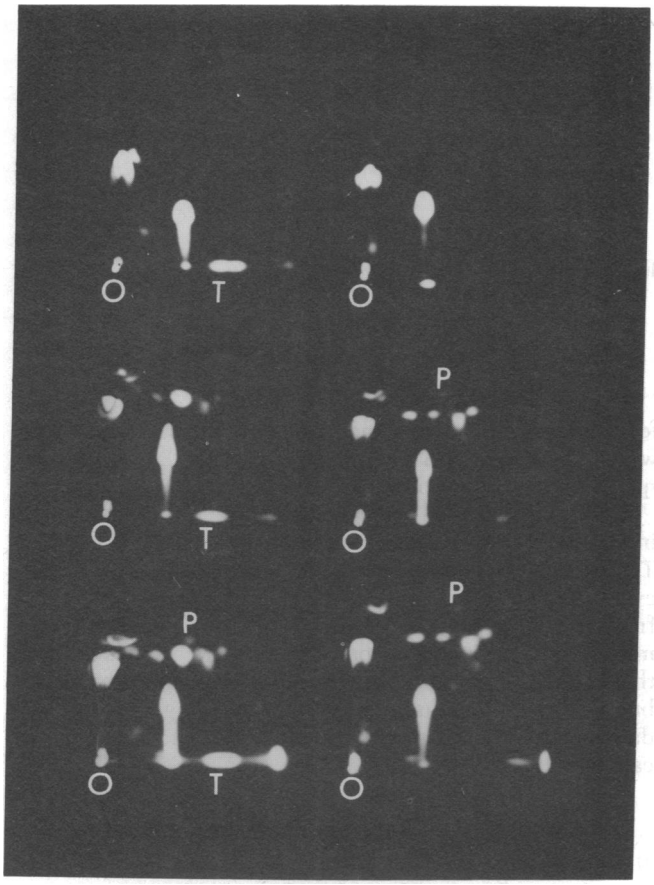

FIG. 2. Use of a dansylation technique to visualize arogenate accumulated in the culture supernatant by auxotroph *tyrB2*. The six thin-layer plates shown were photographed under short-wave UV light. Dansylated supernatant (1 μl) was applied at the origin (O) of each thin-layer micropolyamide plate. Plates were developed in the ascending direction in solvent I, exposed to formic acid vapors (to convert dansylarogenate to dansylphenylalanine), and developed in solvent II (left to right). (*Top Row*) Samples taken from cultures at the initial time of resuspension in accumulation medium. (*Middle Row*) Samples from cultures after 31 hr of incubation in accumulation medium. (*Bottom Row*) Samples were identical to middle row except that authentic arogenate was added prior to dansylation. In each row, left plate represents culture supplemented with 1 mg of L-tyrosine per ml and right plate represents unsupplemented culture. T, migration position of dansyltyrosine; P, position of dansylphenylalanine (acid-converted dansylarogenate).

exacerbated by accumulation of arogenic acid, promoted the nonenzymatic formation of phenylalanine. In mutant *tyrB2* the amount of phenylalanine in the culture supernatant was at least an order of magnitude lower than that of arogenate when pH was constantly adjusted. This possibility is reminiscent of the apparent excretion of L-phenylalanine by a phenylalanine auxotroph of *N. crassa* (20) which was later shown (16) to accumulate arogenate, subsequently seen as phenylalanine owing to the acidic conditions used for amino acid analysis (20).

The *tyrB2* mutant of *B. flavum* should be an ideal choice for the routine microbiological production of arogenate, a procedure which currently uses a phenylalanine auxotroph of *N. crassa* (16). The advantages include: (*i*) the conveniences of faster growth rate, better mass yields during growth, and dealing with unicellular growth of bacteria rather than mycelial growth of fungi; (*ii*) the aspect that arogenate, being a normal intermediary metabolite in *B. flavum*, results in direct accumulation of arogenate behind the mutant block rather than indirect accumulation of a "dead-end" metabolite (16) as in *N. crassa*; (*iii*) the particularly high levels of intracellular glutamate in *B. flavum* should promote maximal transamination of prephenate to form arogenate by prephenate aminotransferase‡; and (*iv*) coryneform species of bacteria have an established history in commercial operations of unmatched capability for the microbiological production of small molecules such as amino acids.

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‡ In a study (21) of aromatic aminotransferases among species of glutamic acid bacteria (coryneform bacteria), glutamate was the best amino-donor substrate in combination with prephenate for prephenate aminotransferase of *B. flavum*.