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Enzymological Basis of Reluctant Auxotrophy for Phenylalanine and Tyrosine in *Pseudomonas aeruginosa**

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Dual biosynthetic pathways to L-phenylalanine and L-tyrosine exist in *Pseudomonas aeruginosa* (Patel, N., Pierson, D. L., and Jensen, R. A. (1977) *J. Biol. Chem.* 252, 5839-5846). Tightly blocked phenylalanine or tyrosine auxotrophs are not obtained as the result of single mutations. Presumably the presence of a mutation that interrupts one pathway is masked by the presence of the alternative pathway. However, a leaky phenylalanine auxotroph (doubling time of 140 min in minimal glucose medium compared to 57 min with wild type) was isolated which completely lacked aminotransferase DE I. This is one of four aromatic aminotransferases of overlapping specificity, each capable of transamination with prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate. A suppressor mutation in the genetic background of the phenylalanine bradytroph was equated with the constitutive synthesis of aminotransferase HA I, normally a catabolic enzyme induced in wild type in the presence of either L-tyrosine or L-phenylalanine. The synthesis of aminotransferase HA II is repressed in the presence of phenylalanine and tyrosine, a result which suggests its probable role in aromatic amino acid biosynthesis. Aminotransferase HA III is unregulated by aromatic metabolites and is thought to function primarily in branched-chain amino acid metabolism.

Although the suppressor mutation restores the wild type growth rate in minimal glucose medium, aromatic biosynthesis is highly stressed in this strain as revealed by its hypersensitivity to antimetabolite analogues of phenylalanine and tyrosine. In fact, the DE I aminotransferase deficiency is no longer suppressed when fructose, a carbon source previously shown in wild type to render aromatic biosynthesis limiting to growth, is used.

It is likely that the phenylalanine bradytroph can be utilized as the genetic background for the isolation of otherwise silent mutations that inactivate the various biosynthetic enzymes of tyrosine and phenylalanine biosynthesis.

Although phenylalanine and tyrosine are synthesized exclusively via phenylpyruvate and 4-hydroxyphenylpyruvate in such widely studied microorganisms as *Escherichia coli* or *Bacillus subtilis*, various (and probably all) species of blue-green algae form L-tyrosine from the amino acid intermediate, pretyrosine (1, 2). In some organisms pretyrosine may also function as a precursor of L-phenylalanine, as described recently in *Pseudomonas aeruginosa* (3). This bacterium was shown to possess dual enzymatic routes to convert prephenate to L-phenylalanine via either phenylpyruvate or pretyrosine, and likewise to possess enzymes converting prephenate to tyrosine via either 4-hydroxyphenylpyruvate or pretyrosine. This enzymatic multiplicity for tyrosine and phenylalanine synthesis in *P. aeruginosa* is illustrated in Fig. 1.

Mutagenic treatment of wild type cultures with nitrosoguanidine has routinely produced nutritional mutants at high frequency in our laboratory. Thus, we readily obtained a full set of tryptophan auxotrophs, determined their enzymological deficiencies (4), and these have now been mapped on the *P. aeruginosa* chromosome.[1] Other aromatic mutants blocked before shikimate or between shikimate and chorismate have been obtained (5)[2] with no difficulty. Nevertheless, we have not managed to isolate mutants having absolute growth requirements for either L-phenylalanine or L-tyrosine. This is consistent with the presence *in vivo* of a second enzymatic option to either L-tyrosine or to L-phenylalanine in any given mutant.

The fractional contributions of the two enzymatic routes to L-phenylalanine or to L-tyrosine under various physiological conditions is uncertain. If any of these four two-step sequences are inadequate in the absence of the alternative pathway to supply the total end product required for protein synthesis, then the imposition of a mutant block in the alternative sequence might produce a phenotype of leaky auxotrophy. Several phenylalanine bradytrophs were indeed obtained, and one was selected for detailed enzymological examination.

MATERIALS AND METHODS

Microbiological Aspects

Strain 1 of *P. aeruginosa* (wild type) was originally obtained from B. W. Holloway (6). Strain 1 is the parent strain from which phenylalanine bradytroph NP72 was derived through *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (100 μg/ml, 30 min) as previously described (7). Mutant NP 76 was derived from mutant NP 72 following its selection as a spontaneous revertant capable of growth at the wild type rate on minimal glucose medium.

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[1] B. W. Holloway, manuscript in preparation.

[2] N. Patel, S. Stenmark-Cox, and R. A. Jensen, unpublished data.

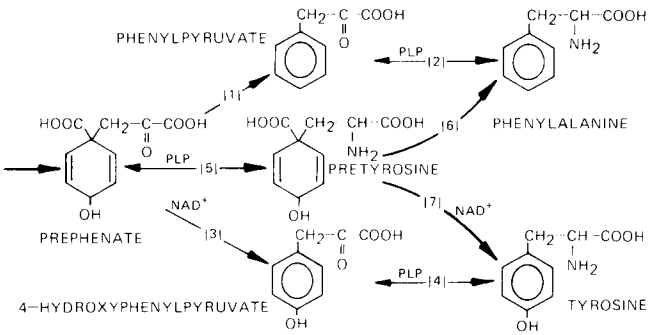

FIG. 1. The upper and lower sequences of reactions show the phenylpyruvate route to phenylalanine biosynthesis and the 4-hydroxyphenylpyruvate route to tyrosine biosynthesis, respectively. Pretyrosine is an amino acid intermediate which is one enzymatic step from phenylalanine or tyrosine, and is formed by transamination of prephenate. Enzyme denotations: |*1*|, prephenate dehydratase; |*2*|, phenylpyruvate aminotransferase; |*3*|, prephenate dehydrogenase (NAD^+-dependent); |*4*|, 4-hydroxyphenylpyruvate aminotransferase; |*5*|, prephenate aminotransferase; |*6*|, pretyrosine dehydratase, |*7*|, pretyrosine dehydrogenase (NAD^+-dependent). *PLP*, pyridoxal 5'-phosphate.

Unless otherwise specified, cultures were grown at 37°C in a minimal salts medium (8) containing 0.5% glucose or fructose (autoclaved separately). Stock cultures were maintained with 6-month longevity between transfers by preparing stab cultures in 0.7% agar, incubating overnight at 30°C, sealing with paraffin wax, and storing the vials at room temperature.

Preparation of Crude Extracts

Cell-free extracts were prepared in 50 mM Tris·HCl, pH 7.9, as previously described (9). Extracts of 2 to 5 ml were prepared from 200-ml volumes of culture in the late exponential phase of growth.

Aromatic Aminotransferase Designations

Four molecular species of aromatic aminotransferases have been characterized in *P. aeruginosa* and designated as DE I, HA I, HA II, and HA III. Each has overlapping specificities for prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate. For convenience, "prephenate aminotransferase," for example, refers to the combined activities of all four transaminases in crude extracts when prephenate is supplied as the keto substrate.

Comparison of Aromatic Aminotransferase Activities in Crude Extracts

Activities of prephenate aminotransferase, phenylpyruvate aminotransferase, and 4-hydroxyphenylpyruvate aminotransferase are expressed as specific activities (nanomoles/min/mg of crude extract) obtained with 5 mM of the keto substrate reactant and 0.25 mM L-[^{14}C]glutamate. Even total aminotransferase activity with a given substrate combination cannot be accurately assessed in crude extracts owing to interfering enzymes which react with the various substrates or products (or both). The method of determination of the fractional contributions of aromatic aminotransferases DE I, HA I, HA II, and HA III to total aminotransferase activity in crude extracts with various keto acceptor substrates under the above assay conditions was detailed before (Table I of Ref. 3).

Phenylalanine bradytroph NP 72 differs from wild type in the absence (by mutation) of aminotransferase DE I. If grown in minimal glucose medium, the bradytroph also differs from wild type in its 3-fold decrease of aminotransferase HA I and its 6-fold increase in aminotransferase HA II. The suppressed revertant NP 76 differs from wild type in the absence of aminotransferase DE I and a 2.5-fold elevation of aminotransferase HA I (in minimal glucose medium).

Analytical Procedures

Aromatic Aminotransferase Assays – In some experiments, a spectrophotometric assay for phenylpyruvate at 320 nm or 4-hydroxyphenylpyruvate at 331 nm was used. In most experiments, radiolabeled amino acids (L-[^{14}C]glutamate or L-[^{14}C]leucine) were used in combination with an aromatic keto acid. Both assay procedures were previously described (3).

Other Enzyme Assays – Chorismate mutase, prephenate dehydrogenase, pretyrosine dehydrogenase, prephenate dehydratase, and pretyrosine dehydratase activities were determined as previously described (3).

Determination of Pretyrosine Concentrations – Several assay techniques for the measurement of pretyrosine concentrations were previously described (3).

Determination of Protein Concentrations – The method of Lowry *et al.* (10) was used.

Enzyme Fractionation Procedures

Procedures of DEAE-cellulose chromatography, hydroxylapatite chromatography, and Sephadex G-200 gel filtration used for the separation of the four aromatic aminotransferases (DE I, HA I, HA II, and HA III) were detailed before (3). When column fractions were pooled and concentrated, an Amicon Diaflow Cell (PM 10 filter) was used.

Source of Biochemicals

Pyridoxal-5'-P, NAD, NADP, L-amino acids, keto acids, 2-amino-2-hydroxymethyl-1,3-propanediol (Trizma) buffer, 4-aminophenylalanine, β-2-thienylalanine, and Sephadex G-200 were obtained from Sigma Chemical Co. DE52 cellulose was from Whatman and hydroxylapatite (Bio-Gel HTP) was from Bio-Rad. L-[^{14}C]Glutamate (270 mCi/mmol) and L-[^{14}C]leucine (324 mCi/mmol) purchased from Amersham/Searle were purified by an organic solvent extraction method (11) before use. Aquasol was obtained from New England Nuclear.

Barium prephenate and either ammonium pretyrosine or barium pretyrosine were isolated from the 5-day accumulation medium of a multiply blocked mutant strain of *Neurospora crassa* (12).

RESULTS

Recovery of Aromatic Auxotrophs in P. aeruginosa – Tightly blocked phenylalanine or tyrosine auxotrophs were not obtained. We did, however, encounter leaky mutants whose growth was restored to the rate of wild type in the presence of L-phenylalanine. Such mutants are recognized if survivors of mutagenesis are screened for faint growth as early as possible following the replica plating technique. Growth data obtained with one such phenylalanine bradytroph, our isolate number NP 72, are illustrated in Table I. In minimal glucose medium, the bradytroph grows about 40% as fast as wild type. The wild type rate of growth was achieved in the presence of L-phenylalanine or phenylpyruvate. L-Tyrosine and its keto acid precursor, 4-hydroxyphenylpyruvate, also increased the growth rate significantly, but did not restore the growth rate seen in the wild type parent. Spontaneous revertants were readily obtained.

Enzymological Deficiency of Phenylalanine Bradytroph NP 72 – When crude extracts of the mutant were compared with wild type extracts for differences in enzyme activities, none were apparent. However, in view of the recent appreciation that enzyme studies with crude extracts are exceedingly complicated owing to the presence of two enzymatic pathways to L-tyrosine and L-phenylalanine (3), fractionation procedures were essential. Using procedures described before (3), mutant NP 72 was found to possess unaltered enzymes for both species of chorismate mutase, prephenate dehydratase, pretyrosine dehydratase, prephenate dehydrogenase, and pretyrosine dehydrogenase.

However, when appropriate chromatographic procedures were used to separate the four aromatic aminotransferase activities previously characterized in wild type, aminotransferase DE I was clearly absent in phenylalanine bradytroph NP 72 (Fig. 2). A revertant derivative of the phenylalanine bradytroph, isolate number NP 76, is seen to have arisen as a

TABLE I

Growth responses of phenylalanine bradytroph NP 72

Small volumes of overnight cultures in minimal glucose medium containing 50 μg/ml of L-phenylalanine in late exponential growth (A_{660} = 0.7) at 37°C were prepared as inocula. The cells were centrifuged and resuspended in 10-ml volumes of fresh medium containing the indicated supplements and incubated with vigorous shaking at 37°C in a shaker. The initial turbidity of the cultures placed in 125-ml side-arm flasks was 0.05 at 600 nm. Optical densities were read at 30-min intervals until stationary phase was approached.

Strain phenotype	Nutritional[a] additions	Doubling time
		min
Wild type	Minimal glucose	57
Phe⁻ bradytroph	Minimal glucose	140
	+4-Hydroxyphenylpyruvate[b]	110
	+L-Tyrosine[b]	70
	+Phenylpyruvate	58
	+L-Phenylalanine	56
Phe⁺ revertant	Minimal glucose	59

[a] Supplements to minimal glucose medium were present at a final concentration of 50 μg/ml.

[b] When 50 μg/ml of 4-hydroxyphenylpyruvate or tyrosine was present, growth proceeded at the indicated rates until a turbidity of about 0.35 at 600 nm was reached, and the growth rate decreased to that obtained in minimal glucose. Apparently this amount of tyrosine is degraded in this time span.

suppressor mutation based upon the transaminase profile shown at the *bottom* of Fig. 2. In revertant NP 76, aminotransferase DE I is absent as is the case with its bradytrophic parent (*middle panel*). This revertant differs genetically from wild type in its absence of aminotransferase DE I (the primary mutation) and its elevated level of aminotransferase HA I (the suppressor mutation).

Regulation of Synthesis of Aromatic Aminotransferases—The data given in Fig. 2 are consistent with an inducible regulation of aminotransferase HA I synthesis. The basal, uninduced level is probably that found in the bradytroph cultured in minimal glucose medium, a condition of end product limitation for the mutant. Presumably, the endogenous level of phenylalanine or tyrosine of wild type (or both) (Fig. 2) are sufficient to partially induce the synthesis of aminotransferase HA I. The data given in Table II illustrate the variation of aminotransferase HA I activity over a 7-fold range. The suppressed mutant (right column) displays constitutive synthesis of aminotransferase HA I. In wild type and in the bradytroph, the presence of L-phenylalanine results in full induction of the HA I enzyme while L-tyrosine promotes partial induction. In the two mutant strains lacking aminotransferase DE I, a certain proportionality between growth rate (Table I) and level of aminotransferase HA I (Table II) exists.

The data given in Fig. 2 are consistent with repression control of aminotransferase HA II synthesis. Thus, in the bradytroph where both phenylalanine and tyrosine are limiting to growth (Table I), the level of aminotransferase HA II was elevated significantly. When the bradytroph was grown in L-phenylalanine-supplemented medium, the level of aminotransferase HA II was repressed to the level of measured in wild type (data not shown). Repression of the synthesis of aminotransferase HA II over about a 6-fold range in the bradytroph is shown in Table III. Apparently in wild type the endogenous levels of end products during growth in minimal glucose are sufficient for maximal repression of aminotransferase HA II.

The level of aminotransferase DE I in wild type is unaffected by culture in the presence of L-phenylalanine or L-tyrosine (or both) (in comparison with unsupplemented cultures). The specific activity of aminotransferase HA III was unaffected by the presence or absence of aromatic amino acids in all these strains.

Total Aminotransferase Activities with Aromatic Substrates in Wild Type and Mutant Strains—In Fig. 3, the combined activities of the four aminotransferase species with prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate in wild type are shown schematically, total activity being proportional to the height of the *leftward bars*. In the bradytroph and its suppressed revertant, the total activity shown is the sum of the three aromatic aminotransferases that remain in these strains, taking into account inducible changes in aminotransferase HA I and repressive changes in aminotransferase HA II.

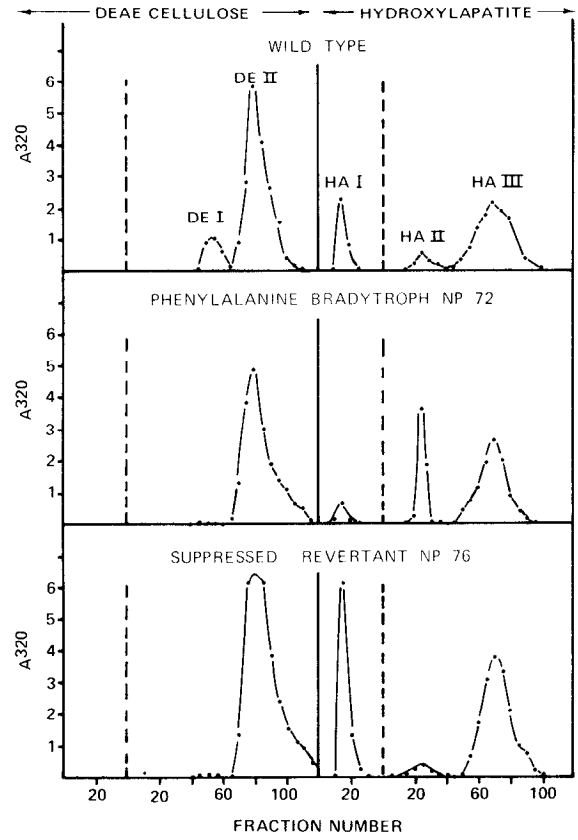

FIG. 2. Separation of aromatic aminotransferases from wild type, phenylalanine bradytroph NP 72, and its revertant derivative NP 76. *Left*, crude extracts were chromatographed on DEAE-cellulose as described under "Materials and Methods." Phenylpyruvate appearance was determined at 320 nm in assays containing 2.5 mM 2-ketoglutarate and 10 mM L-phenylalanine as described under "Materials and Methods." The total activities per fraction per min are plotted against fraction number. An absorbance value of 1.0 corresponds to 57 nmol of phenylpyruvate at 320 nm at alkaline pH. *Right*, the DE II-labeled profile corresponds to three aminotransferases which are separated on hydroxylapatite (HA I, HA II, and HA III). Fractions 66 to 106 recovered from DEAE-cellulose (*i.e.* DE II) were pooled, concentrated to 4.0 ml, and applied to hydroxylapatite as described under "Materials and Methods." Fractions to the *left* of the *vertical dotted line* contain proteins (*e.g.* HA I) which elute in the wash volume.

Total prephenate aminotransferase activity is similar in all three strains, actually being greater in the phenylalanine bradytroph than in wild type. In the phenylalanine bradytroph, the level of phenylpyruvate aminotransferase is 23% of its wild type parent, while the level of 4-hydroxyphenylpyruvate aminotransferase in the bradytroph is 32% of that measured in wild type. Total phenylpyruvate aminotransferase in the revertant is nearly double that of the bradytroph; total 4-hydroxyphenylpyruvate aminotransferase activities of the bradytroph and its revertant are similar.

The total specific activities expressed in Fig. 3 are given in Table IV, together with a detailed breakdown of the fractional contribution of the four aromatic aminotransferase species to each of the three aminotransferase activities involved in aromatic amino acid biosynthesis of *P. aeruginosa*. Aminotransferase DE I of wild type is the major contributor to phenylpyruvate aminotransferase and 4-hydroxyphenylpyruvate aminotransferase activities. In the phenylalanine bradytroph, species HA II accounts for about 60% of all three aminotransferase activities, a result in marked contrast to the low fractional contribution of species HA II to the three aminotransferase activities, of either wild type or the revert-

TABLE II

Inducibility of aromatic aminotransferase HA I

Extracts were prepared from cultures in the late exponential phase of growth as described under "Materials and Methods." Aromatic aminotransferase HA I was isolated as described under Fig. 2 and the appropriate fractions were pooled. Assays were carried out at 37°C for 20 min in 200-μl reaction mixtures containing 50 mM Tris buffer, pH 7.9, 50 mM 2-ketoglutarate, 2.5 mM L-phenylalanine, and enzyme. Reactions were terminated with 0.8 ml of 2.5 N NaOH and absorbance of phenylpyruvate was read at 320 nm.

Growth condition	Specific activity[a]		
	Wild type	Phe⁻ bradytroph	Phe⁺ revertant
	nmol phenylpyruvate/min/mg crude extract		
Minimal glucose	3.2	1.1	8.0
+L-Phenylalanine[b]	8.0	7.2	
+L-Tyrosine[b]	4.0	3.6	

[a] Appropriate calculations were made to extrapolate activities measured in partially purified preparations back to specific activities in crude extracts originally prepared from whole cells.

[b] Added to a final concentration of 50 μg/ml.

TABLE III

Repression of aromatic aminotransferase HA II in phenylalanine bradytroph NP 72

Aminotransferase HA II was recovered from hydroxylapatite as described under Fig. 2.

Growth condition	Specific activity[a]
Minimal glucose	0.68
+L-Phenylalanine[b]	0.18
+L-Tyrosine[b]	0.11

[a] Expressed as nanomoles of L-2-keto[^{14}C]glutarate formed/min/mg of crude extract. The enzyme activity was assayed with prephenate and L-[^{14}C]glutamate as described under "Materials and Methods."

[b] Added to a final concentration of 50 μg/ml.

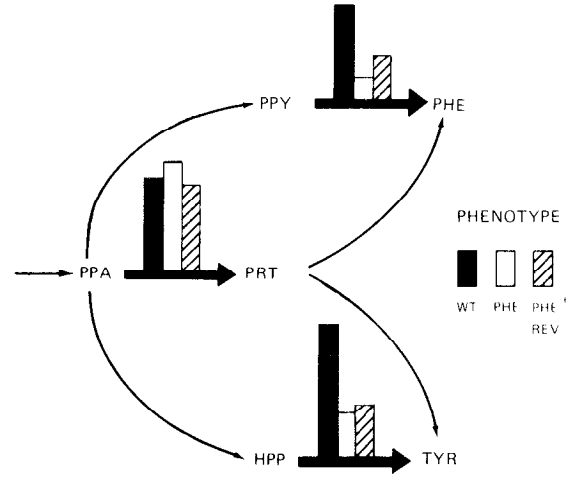

FIG. 3. Levels of aromatic aminotransferase activities in wild type and mutant strains of *P. aeruginosa*. Prephenate (*PPA*) aminotransferase (*left*), phenylpyruvate (*PPY*) aminotransferase (*upper right*), and 4-hydroxyphenylpyruvate (*HPP*) (*lower right*) are represented by *solid arrows*. Other enzyme reactions shown are detailed in Fig. 1. The *bar graphs* indicate the specific activities of the indicated aminotransferase in wild type (*WT*), phenylalanine bradytroph NP 72 (*PHE⁻*), or a suppressed revertant (*PHE⁺REV*.) NP 76 derived from NP 72. Specific activities expressed as nanomoles/min/mg of crude extract protein and determined as described under "Materials and Methods" are: 2.18, 2.14, and 2.94 with prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate, respectively, in wild type; 2.49, 0.50, and 0.95 with prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate, respectively, in phenylalanine bradytroph NP 72; 2.02, 0.77, and 1.15 with prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate, respectively, in the suppressed revertant. *PRT*, pretyrosine.

TABLE IV

Comparison of fractional contributions of various molecular species of aromatic aminotransferases to enzyme reactions in different strains

Aminotransferases DE I, HA I, HA II, and HA III were separated by DEAE-cellulose and hydroxylapatite chromatography, and appropriate calculations were made to extrapolate these completely stable activities back to specific activities of crude extracts originally prepared from whole cells.

Strain phenotype	Prephenate aminotransferase[a]					Phenylpyruvate aminotransferase[a]					4-Hydroxyphenylpyruvate aminotransferase[a]				
	Total specific activity[b]	DE I	HA I	HA II	HA III	Total specific activity[b]	DE I	HA I	HA II	HA III	Total specific activity[b]	DE I	HA I	HA II	HA III
		%	%	%	%		%	%	%	%		%	%	%	%
Wild type	2.18	36	19	12	33	2.14	81	11	2	6	2.94	76	10	3	11
Phe⁻ bradytroph	2.49	0	6	65	29	0.50	0	16	60	24	0.95	0	10	57	33
Phe⁺ revertant	2.02	0	50	14	36	0.77	0	78	6	16	1.15	0	65	8	27

[a] Keto acid used at 5 mM; L-[^{14}C]glutamate at 0.25 mM.

[b] Expressed as nanomoles/min/mg of crude extract (see "Materials and Methods").

ant. In the suppressed mutant, species HA I is the major fractional component of all three aminotransferase activities. Hence, in each of the three strains compared in Table IV, a different species of aromatic aminotransferase contributes the major share of transaminase activity with each of the three aromatic keto substrates.

Recruitment of Catabolic Aminotransferase to Biosynthetic Function – Aminotransferase DE I is reasonably deduced to be a biosynthetic enzyme since its loss by mutation leads to slowed growth in the absence of end products. Aminotransferase HA I is catabolic in function since it is induced by phenylalanine and tyrosine. The kinetic data illustrated in Fig. 4 confirm the opposing equilibria of the enzymes. Aminotransferase DE I exhibits K_m values for the substrate reactants shown that favors catalysis in the rightward directions (*top*) by virtue of order of magnitude differences.

Aminotransferase HA I actually displays greater affinities for phenylpyruvate and 4-hydroxyphenylpyruvate than does aminotransferase DE I. The relative affinities for glutamate and 2-ketoglutarate, however, are dramatically different in comparison of the two enzymes, suggesting catalysis in the rightward direction for aminotransferase DE I and catalysis in the leftward direction for aminotransferase HA I.

The growth of phenylalanine bradytroph NP 72 may be limited more or less equally by phenylalanine and tyrosine synthesis, as suggested by the analysis depicted in Fig. 3. An apparently greater deficiency in endogenous phenylalanine synthesis is suggested by the better growth response to L-phenylalanine or phenylpyruvate (Table I) than to L-tyrosine or 4-hydroxyphenylpyruvate. However, this probably reflects the excellent induction of aminotransferase HA I synthesis by L-phenylalanine, but not by L-tyrosine (Table II).

Comparison of Responses of Wild Type and Pseudo Wild Type to Analogue Stress – In minimal glucose medium the growth rate of wild type *P. aeruginosa* is retarded weakly by β-2-thienylalanine, which is nevertheless one of the most effective phenylalanine antimetabolites for this organism (8). The pseudo wild type derivative (NP 76) of the phenylalanine bradytroph grows in glucose minimal medium at the same rate as wild type (Table I). However, mutant NP 76 is hypersensitive to growth inhibition by β-2-thienylalanine (Table V), indicating that phenylalanine biosynthesis is more stressed in the revertant strain than in wild type. The growth rates of the two strains are only slightly different in the presence of 4-aminophenylalanine, an antimetabolite of L-tyrosine in *P. aeruginosa* (8).

When glucose is replaced with fructose, *P. aeruginosa* grows more slowly (Table VI), and it was previously shown (8) that in the presence of fructose, total aromatic biosynthesis becomes growth-limiting owing to decreased flux of early precursors into the pathway. Under these conditions of growth (minimal fructose), β-2-thienylalanine inhibits the growth rate of wild type better than 4-aminophenylalanine (Table VI), in contrast to results shown in Table V with minimal glucose.

Strikingly, the revertant does not achieve the wild type rate of growth in minimal fructose. In fact, the growth rate of the revertant is as slow as that of the bradytroph. Clearly, the suppressor mutation present in the revertant would never have been detected if selection had been carried out on minimal fructose medium. Presumably aminotransferase HA I cannot operate effectively in the biosynthetic direction under the metabolic conditions that exist during growth on fructose.

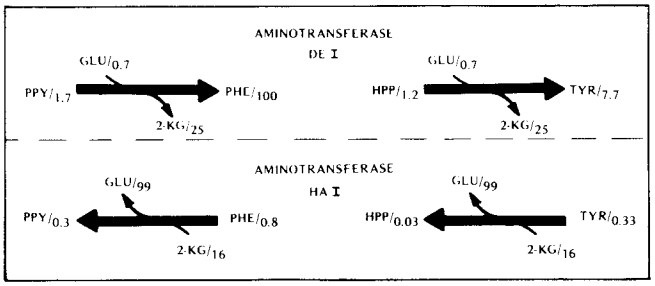

FIG. 4. Comparison of relative affinities of aromatic aminotransferases DE I and HA I for substrate reactants. Aminotransferase DE I is represented as phenylpyruvate (*PPY*) aminotransferase or 4-hydroxyphenylpyruvate (*HPP*) aminotransferase across the *top*. Aminotransferase HA I is represented as phenylalanine (*PHE*) aminotransferase or tyrosine (*TYR*) aminotransferase across the *bottom*. Other amino and keto acid reactants shown are glutamate (*GLU*) or 2-ketoglutarate (*2-KG*). The *numbers* represent the apparent K_m values (millimolar) as determined from double reciprocal plots. Velocities were expressed as nanomoles/min. The K_m for glutamate was determined by maintaining phenylpyruvate or 4-hydroxyphenylpyruvate concentrations constant at 3 mM. To determine K_m values for phenylpyruvate and 4-hydroxyphenylpyruvate, the concentration of glutamate was fixed at 2.5 mM. The reaction mixture (0.2 ml) containing 50 mM Tris·HCl buffer (pH 7.9), appropriate substrates, and enzyme was incubated at 37°C for 20 min. After addition of 4.8 ml of 2.5 N NaOH, the concentration of phenylpyruvate or 4-hydroxyphenylpyruvate was determined by measuring absorbance at 320 or 331 nm, respectively. The K_m value for 2-ketoglutarate was determined using L-phenylalanine at a fixed concentration of 10 mM, and monitoring phenylpyruvate formation at 320 nm. To determine the K_m for L-phenylalanine or L-tyrosine, the concentration of 2-ketoglutarate was fixed at 2.5 mM. The spectrophotometric assay method previously described was used.

TABLE V

Hypersensitivity of suppressed revertant NP 76 to growth inhibition by β-2-thienylalanine

Addition	Doubling time[a]	
	Wild type	Phe$^+$ revertant NP 76
Minimal glucose	57	57
+β-2-Thienylalanine[b]	72	115
+4-Aminophenylalanine[b]	101	108

[a] Doubling times were calculated from the slopes of growth curves carried out essentially as described under Table I.

[b] Initially added to a final concentration of 100 μg/ml.

TABLE VI

Dependence of revertant phenotype upon carbon source

Addition[a]	Doubling time[b]		
	Wild type	Phe$^+$ revertant	Phe$^-$ bradytroph
Minimal fructose	204	281	279
+β-2-Thienylalanine	310	400	
+4-Aminophenylalanine	222	400	

[a] Fructose was added to a final concentration of 0.5%. Analogues, when present, were added to a final concentration of 100 μg/ml.

[b] Doubling times were calculated from the slopes of growth curves carried out essentially as described under Table I.

DISCUSSION

Dual Biosynthetic Pathways and Strategy for Mutant Selection – The simultaneous presence of separate enzymatic pathways leading to a given major metabolite may be more

common in nature than previously suspected, and even *E. coli* is not altogether free of such pathway multiplicity (13). The physiological and evolutionary implications of metabolic ambiguity for synthesis of small molecules has been discussed elsewhere (14). It seems likely that difficulties encountered in the isolation of amino acid auxotrophs from various groups of bacteria of wide distribution in nature may be explained by the compensatory presence in "silent" mutants of a second pathway to a given end product. Aromatic biosynthesis in pseudomonad microorganisms is an excellent example. Since the phenylpyruvate and 4-hydroxyphenylpyruvate pathways of *E. coli* were readily demonstrated in *P. aeruginosa*, the additional presence of the pretyrosine pathways to phenylalanine and tyrosine has remained unsuspected until recently. In such systems, single mutations may at best be expressed as leaky auxotrophs (if the presence of only one intact sequence to end product results in a growth-limiting supply of that end product), or perhaps as mutants that are hypersensitive to inhibition of growth by end product analogues (if the presence of only one intact sequence to end product results in a significantly decreased endogenous pool size of end product). If the presence of a mutation is recognized, either by leaky auxotrophy or by analogue hypersensitivity, a second mutagenesis using this strain should produce tightly blocked derivatives carrying an additional block in the second pathway. When genetic backgrounds containing such combinations of sequentially introduced mutations are subsequently separated by recombination, it is likely that their individual phenotypes may be similar to wild type.

In Vivo Function of Aminotransferase Reactions – The extent to which a given aminotransferase may be shared to carry out transamination reactions in different biochemical pathways is not very well understood. The broadly overlapping specificities of most aminotransferase proteins *in vitro* may or may not reflect the spectrum of reactions actually catalyzed *in vivo*. Even when a particular transaminase reaction can only be catalyzed by one aminotransferase, that aminotransferase may nevertheless function in another pathway, as with histidinol phosphate aminotransferase in *B. subtilis* (15). Under particular, specialized conditions an aminotransferase may function in transamination reactions that do not ordinarily occur in wild type, as with prephenate transaminase in *N. crassa* (12).

The combined activities of aminotransferase enzymes, having different, but overlapping specificities has long been assumed in many biochemical systems to account for the lack of mutant phenotypes that might arise from aminotransferase deficiencies. The fractional contribution of various aminotransferases to particular transamination reactions *in vivo* can best be approached through the systematic and sequential elimination of individual aminotransferases by mutation. Thus, in *E. coli* each of three aminotransferases (specified by genes *tyr*B, *ilv*E, and *asp*C) is sufficient alone to function as phenylpyruvate aminotransferase (16). Absence of aminotransferases specified by *tyr*B or *asp*C does not lead to nutritional requirements unless the genetic background is also deficient in *ilv*E (16). A fourth aminotransferase of *E. coli* appears to participate primarily in L-valine and L-alanine biosynthesis (17).

In *P. aeruginosa*, a comparable systematic approach should be feasible, by exploiting the genetic background of the phenylalanine bradytroph NP 72 for mutagenesis. The absence of aminotransferase DE I in bradytroph NP 72 may permit the recognition of newly arisen aminotransferase deficiencies by the expression of phenotypes that may not be apparent when isolated in wild type backgrounds.

Specialization of Aromatic Aminotransferases in P. aeruginosa – Aminotransferase DE I is clearly essential for normal aromatic biosynthesis in wild type since its loss by mutation results in growth-limiting rates of phenylalanine or tyrosine synthesis. The DE I-negative bradytroph is deficient in overall aminotransferase activity with phenylpyruvate and 4-hydroxyphenylpyruvate, but not in overall aminotransferase activity with prephenate. Since intracellular levels of prephenate should be elevated in the bradytroph owing to relaxation of the regulation of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase (18) in response to end product limitation, intracellular concentrations of pretyrosine may even exceed that of wild type. If so, then pretyrosine dehydratase and pretyrosine dehydrogenase must be rate-limiting reactions for growth in the bradytroph when cultured in minimal glucose medium.

Aminotransferase HA I appears to overlap the catalytic characteristics of aminotransferase DE I most effectively. Since the synthesis of species HA I is induced in the presence of either L-phenylalanine or L-tyrosine, its normal function *in vivo* is undoubtedly catabolic. The inducible regulation of aminotransferase HA I prevents the bradytroph lacking species DE I from exploiting the potential of species HA I to catalyze the reactions normally carried out by species DE I. This interpretation is further supported by the ability of a mutation to constitutive synthesis of aminotransferase HA I to suppress the DE I deficiency. Thus, it appears that elevated levels of a degradative aminotransferase in the absence of its normal substrate (*e.g.* L-phenylalanine) and the probable increase of its normal product (*e.g.* phenylpyruvate) functions in the backward direction in recruitment to biosynthetic function. The ability of a constitutively synthesized aminotransferase HA I to functionally replace the biosynthetic aminotransferase DE I may suggest the evolutionary origin of these aminotransferase proteins from a common protein. The identical molecular weights (70,000) of aminotransferases HA I and DE I (3) are consistent with this possibility. Although a constitutively synthesized aminotransferase HA I can replace aminotransferase DE I when glucose is the carbon source for growth, it cannot do so when aromatic biosynthesis is further stressed by growth on fructose.

Aminotransferase HA II is repressible over a 6-fold range, and this regulation implicates its role in aromatic amino acid biosynthesis. Enzyme HA II works much better as prephenate aminotransferase than as either phenylpyruvate aminotransferase or 4-hydroxyphenylpyruvate aminotransferase (3). The enzymological analysis of the wild type and mutant strains is suggestive of a primary role of aminotransferase HA II as prephenate aminotransferase.

The synthesis of aminotransferase HA III does not appear to vary in response to excess or limitation of aromatic end products. The dramatically better function of L-leucine as an amino-donor reactant in comparison with L-glutamate (3) suggests that aminotransferase HA III may primarily function in branched-chain amino acid metabolism. (In *E. coli* the *tyr*B-coded aminotransferase can function as the sole transamination step required for leucine biosynthesis (16).)

Our present interpretations are that the phenylpyruvate and 4-hydroxyphenylpyruvate sequences to phenylalanine and tyrosine synthesis are fractionally greater than the pretyrosine pathways to phenylalanine and tyrosine synthesis; that aminotransferase DE I is primarily phenylpyruvate/4-

hydroxyphenylpyruvate aminotransferase *in vivo* while aminotransferase HA II functions primarily as a prephenate aminotransferase; that aminotransferase HA I is an inducible enzyme which functions *in vivo* for catabolism of L-tyrosine and L-phenylalanine; and that aminotransferase HA III probably is primarily functional in the branched-chain amino acid pathways, rather than in aromatic metabolism.

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