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Aromatic Aminotransferases in Coryneform Bacteria

AKRAM M. FAZEL[1] AND ROY A. JENSEN[1,2]*

Department of Biological Sciences[1] and Center for Somatic-Cell Genetics and Biochemistry,[2] State University of New York at Binghamton, Binghamton, New York 13901

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Species of coryneform bacteria (*Corynebacterium glutamicum*, *Brevibacterium flavum*, and *B. ammoniagenes*) are capable of transaminating all three of the aromatic pathway intermediates: prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate. Two molecular species of aromatic aminotransferase (denoted aminotransferase I and aminotransferase II) were partially purified from *C. glutamicum* and *B. flavum*, whereas a single aromatic aminotransferase was isolated from *B. ammoniagenes*. In both *C. glutamicum* and *B. flavum*, aromatic aminotransferase I and aromatic aminotransferase II have molecular weights of about 155,000 and 260,000, respectively. The two aromatic aminotransferases from *C. glutamicum* and *B. flavum*, although exhibiting a similar spectrum of overlapping specificities, differ substantially in substrate preference. Pyridoxal-5'-phosphate is tightly associated with these aminotransferases, since little loss of activity was detected when partially purified enzyme preparations were assayed in the absence of exogenous pyridoxal-5'-phosphate. The aminotransferases are quite sensitive to inhibition by phenylhydrazine. This has practical application when assay of prephenate dehydratase is desired in the presence of aromatic aminotransferase activity since potentially trivial interference can be negated by selective phenylhydrazine inhibition of aromatic aminotransferase activity. At 0.1 mM concentrations of phenylhydrazine, 90% inhibitions of aminotransferase activities were achieved in partially purified preparations of *B. flavum* and *C. glutamicum*.

An overall pattern of aromatic biosynthesis whereby the pretyrosine branchlet to L-tyrosine and the phenylpyruvate branchlet to L-phenylalanine is employed has now been documented in cyanobacteria (10, 21), the yeast *Hansenula henricii* (2), and coryneform species of bacteria (6). Since pretyrosine may also serve as a precursor of L-phenylalanine in other organisms, e.g., *Pseudomonas aeruginosa* (17, 18), the transamination of prephenate to form pretyrosine may be an essential enzymatic reaction for biosynthesis of one or both of these aromatic aminotransferases in nature. The biochemical alternatives for tyrosine and phenylalanine biosynthesis are illustrated in Fig. 1. Transamination of prephenate has been studied in plants (J. L. Rubin and R. A. Jensen, Plant Physiol., in press) as well as in a growing list of microorganisms (2, 10, 17, 18, 21, 22).

Aminotransferases may possess sufficient specificity to confine function to a particular biochemical pathway (as with biotin synthesis). On the other hand, an enormous breadth of aminotransferase specificity may provide functional overlap of otherwise distinct biochemical pathways (7). A single aromatic aminotransferase has been partially purified and characterized from *Escherichia coli* (5, 7, 13, 19, 21). Two species of aromatic aminotransferase were identified in two species of cyanobacteria (E. DeFuria, unpublished data), in *Bacillus subtilis* (16), and in mung beans (20), whereas four species of aromatic aminotransferase were described in *P. aeruginosa* (17, 18).

Coryneform bacteria possessing appropriate genetic blocks have excellent potential to replace *Neurospora crassa* (11) as a biological source of pretyrosine. These organisms (glutamic acid bacteria) accumulate high intracellular levels of glutamate, a preferred amino donor for transamination of prephenate. The multiplicity and specificity of aromatic aminotransferases in coryneform bacteria were of special interest in this context. Although the species included in this study carry two generic designations (*Brevibacterium* and *Corynebacterium*), the dubious status of *Brevibacterium* has been cited previously (6). For convenience, an aminotransferase capable of reaction with prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate as keto acid

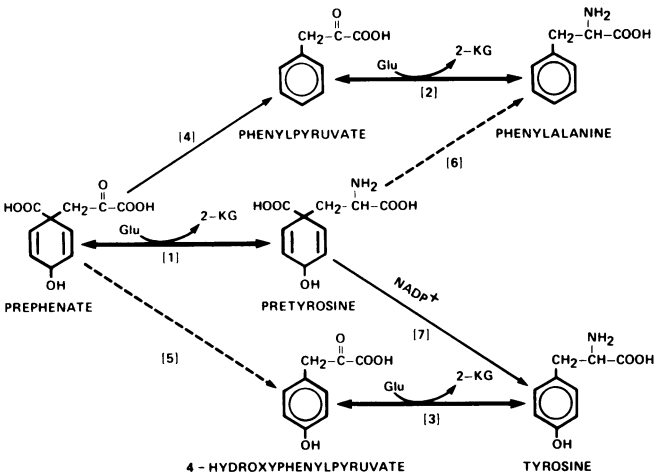

FIG. 1. *Biosynthetic routes to L-phenylalanine and L-tyrosine. Aminotransferase reactions with prephenate reaction [1], phenylpyruvate reaction [2], and 4-hydroxyphenylpyruvate reaction [3] are highlighted by representation as bold arrows. All reactions shown are simultaneously present in P. aeruginosa, but the reactions represented by dotted arrows (prephenate dehydrogenase [5] and pretyrosine dehydratase [6]) are absent in coryneform microorganisms. Reaction [4] is prephenate dehydratase and reaction [7] is pretyrosine dehydrogenase.*

substrates is denoted as an aromatic aminotransferase even though prephenate (and pretyrosine) are not aromatic structures.

MATERIALS AND METHODS

Microbial strains. Lyophile stocks of *B. flavum* (ATCC 14067), *B. ammoniagenes* (ATCC 6872), *B. ammoniagenes pheA1* (ATCC 19351), and *C. glutamicum* (ATCC 13032) were obtained from the American Type Culture Collection (Rockville, Md.). *B. flavum pheA5* (a prephenate dehydratase-deficient phenylalanine auxotroph) and *B. flavum tyrB2* (a pretyrosine dehydrogenase-deficient tyrosine auxotroph) were isolated as mutant derivatives of *B. flavum* (ATCC 14067) (6).

Nutritional procedures. Cells were cultured in a defined mineral salts medium containing 3.6% glucose (carbon source) and 1% urea (nitrogen source) as detailed previously (6). Tryptic soy broth was used for complete medium. Solid media were solidified with 1.5% (wt/vol) agar (Difco). Liquid cultures were aerated by gyratory action of a New Brunswick shaker at 175 rpm. The temperature during growth was maintained at 32°C.

Growth rates were determined in the following manner. Wild-type or mutant populations were grown at 32°C to the late exponential phase of growth in minimal glucose medium supplemented with 50 μg of any amino acids appropriate to the nutritional requirements of auxotrophic mutants per ml. A 0.1-ml portion of this culture was the inoculum for 10 ml of sterile medium contained within a 125-ml Erlenmeyer flask fitted with a side-arm tube sized to match the aperture of a Klett photometer. Klett readings of turbidity were carried out with a red filter (no. 64). Culture samples were diluted to yield turbidity measurements in the range of 25 to 250 Klett units to prepare a correction curve for high turbidity values measured in growth flasks. Turbidities giving an optical density of 0.1 at 600 nm correspond to 13 Klett units, a wet weight of 0.2 g/liter, and a viable count of approximately 2×10^7 plating units per ml. Final mass yields from wild type after growth on minimal glucose medium approximated 2,000 Klett units.

Enzyme preparations. Cell-free extracts were prepared by sonic disruption of cell pellets resuspended in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5), as previously described (6). Rigorously dialyzed preparations are denoted as crude extract. All subsequent steps of enzyme purification were carried out at 4°C by using the following order of procedural steps for fractionation.

An amount of crude extract equivalent to 100 μg of protein was applied to a column (25 by 1.5 cm) of diethylaminoethyl (DEAE)-cellulose equilibrated with 50 mM Tris-hydrochloride (pH 7.5). After washing with 100 ml of buffer, an elution profile was developed with 300 ml of NaCl, applied as a linear concentration gradient between 0.1 and 1.0 M. Fractions of 2.2-ml volume were collected.

Fractions corresponding to the peak area of a given enzyme were combined and concentrated to 5 ml by passage through an Amicon PM 10 membrane. The protein concentrate was applied to a Bio-Gel hydroxylapatite (Bio-Rad) column (20 by 1.5 cm) that was previously equilibrated with 10 mM phosphate buffer (pH 7.03). After washing with 100 ml of the potassium phosphate buffer, protein was eluted from the column in 300 ml of a linear gradient (between 10 and 200 mM) potassium phosphate (pH 7.03). Fractions of 2.2 ml were collected.

Appropriate eluate fractions were combined as above and concentrated to 4 ml by passage through

an Amicon PM 10 membrane. This protein concentrate was layered on a column of LKB Ultrogel AcA34 (55 by 2.25 cm) or on a Sephadex G-200 column that was equilibrated with 50 mM Tris-hydrochloride (pH 7.5). Fractions of 2.2 ml were collected.

The factors of enzyme purification given are undoubtedly underestimates because the activities cited for crude extracts are the combined activities of species aminotransferase I (AT-I) and aminotransferase II (AT-II), these aminotransferases exhibiting substantial overlap in substrate specificity.

Analytical procedures. Aminotransferase activities were assayed by an isotopic labeling procedure (15) employing ^{14}C-labeled amino acid substrates and measuring ^{14}C-labeled keto acid product. Reaction mixtures (100 μl) contained 50 mM Tris-hydrochloride (pH 7.5), 0.25 mM pyridoxal-5'-phosphate, 0.125 μCi of 1 mM L-^{14}C-labeled amino acid, 5 mM keto acid, 25 μg of bovine serum albumin, and enzyme. After incubation at 37°C for 20 min, the reaction was stopped by addition of 1 N HCl. The radioactive keto acid product was extracted into 1.0 ml of ethylacetate-toluene (4:1, vol/vol). A 200-μl sample of the organic phase was transferred into scintillation vials containing 10 ml of New England Nuclear liquifluor, and the radioactivity was measured in a Packard scintillation counter. Correction factors were established to account for the fractional extractabilities of each keto acid into the extraction solvent. [α-^{14}C]ketoglutarate was used to determine an extraction efficiency of 25%. Extraction efficiencies of α-ketoisocaproate (9) and oxaloacetate (8) were determined through the measurement of phenylhydrazone derivatives of keto acid distributed in each phase of the extraction system. Extraction efficiency for phenylpyruvate was determined by extracting phenylpyruvate into 1 ml of ethylacetate-toluene (4:1, vol/vol) and measuring the aqueous phase for the presence of phenylpyruvate. Extraction efficiences used for α-ketoisocaproate, 4-hydroxyphenylpyruvate, phenylpyruvate, and oxaloacetate were 70, 88, 90, and 20%, respectively.

Prephenate dehydratase was assayed in 100-μl reaction mixtures containing 1 mM prephenate, 50 mM Tris-hydrochloride (pH 7.5), and enzyme. After 20 min at 37°C, 0.9 ml of 2.5 N NaOH was added, and phenylpyruvate was measured as absorbance at 320 nm with a Gilford model 250 spectrophotometer. Calculations of phenylpyruvate concentration were based upon a molar extinction coefficient of 17,500 (5).

Protein concentrations were determined by the method of either Lowry et al. (12) or Bradford (3) as described in Bio-Rad Technical Bulletin 1051. All spectrophotometric measurements were carried out at a light path of 1 cm.

Pretyrosine concentrations were estimated by a dansylation procedure (J. Bowen et al., manuscript in preparation). Dansylated pretyrosine was converted nonenzymatically at acidic pH to dansyl-phenylalanine, and its fluorescence was measured using an Aminco Bowman spectrophotofluorometer. The concentration of acid-converted dansyl-pretyrosine (dansyl-phenylalanine) was determined from a dansyl-phenylalanine standard curve. The pretyrosine preparations used were contaminated with shikimate, phenylalanine (20%), and several unidentified compounds detected by high-pressure liquid chromatography.

Molecular weight estimation by gel filtration. Molecular weights were estimated by the method of Andrews (1) by using LKB Ultrogel AcA34 (protein fractionation range of 20,000 to 350,000) or Sephadex G-200. Gel filtration columns were equilibrated with Tris-hydrochloride (pH 7.5). The column (55 by 2.25 cm) was calibrated with the following protein standards: aldolase (158,000 daltons), ovalbumin (45,000 daltons), chymotrypsinogen A (25,000 daltons), and ribonuclease A (13,700 daltons). Void volumes were determined by use of dextran blue. Enzyme preparations (4.0 ml) were layered on the column at 4°C, and 2.2-ml fractions were collected.

Biochemicals and materials. Sephadex G-200 and protein standards for calibration of gel filtration columns were obtained from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52), hydroxylapaptite, and Ultrogel were from Whatman, Calbiochem, and LKB Instruments, respectively. Micropolyamide plates were from Schleicher and Schuell. ^{14}C-labeled compounds were purchased from Amersham and were purified by an extraction procedure (15) before use. Liquifluor was purchased from New England Nuclear. *p*-Hydroxymercuribenzoic acid was from Mann Research Laboratories and L-aspartate was from Calbiochem. Other amino acids and keto acids were from Sigma Chemical Co. 2-Amino-2-hydroxymethyl-1,3-propanediol (Trizma) was also from Sigma. The barium salt of prephenate (79% purity) and potassium pretyrosine (about 50%, pure) were isolated from a multiply blocked mutant of *N. crassa* (11).

RESULTS

Purification of aromatic aminotransferases. In both *C. glutamicum* and *B. flavum* two molecular species of aromatic aminotransferase active with prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate were separated when crude extracts were fractionated on a DEAE-cellulose column. Figure 2 illustrates the elution profile obtained for *C. glutamicum*. The leading DEAE-cellulose peak which eluted at 0.36 M NaCl is designated AT-I, whereas the second peak, which eluted at 0.56 M NaCl is designated AT-II. In Fig. 2 the leading peak of activity (shaded area) was pooled and concentrated, as was described in Materials and Methods, and applied on a hydroxylapatite column to determine whether additional aminotransferase activities might be resolved. A single aminotransferase activity was recovered. Similar results were obtained for the second peak of activity (AT-II). To further purify each aminotransferase, active fractions of each from hydroxylapatite columns were pooled and separately applied to a Sephadex G-200 column. *B. flavum* differed from *C. glutamicum* in its relatively low (about 10-fold lower) specific activity for AT-I

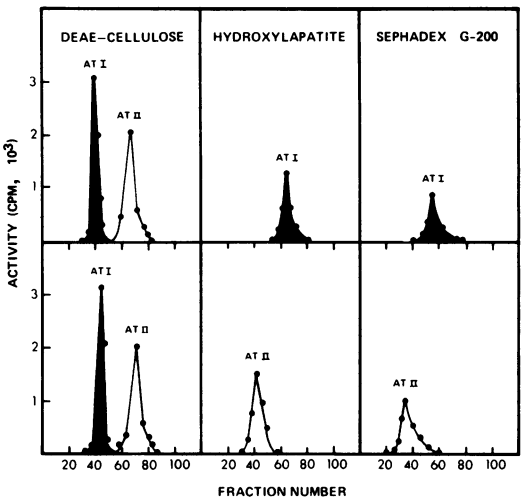

FIG. 2. *Separation of aromatic aminotransferases in C. glutamicum. Crude extracts were chromatographed over DEAE-cellulose as described in Materials and Methods. Two different species of prephenate-glutamate and aminotransferase were separated and designated as AT-I and AT-II. In the top panels, fractions corresponding to AT-I (shaded area) were concentrated and further fractionated over hydroxylapatite. Fractions possessing aminotransferase activity (upper middle panel) were concentrated and applied to a Sephadex G-200 column, appropriate eluates yielding a final preparation with a specific activity of 59.73 nmol/min per mg of protein. In the bottom sequence of panels analogous procedures were carried out with eluate fractions from DEAE-cellulose corresponding to AT-II (unshaded). The pooled eluates of AT-II recovered from the gel filtration step yielded a final preparation having a specific activity of 42 nmol/min per mg of protein. Enzyme activity was assayed as described in Materials and Methods, [L-^{14}C]glutamate and prephenate serving as substrates.*

(compare footnotes of Tables 1 and 2). In *B. ammoniagenes*, in contrast to the other two species of coryneform bacteria, only one aromatic aminotransferase which eluted at 0.54 M NaCl was isolated (apparently most like species AT-II) when crude extracts were chromatographed over DEAE-cellulose. When active DEAE-cellulose fractions were pooled, concentrated, and rechromatographed on hydroxylapatite, a single peak of activity was again recovered.

Molecular weights. Molecular weights were determined as described in Materials and Methods. AT-I from *C. glutamicum* and *B. flavum* possessed similar molecular weights, 160,000 and 152,000, respectively, whereas AT-II was identical in both species, 260,000. The molecular weight of the *B. ammoniagenes* aminotransferase was also 260,000. The molecular weight of each aminotransferase was unaltered when results of enzyme samples recovered from different stages of purification were compared.

Substrate specificity of aromatic aminotransferases. The aminotransferases partially purified from the coryneform bacteria were capable of utilizing a variety of substrate combinations (Tables 1 through 3). In every case the combination of prephenate-glutamate supported the highest specific activity. AT-I and AT-II (Tables 1 and 2), in addition to reacting with phenylpyruvate and 4-hydroxyphenylpyruvate, also reacted with branched-chain keto acids. Both aromatic aminotransferases from *C. glutamicum* and *B. flavum* and the single aromatic aminotransferase from *B. ammoniagenes* utilized glutamate, leucine, phenylalanine, and tyrosine as amino donors (Tables 1 through 3).

Although the aromatic aminotransferases showed overlapping specificities, they exhibited substantial differences in their relative utilization of amino-keto acids that were tested. When prephenate was the keto acceptor, glutamate served as the best amino donor. Species AT-II (Table 1) was also very active on the substrate combination of phenylpyruvate-leucine. In contrast, species AT-I (Table 2) was completely inactive with the phenylpyruvate-leucine combination. Hence, AT-II from both *C. glutamicum* and *B. flavum* differs in specificity, depending on the substrate combination. The specific activity of AT-II for the leucine-phenylpyruvate pair was about fivefold higher than the leucine-prephenate pair or the leucine-4-hydroxyphenylpyruvate pair in *C. glutamicum* and about twofold higher in *B. flavum*. However, when glutamate served as the amino donor, AT-II reacted best with prephenate as the keto acceptor. The specific activity of the glutamate-prephenate pair was about fourfold higher than the glutamate-phenylpyruvate or glutamate–4-hydroxyphenylpyruvate pairs in *C. glutamicum*. The phenomenon is more dramatic in *B. flavum* where the specific activity of the glutamate-prephenate pair is about 15-fold higher than the glutamate-phenylpyruvate combination. Generally, the relative activities seen with various substrate combinations varied in parallel comparing *C. glutamicum* and *B. flavum* enzymes. An exception was any combination with oxaloacetate (Tables 1 through 3) or aspartate (data not shown).

Effect of pyridoxal-5'-phosphate and carbonyl reagents. Pyridoxal-5'-phosphate appears to be tightly bound since the activity of partially purified preparations (DEAE-cellulose) in *B. flavum* was diminished only about

TABLE 1. *Substrate specificity of AT-II from wild-type strains of C. glutamicum and B. flavum*[a]

Keto acid substrate	L-^{14}C-labeled amino acid substrate							
	Glutamate		Leucine		Tyrosine[b]		Phenylalanine	
	Cg	Bf	Cg	Bf	Cg	Bf	Cg	Bf
Prephenate	100	100	12	39			17	10
Phenylpyruvate	13	7	65	93	26	28	60	51
4-Hydroxyphenylpyruvate	10	23	13	50	55	40	15	15
Oxaloacetate	44	32	3	0.6	26	0.2	12	0.2
α-Ketomethylvalerate	20	40	47	42	50	25	35	35
α-Ketoisocaproate	35	34	60	45	33	18	38	43
α-Ketoisovalerate	15	44	57	37	41	14	28	39

[a] Enzyme activities are expressed as relative numbers where a value of 100 for *C. glutamicum* (Cg, left columns) corresponds to a specific activity of 24.0 nmol/min per mg of protein and a value of 100 for *B. flavum* (Bf, right columns) corresponds to a specific activity of 32.8 nmol/min per mg of protein. Assay technique and conditions are specified in Materials and Methods. The purity and yield of *C. glutamicum* AT-II (from hydroxylapatite step of purification) were 45-fold and 62%, respectively. The purity and yield of *B. flavum* AT-II were 52-fold and 68%, respectively.

[b] Aminotransferase assays with substrate combinations of prephenate and glutamate are complicated by the presence of tyrosine-activated prephenate dehydratase.

TABLE 2. *Substrate specificity of AT-I from wild-type strains of C. glutamicum and B. flavum*[a]

Keto acid substrate	L-^{14}C-labeled amino acid substrate							
	Glutamate		Leucine		Tyrosine		Phenylalanine	
	Cg	Bf	Cg	Bf	Cg	Bf	Cg	Bf
Prephenate	100	100	0	0	42	13	22	11
Phenylpyruvate	41	4	0	0	9	14	57	33
4-Hydroxyphenylpyruvate	46	5	0	0	25	19	22	22
Oxaloacetate	0.3	22	0.5	11	0.5	0.3	1	0.6
α-Ketomethylvalerate	6	33	4	22	5	8	9	17
α-Ketoisocaproate	11	89	3	8	6	11	7	19
α-Ketoisovalerate	3	78	3	19	7	22	12	4

[a] Enzyme activities are expressed as relative numbers where a value of 100 for *C. glutamicum* (Cg, left columns) corresponds to a specific activity of 44.0 nmol/min per mg of protein and a value of 100 for *B. flavum* (Bf, right columns) corresponds to a specific activity of 3.6 nmol/min per mg of protein. Assay techniques and conditions are specified in Materials and Methods. The purity and yield of *C. glutamicum* AT-I (from hydroxylapatite step of purification) were 81-fold and 52%, respectively. Purity and yield of *B. flavum* AT-I were 69-fold and 60%, respectively.

30% in the absence of pyridoxal-5'-phosphate.

Under standard assay conditions, phenylhydrazine inhibited each of the aminotransferases studied in both crude and partially purified enzyme preparations. At 0.1 mM concentrations of phenylhydrazine, 90 to 95% of aminotransferase activities were inhibited in all three species of the coryneform bacteria under study. This is exemplified by the data given in Fig. 3. The selective inhibition of aminotransferase activity is useful when it is desirable to assay prephenate dehydratase, an enzyme requiring L-tyrosine as an activator ligand (6). Since prephenate dehydratase copurifies with AT-II (which does not require added pyridoxal-5'-phosphate), both reactions will occur in the presence of prephenate and L-tyrosine. On the other hand, the presence of prephenate dehydratase in partially purified preparations does not complicate aminotransferase assays as long as L-tyrosine is absent.

Growth response of tyrosine- and phenylalanine-requiring mutants in the presence of 4-hydroxyphenylpyruvate or phenylpyruvate. *B. flavum tyrB2* (6) and *B. flavum pheA5* (6) cells were grown in the presence of 50-μg/ml concentrations of 4-hydroxyphenylpyruvate and phenylpyruvate, respectively, as described in the legend of Fig. 4. *tyr*B2 cells lacking pretyrosine dehydrogenase were capable of growing at the wild-type rate when either L-

TABLE 3. *Substrate specificity of aromatic aminotransferase from wild-type B. ammoniagenes*[a]

Keto acid substrate	L-^{14}C-labeled amino acid substrate			
	Glutamate	Leucine	Tyrosine	Phenylalanine
Prephenate	66.0	9.6	—[b]	10.4
Phenylpyruvate	4.8	25.0	4.1	25.6
4-Hydroxyphenylpyruvate	3.6	6.0	22.6	3.8

[a] The purity and yield of enzyme (obtained from the hydroxylapatite step of purification) were 110-fold and 48%, respectively. Datum values are specific activities expressed as nanomoles per minute per milligram of protein. Assays were carried out as described in Materials and Methods.

[b] —, This aminotransferase, being similar to AT-II of *C. glutamicum* or *B. flavum*, yields unreliable results with the prephenate-tyrosine combination of substrates owing to the co-purification of a tyrosine-activated prephenate dehydratase.

tyrosine or 4-hydroxyphenylpyruvate was used as a growth supplement, as shown in Fig. 4. However, mutant *pheA5* (lacking prephenate dehydratase) did not achieve the wild-type rate of growth in the presence of phenylpyruvate.

DISCUSSION

Prephenate aminotransferase. The transamination reaction whereby prephenate is converted to pretyrosine was first reported (21) in 1974. This reaction is significant in vivo with organisms that employ either the pretyrosine branchlet of L-tyrosine biosynthesis or the pretyrosine branchlet of L-phenylalanine biosynthesis. The fractional flow of intermediates through pretyrosine on the way to L-phenylalanine and to L-tyrosine biosynthesis in an organism such as *P. aeruginosa* (17, 18) is unknown because of the complication of multiple pathways existing to each end product. In *P. aeruginosa* four aminotransferases were resolved, each capable of functioning in vitro as prephenate aminotransferase (18). In mung bean a similar duality of pathways to L-tyrosine complicates assessment of the fractional contribution of prephenate aminotransferase to L-tyrosine biosynthesis (20). At the other extreme (11) *N. crassa* cannot convert pretyrosine to either L-phenylalanine (i.e., lacks pretyrosine dehydratase) or L-tyrosine (i.e., lacks pretyrosine dehydrogenase). Nevertheless, auxotrophic mutants which accumulate abnormally high intracellular concentrations of prephenate transaminate a fraction of this to pretyrosine, a dead-end metabolite for this organism (11).

The in vivo role of prephenate aminotransferase is more easily deduced in organisms where pretyrosine is an obligatory precursor of end product. All species of cyanobacteria studied (10, 21, 22) and the three species of corynebacteria (6) studied here synthesize L-tyrosine from pretyrosine. It is clear that the two aromatic pathway aminotransferase activities functioning in vivo with coryneform bacteria are phenylpyruvate aminotransferase (phenylalanine branchlet) and prephenate aminotransferase (tyrosine branchlet).

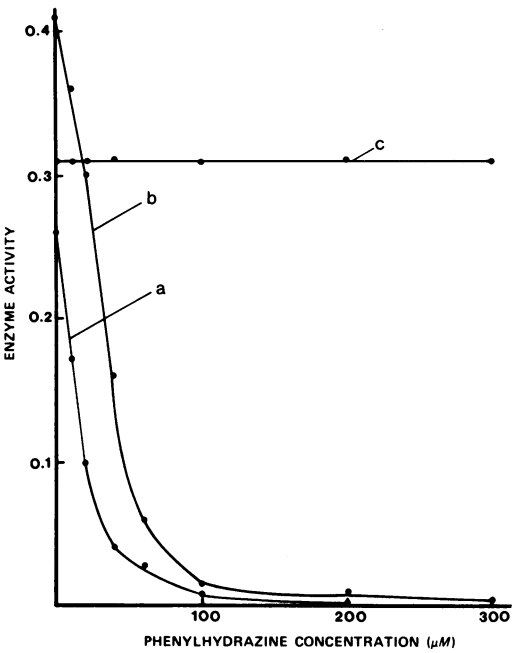

FIG. 3. *Inhibition of AT-II but not prephenate dehydratase by phenylhydrazine. (a) A 15-fold partially purified preparation of AT-II from B. flavum was incubated in the presence of 2.5 mM α-ketoglutarate and phenylhydrazine. After 10 min, 2.5 mM phenylalanine was added. The reaction was stopped by addition of 0.9 ml of 2.5 N NaOH after 10 min, and phenylpyruvate was measured spectrophotometrically at 320 nm (ordinate). (b) Aminotransferase inhibition in the presence of 0.25 mM pyridoxal-5'-phosphate, other assay conditions being the same as above. (c) Activity of prephenate dehydratase in the presence of phenylhydrazine. The enzyme was incubated with phenylhydrazine for 10 min. Then 1.0 mM prephenate and 0.25 mM L-tyrosine were added to the reaction vessels, and incubation was continued for 10 min. The reaction was stopped by addition of 0.9 ml of 2.5 N NaOH, and phenylpyruvate was measured spectrophotometrically at 320 nm.*

In vivo roles of aminotransferase multiplicity. The tendency of many aminotransferases to exhibit considerable ambiguity for substrates is well known (4, 6, 11). In some cases an aminotransferase that functions mainly in one

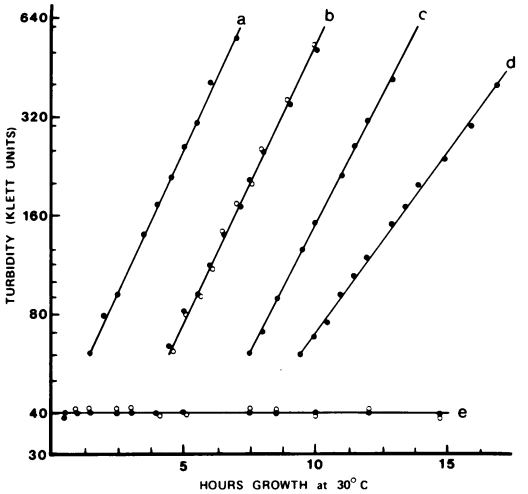

FIG. 4. *Nutritional responses of phenylalanine and tyrosine auxotrophs. Procedures for obtaining growth curves are given in Materials and Methods. For convenience, the major portion of the exponential phase of growth is shown. (a) Wild-type B. flavum in minimal glucose medium. (b) Tyrosine auxotroph B. flavum tyrB2 cultured in minimal glucose medium in the presence of 50 μg of L-tyrosine (●) or 50 μg of 4-hydroxyphenylpyruvate (○) per ml. (c) Phenylalanine auxotroph B. flavum pheA1 cultured in minimal glucose medium containing 50 μg of L-phenylalanine per ml. (d) Phenylalanine auxotroph pheA1 cultured in minimal glucose medium containing 50 μg of phenylpyruvate per ml. (e) Mutant pheA1 (●) or tyrB2 (○) resuspended in unsupplemented minimal glucose medium.*

pathway may realize its in vitro potential to substitute in mutant strains for one or more other aminotransferases that mainly function in different pathways (7, 19). Since in *C. glutamicum* and *B. flavum* there are two aminotransferases to correspond with two physiological aminotransferase reactions (prephenate aminotransferase and phenylpyruvate aminotransferase), it is tempting to consider the possibility of in vivo aminotransferase specialization for these two reactions. Aromatic AT-II copurifies with prephenate dehydratase following DEAE-cellulose, hydroxylapatite, and Sephadex G-200 fractionation steps. Since prephenate dehydratase forms phenylpyruvate, the possible protein-protein association of prephenate dehydratase and aromatic AT-II would favor the spatial access of phenylpyruvate to the latter enzyme. However, several phenylalanine auxotrophs lacking prephenate dehydratase retained an unaltered aromatic AT-II. Furthermore, regulatory mutants derepressed for prephenate dehydratase possessed the same specific activity of aromatic AT-II as wild type. Because the latter results with auxotrophic and regulatory mutants ruled out a multifunctional protein or an enzyme complex, any postulated protein-protein interactions must be fragile associations. Perhaps the inability of a phenylalanine auxotroph lacking prephenate dehydratase to sustain the wild-type rate of growth on phenylpyruvate reflects disruption of such a fragile association in vivo. It is intriguing that aromatic AT-II functions better with phenylpyruvate than with prephenate provided L-leucine is the amino donor reactant. However, the slow growth rate of the phenylalanine auxotroph on phenylpyruvate (curve d of Fig. 4) was not increased in the presence of exogenous L-leucine at 100 μg/ml. Aromatic AT-I functioned best with prephenate under all assay conditions tested. At this time no compelling case can be made to identify separate in vivo roles of the two aromatic aminotransferases.

Amazingly, a tyrosine auxotroph of *B. flavum* does sustain the wild-type rate of growth in the presence of 4-hydroxyphenylpyruvate. The transamination of 4-hydroxyphenylpyruvate is presumed to be unphysiological since wild type lacks prephenate dehydrogenase, relying instead on the pretyrosine sequence for L-tyrosine biosynthesis (6). In *B. flavum* aromatic AT-II seems to have the greatest potential for transamination of 4-hydroxyphenylpyruvate.

ACKNOWLEDGMENTS

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