Regulation at Metabolic Branch Points of Aromatic Amino Acid Biosynthesis in *Brevibacterium flavum*

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In the biosynthetic pathway of aromatic amino acids of *Brevibacterium flavum*, ratios of each biosynthetic flow at the chorismate branch point were calculated from the reaction velocities of anthranilate synthetase for tryptophan and chorismate mutase for phenylalanine and tyrosine at steady state concentrations of chorismate. When these aromatic amino acids were absent, the ratio was 61, showing an extremely preferential synthesis of tryptophan. The presence of tryptophan at 0.01 mM decreased the ratio to 0.07, showing a diversion of the preferential synthesis to phenylalanine and tyrosine. Complete recovery by glutamate of the ability to synthesize the Millon-positive substance in dialyzed cell extracts confirmed that tyrosine was synthesized *via* pretyrosine in this organism. Partially purified prephenate aminotransferase, the first enzyme in the tyrosine-specific branch, had a pH optimum of 8.0 and K_m 's of 0.45 and 22 mM for prephenate and glutamate, respectively, and its activity was increased 15-fold by pyridoxal-5-phosphate. Neither its activity nor its synthesis was affected at all by the presence of the end product tyrosine or other aromatic amino acids. The ratio of each biosynthetic flow for tyrosine and phenylalanine at the prephenate branch point was calculated from the kinetic equations of prephenate aminotransferase and prephenate dehydratase, the first enzyme in the phenylalanine-specific branch. It showed that tyrosine was synthesized in preference to phenylalanine when phenylalanine and tyrosine were absent. Furthermore, this preferential synthesis was diverted to a balanced synthesis of phenylalanine and tyrosine through activation of prephenate dehydratase by the tyrosine thus synthesized. The feedback inhibition of prephenate dehydratase by phenylalanine was proposed to play a role in maintaining a balanced synthesis when supply of prephenate was decreased by feedback inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP*) synthetase, the common key enzyme. Overproduction of the end products in various regulatory mutants was also explained by these results.

Common key enzymes of branched biosynthetic pathways have often been found to be regulated by some of the metabolic end products, but not by all of them. This regulation would not disturb biosynthesis of the end products that do not take part in the regulation only when they are synthesized in preference to those which take part. It has also often been found that one of the specific branches for each end product in a branched biosynthetic pathway does not have any feedback control mechanism. Even in this situation, the appropriate combination of biosynthetic preference at the branch point, with

regulation in the other specific branches, would prevent an overproduction of the former end product.¹⁾ A typical example is the regulatory mechanism for aromatic amino acid biosynthesis in *Brevibacterium flavum*. That is, DAHP synthetase of the common pathway is feedback-inhibited by only two of the three end products, phenylalanine and tyrosine.^{2,3)} At the chorismate branch point, anthranilate synthetase of the tryptophan-specific branch is strongly feedback-inhibited⁴⁾ and repressed by tryptophan,⁵⁾ whereas chorismate mutase of the phenylalanine- and tyrosine-specific branch is not effectively regulated by phenylalanine and tyrosine.⁶⁾ Moreover, preliminary experiments showed that at the prephenate branch point, pre-

* Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid.

phenate aminotransferase of the tyrosine-specific branch was not regulated by tyrosine, while prephenate dehydratase of the phenylalanine-specific branch is inhibited by phenylalanine and activated by tyrosine.⁷⁾ The present study deals with enzymatic evidence for the preferential order of these amino acid biosyntheses at the chorismate- and prephenate-branch points, regulatory mechanisms based on this enzymatic evidence, and the relationship of these mechanisms to overproduction of certain amino acids in regulatory mutants. The results obtained showed preferential synthesis of tryptophan and tyrosine at these branch points, respectively, and explained, as well, their overproduction in regulatory mutants.

MATERIALS AND METHODS

Organism. *Brevibacterium flavum* No. 2247 (ATCC 14067) and its mutants were used. Strains No. 3⁸⁾ and 1-147⁹⁾ were phenylalanine tyrosine double auxotrophs. Strain D-32 was a tyrosine auxotroph and BN₂₋₄¹⁰⁾ was a phenylalanine auxotroph. Strain 187 was a tyrosine methionine double auxotroph. Strain TA-24⁵⁾ was a tryptophan auxotroph.

Chemicals. Chorismic acid and barium prephenate were prepared as described previously.¹⁰⁾ DEAE-Cellulose and hydroxyapatite were purchased from Midori-juji K.K. and BDH chemicals, respectively. Sources of the other chemicals used were as described previously.³⁾

Cultivation. Strains No. 2247, BN₂₋₄, No. 3 and TA-24 were aerobically cultured at 30°C for 24 hr in medium 5,⁵⁾ supplemented with the required amino acid(s). Strain D-32 and 1-147 were cultured in medium 13,¹³⁾ supplemented with the required amino acid(s), including 0.3% Casamino acids for strain 1-147.

Enzyme preparation. The cultured cells were harvested by centrifugation, washed twice with 0.2% KCl, and suspended either in 0.1 M Tris-HCl buffer, pH 7.5 for measurements of prephenate aminotransferase, pretyrosine dehydrogenase and prephenate dehydratase activities or in 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)-NaOH buffer, pH 7.0, containing 5 mM MgCl₂, 2 mM dithiothreitol and 1 mM tryptophan, for measurements of chorismate mutase, DAHP synthetase and anthranilate synthetase activities. The cell suspension was then sonically disrupted at 10 kc for 20 min. For prephenate aminotransferase

and pretyrosine dehydrogenase assays, the supernatant solution of the sonicate, after centrifugation at 32,000 × *g* for 30 min (crude extract), was dialyzed overnight against 2,000-volumes of 0.05 M Tris-HCl buffer, pH 7.5. Gel filtration of the same supernatant was carried out on a Sephadex G-50 column (1.5 × 10 cm) equilibrated either with 0.05 M Tris-HCl buffer, pH 7.5 for the prephenate dehydratase assay, with 0.05 M TES-NaOH buffer, pH 7.0, for anthranilate synthetase and chorismate mutase assays, or with 0.05 M TES-NaOH buffer, pH 7.0, containing 1 mM tryptophan, for DAHP synthetase.

Prephenate aminotransferase and pretyrosine dehydrogenase were purified from the dialyzed extracts of strain BN₂₋₄, cultured in 250 ml of medium 5 supplemented with 200 mg/liter of phenylalanine. The dialyzed extracts were applied to a DEAE-cellulose column (2.2 × 22 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The column was washed with 100 ml of the same buffer, and then the enzymes were eluted using KCl concentration gradient consisting of 300 ml of the same buffer plus 300 ml of the same buffer containing 0.5 M KCl. Fractions of 6 ml were collected. The fractions containing prephenate aminotransferase or pretyrosine dehydrogenase were pooled separately and each concentrated to 3.8 ml with an ultrafiltration apparatus (Amicon PM-10). The solution containing prephenate aminotransferase or pretyrosine dehydrogenase was gel-filtered on a Sephadex G-50 column (1.5 × 17.5 cm) equilibrated with 0.005 M Tris-HCl buffer, pH 7.5 and then applied to a hydroxyapatite column (1.5 × 20 cm) equilibrated with the same buffer. The enzymes were then eluted using potassium phosphate concentration gradient consisting of 150 ml of the same buffer plus 150 ml of the same buffer containing, in the case of prephenate aminotransferase, 0.05 M potassium phosphate buffer, pH 7.5, or 0.2 M potassium phosphate buffer, pH 7.5, for pretyrosine dehydrogenase. Fractions of 3 ml were collected, and those containing prephenate aminotransferase or pretyrosine dehydrogenase activity were pooled and concentrated to 3.3 or 3.6 ml, respectively, by ultrafiltration. These partially purified enzymes had protein concentrations of 4.9 and 1.7 mg/ml and specific activities of 4.22 and 29.0 units/mg protein, respectively.

Enzyme assay. Prephenate aminotransferase was assayed by measuring the decrease of prephenate (Method I) or NADH formation (Method II) in the presence of excess pretyrosine dehydrogenase. The reaction mixture for Method I contained 50 mM Tris-HCl buffer, pH 7.5, 2 mM prephenate, 50 mM glutamate, 0.1 mM pyridoxal-5-phosphate and the enzyme in a total volume of 0.5 ml. The reaction was carried out at 37°C for 20 min and stopped by the addition of 0.5 ml of 1 N HCl. The mixture underwent a further incubation for 10 min. After 50-fold dilution of the incubation mixture with 1 N NaOH, the absorbancy at 320 nm was observed. The reaction mixture

for Method II was the same as that for Method I supplemented with 2 mM NAD and 2.5 units of partially purified pretyrosine dehydrogenase. The reaction was started by the addition of prephenate aminotransferase. After incubation for 10 min at room temperature, the increase in absorbancy of the reaction mixture at 340 nm was determined.

Pretyrosine dehydrogenase was assayed as follows: 0.1 ml of the crude extract of strain 1-147 which lacks pretyrosine dehydrogenase and prephenate dehydratase, containing 0.2 unit of prephenate aminotransferase, was added to the reaction mixture for assay Method I, and incubated at 37°C until the reaction had gone to completion (for 40 to 60 min). Then, the pretyrosine dehydrogenase reaction was started at 37°C by adding the enzyme plus 2 mM NAD to the mixture (final volume, 0.6 ml). After 20 min, tyrosine formed was determined by the Millon reaction.¹²⁾

DAHP synthetase,⁶⁾ chorismate mutase,¹¹⁾ anthranilate synthetase,⁴⁾ prephenate dehydratase⁷⁾ and protein⁴⁾ were assayed by the methods described previously.

For prephenate aminotransferase or pretyrosine dehydrogenase, one unit of enzyme activity was defined as that amount catalyzing decrease or increase of 1 unit in the absorbancy at 320 nm or 490 nm per 20 min, respectively, and as that catalyzing formation of 1 nmol of the product per min for DAHP synthetase, chorismate mutase, anthranilate synthetase and prephenate dehydratase.

RESULTS

Chorismate branch point

At this branch point, the ratio of biosyn-

thetic velocity of phenylalanine and tyrosine to that of tryptophan depends on the V_m and K_m values of the first enzymes of each specific branch, anthranilate synthetase and chorismate mutase, and on the velocity of the chorismate supply. Table I shows these values.

When all these amino acids are absent, the V_m 's of anthranilate synthetase and chorismate mutase, the data under the limitation of tryptophan and tyrosine which repressed each enzyme, are 28.8 and 57.6 units/mg protein, respectively. Thus, the V_m of chorismate mutase is higher than that of anthranilate synthetase, while the affinity of the latter enzyme for the common intermediate, chorismate, is higher than that of the former, as the K_m or $S_{0.5}$ value indicates. Therefore, the velocity of tryptophan synthesis (reaction velocity of anthranilate synthetase (V_{AS})) is larger than that of phenylalanine and tyrosine biosynthesis (reaction velocity of chorismate mutase (V_{CM})), if the concentration of chorismate is less than 4.8 mM, as is shown in Fig. 1(A). The chorismate concentration can be obtained as that at a steady state, in which the velocities of consumption and supply are equal to each other. Assuming that the velocity of its supply is that of DAHP synthetase, the key regu-

TABLE I. MAXIMUM VELOCITIES AND MICHAELIS CONSTANTS OF ANTHRANILATE SYNTHETASE, CHORISMATE MUTASE AND DAHP SYNTHETASE IN CELL EXTRACTS

Enzyme	V_m (unit/mg protein)			K_m (or $S_{0.5}$) for chorismate (mM)
	Normal ^a	Limited Trp ^b	Limited Tyr ^c	
Anthranilate synthetase	1.26	28.8	1.38	0.04 ^f
Chorismate mutase ^d	27.7	29.4	57.6	5.0 ^g
DAHP synthetase ^e	15.3	14.7	18.3	—

^a Data from crude extracts of the wild stain No. 2247, cultured in medium 5.

^b Data from crude extracts of the tryptophan auxotroph, TA-24, cultured in medium 5 supplemented with 15 mg/liter of tryptophan.

^c Data from crude extracts of the phenylalanine tyrosine double auxotroph, 1-147, cultured in medium 13 supplemented with 80 mg of tyrosine, 300 mg of phenylalanine and 3 g of Casamino acids per liter.

^d Data at a high enzyme concentration where the linearity of the activity against the enzyme concentration is obtained.⁶⁾

^e Specific activity under standard assay conditions.

^{f, g} Data from refs. 4 and 5, respectively.

FIG. 1. Anthranilate Synthetase and Chorismate Mutase Reaction Velocities at Various Concentrations of the Substrate, Chorismate.

(A) Reaction velocities under limitation of aromatic amino acids. The reaction velocity of chorismate mutase (V_{CM} , $\triangle$) was measured using crude extracts of strain 1-147 at high enzyme concentrations where linearity of the activity against the enzyme concentration was obtained. The reaction velocity of anthranilate synthetase (V_{AS} , $\circ$) was calculated by Michaelis-Menten-type equation using the values 0.04 mM for the K_m and 28.8 units/mg protein for the V_m (Table I). Velocity of chorismate consumption ($\bullet$) was expressed as the sum of the velocities of anthranilate synthetase and chorismate mutase ($V_{AS} + V_{CM}$). Straight line (I) shows the specific activity of DAHP synthetase (18.3 units/mg protein, Table I).

(B) The reaction velocities of chorismate mutase were measured in a way similar to that in (A), but also in the presence of 0.01 ($\triangle$), 0.1 ($\circ$) and 1.0 mM tryptophan ($\square$). The reaction velocity of anthranilate synthetase was calculated in a similar manner to (A) using 1.32 unit/mg protein as V_m , 0.0048 mM as K_i and 0.04 mM as K_m . Then, velocities of the chorismate consumption were expressed as sums of those of anthranilate synthetase and chorismate mutase in the presence of 0.01 ($\triangle$), 0.1 ($\bullet$) and 1.0 mM tryptophan ($\blacksquare$).

latory enzyme of the common pathway, under the standard assay conditions, this velocity

would be 18.3 units/mg protein, the value under tyrosine-limitation. Thus, the steady state concentration of chorismate was estimated to be 0.07 mM from the plots of Fig. 1(A). In addition, the ratio of V_{AS}/V_{CM} was calculated to be 61 at the steady state concentration, showing an extremely preferential synthesis of tryptophan.

The metabolic flow of chorismate would be diverted from tryptophan to phenylalanine and tyrosine synthesis, mainly through the feedback inhibition and repression of anthranilate synthetase by the tryptophan thus synthesized,⁴⁾ and partially through the activation of chorismate mutase by tryptophan.^{6,11,14)} Under these circumstances, the V_m of anthranilate synthetase is 1.32, the average value of 1.26 and 1.38, unit/mg protein under the non-tryptophan limiting conditions in Table I, and the V_{AS} in the presence of the competitive inhibitor, tryptophan, can be calculated by a Michaelis-Menten-type equation, described in a previous paper.⁶⁾ On the other hand, V_{CM} was experimentally estimated in the presence of tryptophan, using enzyme extracts from cells cultured under the tyrosine-limiting conditions (see Table I). Figure 1(B) shows plots of V_{CM} and $V_{CM} + V_{AS}$ in the presence of 0.01, 0.1 or 1.0 mM tryptophan. Chorismate concentrations, which gave equal velocities of consumption ($V_{CM} + V_{AS}$) and of supply (18.3 units/mg protein) were determined to be 2.9, 2.6 and 2.3 mM, respectively. At these steady state concentrations, the ratios of V_{AS}/V_{CM} were calculated to be 0.073, 0.056 and 0.017, respectively. These results show a diversion of the major metabolic flow from tryptophan to phenylalanine and tyrosine synthesis. It should be noted that the chorismate concentration markedly increased during this change. This increase is due to the low substrate affinity of chorismate mutase.

Enzymes of phenylalanine- and tyrosine-specific branches

The presence in *B. flavum* of an amino-transferase using prephenate as an amino-acceptor, and of pretyrosine dehydrogenase,

TABLE II. FORMATION OF MILLON-POSITIVE SUBSTANCE(S) FROM PREPHENATE BY CRUDE CELL EXTRACTS

The reaction mixture contained 0.05M Tris-HCl buffer, pH 7.5, 2mM prephenate, 1mM NAD, given concentrations of glutamate and pyridoxal-5-phosphate and the crude extract of strain No. 2247 in a total volume of 0.5 ml. After the reaction was carried out at 37°C for 20 min, the Millon-positive substances formed were determined.¹²⁾

Cell extract	Pyridoxal-5-phosphate (mM)	Millon-positive substance formed in the presence of glutamate at		
		0	1	50 mM
		(nmol/min/mg protein)		
Undialyzed	0	3.0	3.6	12
	0.1	3.9	4.6	13
Dialyzed	0	0.0	0.9	11
	0.1	0.0	1.2	12

TABLE III. PREPHENATE AMINOTRANSFERASE AND PRETYROSINE DEHYDROGENASE ACTIVITIES IN TYROSINE AUXOTROPHS

The amino acid(s) required to supplement the culture medium were 300mg of phenylalanine and 300mg of tyrosine per liter for strain 1-147, 300mg/liter of tyrosine for strain D-32, 600 mg/liter of tyrosine for strain 187 and 300 mg/liter of phenylalanine for strain BN₂₋₄. Prephenate aminotransferase was assayed by Method I, except that 1mM phenylalanine was added to the mixture in order to prevent a decrease in the prephenate concentration by action of the prephenate dehydratase present in the crude extracts.

Strain	Prephenate aminotransferase (unit/mg protein)	Pretyrosine dehydrogenase
1-147	0.084	-0.006
D-32	0.126	-0.006
187	0.157	-0.009
BN ₂₋₄ (Wild-type)	0.107	0.263

as well as the lack of the latter enzyme in a tyrosine auxotroph, suggested that tyrosine might be synthesized *via* pretyrosine in this organism, in a manner similar to that in blue-green bacteria.¹⁵⁾ However, it is possible that the prephenate may also be metabolized by pretyrosine dehydrogenase, since its structure is analogous to that of pretyrosine, and the substrate specificity of this enzyme has not been clarified. Furthermore, an aminotransferase activity, using 4-hydroxyphenyl-

pyruvate as an amino-acceptor, is present in extracts of *B. flavum*. Therefore, tyrosine could be synthesized, at least partially, *via* 4-hydroxyphenylpyruvate, as is the case in *Escherichia coli*. As reported previously,¹⁰⁾ crude extracts of *B. flavum* catalyzed a NAD-dependent conversion of prephenate to Millon-positive substance(s), a group to which tyrosine and 4-hydroxyphenylpyruvate, but not pretyrosine, belong. As shown in Table II, this activity disappeared after dialysis but was completely restored by the addition of a high concentration of glutamate. The activity of the undialyzed extracts also increased 4-fold with the addition of glutamate. These results show that the activity of the extracts depends completely on the presence of glutamate, and that tyrosine is synthesized only through the pretyrosine pathway, where a transamination reaction is essential for the formation of Millon-positive substance(s). Table III shows that all tyrosine auxotrophs of *B. flavum* tested lacked pretyrosine dehydrogenase activity. Since a prephenate aminotransferase-lacking mutant might require both phenylalanine and tyrosine for its growth, eight strains of phenylalanine tyrosine double auxotrophs having chorismate mutase were tested, but none lacked prephenate aminotransferase.

In order to examine some properties of prephenate aminotransferase, the first enzyme

FIG. 2. Double Reciprocal Plots of Prephenate Aminotransferase Reaction Rate against Substrate Concentration at Fixed Concentrations of a Second Substrate.

Prephenate aminotransferase was assayed using the purified preparation, containing 24 μ g of protein, by Method II except for variations in the substrate concentration. In (A), the concentrations of glutamate were 50 ($\text{---}\bigcirc\text{---}$), 14.3 ($\text{---}\triangle\text{---}$), 8.33 ($\text{---}\square\text{---}$), 5.88 ($\text{---}\bullet\text{---}$) and 4.5 mM ($\text{---}\blacktriangle\text{---}$). The symbol, $\text{---}\times\text{---}$, indicates secondary plots of the intercept on the ordinate of (B). In (B), the concentrations of prephenate were 1.5 ($\text{---}\bigcirc\text{---}$), 1.0 ($\text{---}\triangle\text{---}$), 0.57 ($\text{---}\square\text{---}$), 0.4 ($\text{---}\bullet\text{---}$) and 0.3 mM ($\text{---}\blacktriangle\text{---}$). The symbol, $\text{---}\times\text{---}$, indicates secondary plots of the intercept on the ordinate of (A).

in the tyrosine-specific branch, it was purified approximately 12-fold by the method described in MATERIALS AND METHODS from cells of strain BN₂₋₄. This strain lacks prephenate dehydratase, which also is capable of metabolizing prephenate and disturbing the aminotransferase assay. Only one peak showing prephenate aminotransferase activity was obtained in the DEAE-cellulose column chromatography, in contrast to the results of Fazel and Jensen.^{15,16} They obtained two different species of prephenate aminotransferase, AT-I and AT-II, having an abundance ratio of one to ten. The prephenate aminotransferase obtained in the present experiments was identified as AT-II because of its substrate specificity. Since the glutamate concentration in the assay medium of Fazel and Jensen is 1 mM, which is much lower than the *K_m* of AT-II, as described later, the AT-II activity reported seems to be about 1/10 of that found in the present assay. Therefore, it is likely that the AT-I activity was really about 1/100 of the AT-II, and, therefore, negligible under the present assay conditions. Since these preparations did not contain NADH oxidase, the activity of the partially purified enzyme was measured by Method II in the presence of

TABLE IV. EFFECT OF AROMATIC AMINO ACIDS ON PREPHENATE AMINOTRANSFERASE ACTIVITY

Amino acid added	Concentration (mM)	Relative activity (%)
None	—	100
Tyrosine	0.1	99
	0.2	101
	0.5	102
	1.0	105
Phenylalanine	1.0	98
	2.0	98
	5.0	99
	10	97
Tryptophan	1.0	100
	2.0	102
	5.0	101
	10	103

excess amounts of pretyrosine dehydrogenase, purified 24-fold by the method described in MATERIALS AND METHODS.

The optimum pH was 8.0 with Tris-HCl buffer. Enzyme activity was stimulated 15-fold by pyridoxal-5-phosphate at 0.01 mM, which is an almost saturating concentration for this activity. This effect of pyridoxal-5-phosphate was observed with the enzyme preparations after chromatography on DEAE-cellulose, but not with the dialyzed crude extracts, as is shown in Table II.

As shown in Fig. 2 by the double reciprocal plots of reaction rate against substrate concentration at fixed concentrations of a second

substrate, the mechanism of the enzyme reaction is of a ping pong-type, similar to that observed for other aminotransferases. From the secondary plots of the intercepts on the ordinate against the concentrations of the second substrate, the *K_m*'s for prephenate and glutamate were estimated to be 0.45 and 22 mM, respectively.

The enzyme activity was not affected by the end product, tyrosine, or by the other aromatic amino acids, phenylalanine and tryptophan (Table IV). Synthesis of the enzyme

TABLE V. EFFECTS OF PHENYLALANINE AND TYROSINE CONCENTRATION IN THE CULTURE MEDIUM ON THE SPECIFIC ACTIVITIES OF PREPHENATE AMINOTRANSFERASE AND PREPHENATE DEHYDRATASE

Prephenate aminotransferase was assayed by Method I, in the dialyzed crude extracts of the phenylalanine tyrosine double auxotroph, 1-147, which lacked prephenate dehydratase and pretyrosine dehydrogenase. This mutant was cultured in medium 13 supplemented with 3 g/liter of Casamino acids and various concentrations of phenylalanine and tyrosine. Prephenate dehydratase in the dialyzed crude extract of the phenylalanine tyrosine double auxotroph, No. 3, which lacked chorismate mutase component B, was assayed under standard conditions except that 1 mM tyrosine was added to the mixture. This auxotroph, No. 3, was cultured in medium 5 supplemented with various concentrations of phenylalanine and tyrosine.

Addition (mg/liter)		Growth (<i>A</i> _{562 nm})	Specific activity (unit/mg protein)	
Tyrosine	Phenylalanine		Prephenate aminotransferase	Prephenate dehydratase
50	300	0.145	0.067	
75	300	0.230	0.071	
100	300	0.400	0.081	
125	300	0.415	0.099	
150	300	0.390	0.088	
250	25	0.115	0.091	
250	50	0.148	0.097	
250	100	0.216	0.095	
250	150	0.214	0.092	
250	200	0.193	0.086	
250	250	0.200	0.095	
200	25	0.170		8.1
200	50	0.280		13
200	75	0.405		12
200	100	0.480		11
200	125	0.530		9.2
200	200	0.510		11
24	200	0.110		5.1
50	200	0.210		8.3
75	200	0.300		10
100	200	0.415		9.9
125	200	0.520		9.1
150	200	0.530		9.5

was also not affected by tyrosine or phenylalanine (Table V).

As reported already, neither the synthesis nor the activity of pretyrosine dehydrogenase, the second enzyme of the tyrosine-specific branch, was affected by tyrosine.¹⁵⁾

In contrast, prephenate dehydratase, the first enzyme of the phenylalanine-specific branch, was inhibited by phenylalanine and activated by tyrosine.⁷⁾ Table V also shows that synthesis of prephenate dehydratase was not at all affected by either phenylalanine or

TABLE VI. MAXIMUM VELOCITIES AND MICHAELIS CONSTANTS OF PREPHENATE AMINOTRANSFERASE AND PREPHENATE DEHYDRATASE IN CELL EXTRACTS

Enzyme	1 mM Tyrosine	V_m (or V_m') ^a (nmol/min/mg protein)	K_m (or K_m') for prephenate (mM)
Prephenate aminotransferase	—	10.3	0.45
Prephenate dehydratase	—	2.3	0.054 ^b
	+	10.6	0.0095 ^c

^a Data at 30°C with cell extracts from strain No. 2247 cultured in medium 5.

^b Data from ref. 7.

^c K_m' calculated from kinetic constants in ref. 14.

TABLE VII. PREPHENATE AMINOTRANSFERASE AND PREPHENATE DEHYDRATASE REACTION VELOCITIES IN STEADY STATE UNDER THE REGULATION BY PHENYLALANINE AND TYROSINE

Data were calculated by the method described in the text.

Type of regulation	Tyr (mM)	Phe (mM)	DAHP synthetase		Steady state conc. of prephenate (mM)	V_{PAT} (nmol/min/mg protein)	V_{PDT} (nmol/min/mg protein)	V_{PAT}/V_{PDT}
			Level ^a	Inhibition ^b (%)				
Normal	0	0	Derepressed	0	— ^c	10.3	2.0	5.2
	0.01	0	Derepressed	0	3.6	9.3	9.0	1.03
			Repressed	0	0.7	6.4	8.9	0.72
	0.05	0.05	Derepressed	48	0.36	4.5	5.0	0.90
			Repressed	48	0.21	3.3	4.5	0.74
Feedback-insensitive prephenate dehydratase	0.05	0.05	Derepressed	48	0.052	1.1	8.5	0.124
			Repressed	48	0.027	0.60	7.2	0.083
Feedback-insensitive DAHP synthetase	0.05	0.05	Derepressed	0	— ^c	10.3	5.9	1.75
			Repressed	0	4.0	9.2	5.8	1.59
	0.15	0.10	Derepressed	0	— ^c	10.3	7.0	1.47
			Repressed	0	1.8	8.2	6.8	1.21

^a Derepressed or repressed level of DAHP synthetase was 18.3 or 15.0 nmol/min/mg protein, respectively (Table I).

^b see ref. 3.

^c Steady state concentration was not obtained.

tyrosine.

Prephenate branch point

The V_m 's of prephenate aminotransferase and prephenate dehydratase of the tyrosine- and phenylalanine-specific branches, respectively, were estimated in cell extracts of the wild strain No. 2247. Table VI summarizes these V_m 's and K_m 's, and gives as well, the maximum velocity (V_m') and apparent K_m (K_m') in the presence of tyrosine. When tryptophan is synthesized in a sufficient amount, but phenylalanine and tyrosine are still absent, the velocity of prephenate supply is nearly equal to the specific activity of DAHP synthetase under the tyrosine limitation (18.3 units/mg protein, see Table I). Since the velocity of supply is much higher than the maximum value of 12.3 units/mg protein (in the absence of tyrosine) for the velocity of

prephenate consumption, which is sum of the reaction velocities of prephenate aminotransferase (V_{PAT}) and prephenate dehydratase (V_{PDT}), the prephenate seems to accumulate at a high concentration. Under these conditions, the ratio of biosynthetic velocity of tyrosine to that of phenylalanine (V_{PAT}/V_{PDT}) would be determined by their V_m values, but not by their K_m 's. The ratio in Table VII indicates that tyrosine is synthesized in preference to phenylalanine. Increasing the concentration of tyrosine alone, as a result of preferential synthesis, increases the velocity of prephenate consumption ($V_{PAT} + V_{PDT}$) through activation of prephenate dehydratase and, therefore, makes it possible to achieve a steady state concentration of prephenate such that the velocities of prephenate consumption and the supply (V_{DS}) are equal. Thus, V_{PAT} and V_{PDT} ^{7,14)} are calculated from the following equation;

$$V_{PAT} = \frac{V_m}{1 + K_m/S} \text{ (at a saturated concentration of glutamate)} \quad (1)$$

and

$$V_{PDT} = \frac{V_m + V_m' \cdot A/Ka'}{(1 + A/Ka' + I/Ki') + (1 + A/Ka + I/Ki)Km/S}, \quad (2)$$

where S , A and I are the prephenate, tyrosine and phenylalanine concentrations, respectively. Ka and Ka' are the kinetic constants related to tyrosine and have been calculated to be 0.012 and 0.0021 mM, respectively.¹⁴⁾ Ki and Ki' are those related to phenylalanine; their respective values were calculated to be 0.0010 and 0.027 mM.¹⁴⁾ V_m , V_m' , as well as the K_m 's, are given in Table VI.

If phenylalanine is absent, eq. (2) can be simplified as follows:

$$V_{PDT} = \frac{V_m + V_m' \cdot A/Ka'}{(1 + A/Ka') + (1 + A/Ka)Km/S} \quad (3)$$

When V_{PAT} , V_{PDT} and $V_{PAT} + V_{PDT}$ were plotted against prephenate concentrations according to these equations, the steady state concentration of prephenate, as well as V_{PAT} , and V_{PDT} at the steady state, were obtained graphi-

cally from the V_{DS} of Table I. As shown in Table VII, V_{PAT}/V_{PDT} in the presence of 0.01 mM tyrosine, a concentration which did not inhibit DAHP synthetase²⁾ was estimated to be between 1.03 and 0.72, depending on a possible repression of DAHP synthetase. This means a balanced synthesis of phenylalanine and tyrosine. When both the tyrosine and phenylalanine concentrations increased to 0.05 mM as a result of their balanced synthesis, the ratio of V_{PAT}/V_{PDT} was between 0.90 and 0.74. Accordingly, this biosynthetic reaction was still considered balanced. Furthermore, since the steady state concentration of prephenate was markedly decreased by increasing concentrations of tyrosine and phenylalanine, it can be concluded that the biosynthesis of both tyrosine and phenylalanine was controlled at the supply side of prephenate and,

therefore, by feedback inhibition of DAHP synthetase. If the feedback inhibition by phenylalanine of prephenate dehydratase was absent, the same conditions as described above gave a V_{PAT}/V_{PDT} of about 0.1. Such a value was obtained because of an increase in the apparent V_m and a decrease in apparent K_m of the enzyme caused by the tyrosine, thus, showing a preferential synthesis of phenylalanine. These results show that the mechanism maintaining a balanced synthesis of tyrosine and phenylalanine is actually a feedback inhibition of prephenate dehydratase when prephenate supply *via* the DAHP synthetase reaction, is considerably depressed by the presence of phenylalanine and tyrosine. These results are also consistent with overproduction of phenylalanine by mutants having a prephenate dehydratase which is insensitive to the feedback inhibition.¹⁰⁾ Table VII shows that the V_{PAT}/V_{PDT} increases to more than 1.0 if the feedback inhibition of DAHP synthetase is absent. However, when the tyrosine concentration increases more rapidly than does the phenylalanine concentration, as a result of imbalanced synthesis, and their concentrations reach to 0.15 and 0.1 mM, respectively, then the V_{PAT}/V_{PDT} decreases toward 1.0. As a result, the imbalance of their formation does not expand further. These results are virtually consistent with the overproduction of phenylalanine and tyrosine, at ratio of approximately 1, by mutants having a DAHP synthetase insensitive to the feedback inhibition.¹⁰⁾

DISCUSSION

One of purposes of the present study was to examine the possibility of whether regulatory mechanisms in a complicated and branched biosynthetic pathway can be constructed from enzymatic properties of the key enzymes involved or whether *in vivo* results, such as overproduction, can be predicted or explained by these mechanisms.

Results of the present study showed that tryptophan was synthesized in preference to

phenylalanine and tyrosine. Since tryptophan does not take part in the feedback control of DAHP synthetase, the key enzyme of the common pathway for aromatic amino acid biosynthesis in *B. flavum*, the results are consistent with a general regulatory mechanism that includes preferential synthesis.¹⁾ The preferential synthesis of tryptophan is also consistent with the evidence that, *in vivo*, mutants lacking regulation in the tryptophan-specific branch overproduce tryptophan and that their overproduction is depressed by the addition of phenylalanine and tyrosine.¹⁷⁾

The present results confirmed the suggestion of Fazel and Jensen¹⁵⁾ that tyrosine was synthesized *via* pretyrosine in *B. flavum*. The data showed, further, that prephenate aminotransferase, the first enzyme of the tyrosine-specific branch, was not regulated at all by tyrosine nor by the other aromatic amino acids, while prephenate dehydratase, the enzyme for phenylalanine synthesis, was inhibited, but not repressed, by phenylalanine and activated by tyrosine. Moreover, this inhibition by phenylalanine was competitively recovered by the presence of tyrosine.⁷⁾ Under these circumstances, there are two possible regulatory mechanism. One is that tyrosine is preferentially synthesized, and the activation of prephenate dehydratase by tyrosine thus synthesized turns the preferential synthesis to phenylalanine. The other is that phenylalanine is synthesized preferentially, and the feedback inhibition of prephenate dehydratase by phenylalanine thus synthesized turns the preferential synthesis to tyrosine. The present results showed a preferential synthesis of tyrosine. Therefore, activation of the prephenate dehydratase by tyrosine was confirmed as one of the physiologically significant regulatory mechanisms. On the other hand, feedback inhibition of prephenate dehydratase by phenylalanine plays a role in maintaining a balanced synthesis of phenylalanine and tyrosine when the supply of the common intermediate, prephenate, is decreased by the synergistic inhibition of DAHP synthetase by phenylalanine plus tyrosine. Therefore, mu-

FIG. 3. Regulation of Aromatic Amino Acid Biosynthesis in *Brevibacterium flavum*.

1, DAHP synthetase; 2, anthranilate synthetase; 3, chorismate mutase; 4, prephenate aminotransferase; 5, pretyrosine dehydrogenase; 6, prephenate dehydratase; **→**, preferential synthesis; **⇨**, feedback inhibition; **⇩**, activation.

tants having a prephenate dehydratase insensitive to the feedback inhibition can not maintain a balanced synthesis of phenylalanine and tyrosine and are under the limitation of tyrosine, which, in turn, causes the overproduction of phenylalanine.¹⁰ This regulatory mechanism also suggests that a mutant having prephenate dehydratase and DAHP synthetase which are both insensitive to feedback inhibitions, overproduces both phenylalanine and tyrosine. Since such a result contradicts conclusion based on the assump-

tion that phenylalanine is synthesized in preference to tyrosine, this mutant will provide critical evidence that the enzymatic conclusion described here is correct.

Figure 3 summarizes the major regulatory mechanisms for aromatic amino acid biosynthesis in *B. flavum*.

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