Regulation of Tryptophan Biosynthesis by Feedback Inhibition of the Second-Step Enzyme, Anthranilate Phosphoribosyltransferase, in *Brevibacterium flavum*

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Anthranilate phosphoribosyltransferase (PRT), the second-step enzyme in the tryptophan specific biosynthetic pathway of *Brevibacterium flavum*, was inhibited by L-tryptophan but not by D-tryptophan, L-phenylalanine or L-tyrosine at all. The pattern of the inhibition by various tryptophan analogs was quite different from that of anthranilate synthase (AS), the first-step enzyme in the pathway. The concentration of tryptophan giving 50% inhibition of PRT and the inhibitor constant for tryptophan, K_i , were 0.15 and 0.26 mM, respectively. The inhibition of PRT was noncompetitive for both the substrates, anthranilate and phosphoribosylpyrophosphate. Glycerol increased the sensitivity to the tryptophan inhibition as well as the activity, whereas KCl and NaCl stimulated the PRT activity alone. In all the mutants tested, the feedback inhibition of PRT was removed concomitantly with that of AS. When phenylalanine tyrosine double auxotrophs having derepressed levels of the tryptophan enzymes were cultured under tyrosine-limitation, they accumulated anthranilate but not tryptophan, as did those having normal repression control. These results were concluded to be due to the presence of the feedback inhibition by tryptophan of PRT. In *Bacillus subtilis* K, the inhibition by tryptophan of PRT was much weaker than that in *B. flavum* and almost the same as that in *B. flavum* mutants having PRT resistant to the feedback inhibition. PRT in a tryptophan-producing mutant of *B. subtilis* was similar to the wild-type enzyme.

Tryptophan biosynthesis in *Brevibacterium flavum* has been found to be regulated by two mechanisms in its specific pathway; feedback inhibition by tryptophan of the first-step enzyme, anthranilate synthase (AS),¹⁾ and repression by tryptophan of synthesis of AS and other tryptophan enzymes²⁾ (Fig. 1). In fact, tryptophan is overproduced only when both the two regulations are mutationally removed.^{3,4)} When the feedback inhibition alone is removed, the cells do not overproduce tryptophan but anthranilate, an intermediate in the tryptophan pathway.^{3,4)}

On the other hand, synthesis of a common intermediate of aromatic amino acid biosynthesis, chorismate, is controlled through synergistic inhibition by phenylalanine and tyrosine

of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase,⁵⁾ the first enzyme in the common pathway (Fig. 1). Thus, tyrosine auxotrophs accumulate phenylalanine under tyrosine-limitation.⁶⁾ Under these conditions, the inhibition does not operate and in consequence, common intermediates, chorismate and prephenate, would be accumulated up to a high concentration, which, in turn, may compete with the feedback inhibition in the phenylalanine-pathway and causes overproduction of phenylalanine. Similarly, it is very likely that tryptophan is overproduced by phenylalanine tyrosine double auxotrophs under tyrosine-limitation. However, preliminary experiments did not show accumulation of tryptophan but of anthranilate. This could be

Abbreviations: PRT, anthranilate phosphoribosyltransferase; AS, anthranilate synthase; PRPP, phosphoribosyl pyrophosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; CTAB, cetyltrimethylammonium bromide; NG, N-methyl-N'-nitro-N-nitrosoguanidine.

FIG. 1. Regulation of Aromatic Amino Acid Biosynthesis in *Brevibacterium flavum*.

↔, feedback inhibition; ←---, repression; 1, DAHP synthase; 2, chorismate mutase; 3, anthranilate synthase (AS); 4, anthranilate phosphoribosyltransferase (PRT); 5, phosphoribosylanthranilate isomerase (PRAI); 6, indole-3-glycerol phosphate synthase (InGPS); 7, tryptophan synthase (TS).

due to presence of the repression control in the tryptophan pathway. However, further mutational removal of the repression did not cause any conversion of anthranilate to tryptophan. These results strongly suggest the presence of a regulatory mechanism other than the repression in the tryptophan pathway after anthranilate. For example, feedback inhibition of anthranilate phosphoribosyltransferase (PRT), the second-step enzyme which uses anthranilate as a substrate. Several reports have already dealt with feedback inhibition of PRT. The enzymes of enteric bacteria⁷⁻⁹ are in complex forms with AS and are partially inhibited by tryptophan. However, they lose sensitivity to the inhibition if they are separated from AS.⁹ The feedback inhibition of PRT that did not form a complex with AS has been found in *Acinetobacter calcoaceticus*,¹⁰ but with use of crude enzyme extracts. Further details of the properties have not been studied, probably because PRTs forming no complex with AS as in this bacterium are generally unstable.¹¹ PRT of *B. flavum* also does not

associate with AS and the activity was only detected with extraction in the presence of glycerol.²

This paper deals with the accumulation in phenylalanine tyrosine double auxotrophs of *B. flavum*, assay conditions and feedback inhibition of the PRT activity and mutational desensitization. PRT of *Bacillus subtilis* K was also studied and compared with that of *B. flavum*.

MATERIALS AND METHODS

Organisms. *Brevibacterium flavum* wild strain, No. 2247 (ATCC 14067), 5-fluorotryptophan-resistant tryptophan-producing mutant strains, No. 187,¹² No. 695,³ No. 833,³ and No. 404,³ a 5-fluorotryptophan-resistant anthranilate-producing mutant strain, No. 251,³ and a phenylalanine tyrosine histidine triple auxotroph, No. 10-1,³ were used.

Bacillus subtilis TD-9¹³ and FT-39¹⁴ are a tryptophan auxotroph and a 5-fluorotryptophan-resistant tryptophan-producing mutant derived from *B. subtilis* K, respectively.

Chemicals. Anthranilic acid, phosphoribosylpyrophosphate (PRPP) and DEAE-cellulose (DE-52) were purchased from ICN Pharmaceuticals, Boehringer-Mannheim and Whatman, respectively. Analogs of tryptophan were purchased from Sigma Chemicals except that L-3-(2'-naphthyl)-alanine was from Chemical Dynamical Corp. All L-amino acids used were products of Ajinomoto Co., Inc.

Derivation of mutants. Mutants having a derepressed level of AS, No. 27-1 and No. 33-2, were obtained as follows: Strain No. 10-1 grown overnight in Medium 6² supplemented with phenylalanine, tyrosine and histidine (300 mg/liter each) was harvested and washed once with 10 ml of 0.1 M sodium phosphate buffer, pH 7.0. Then, it was treated with 750 to 1000 µg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) at 30°C for 15 min to give 11 to 22% survivors. The surviving cells were spread on Medium 6 agar plates containing phenylalanine, tyrosine and histidine (300 mg/liter each) and 200 to 700 mg/liter of DL-5-fluorotryptophan. Colonies appearing during 8 days' incubation were picked up as 5-fluorotryptophan-resistant mutants. The mutants (about 1600 strains) were cultured in test tubes containing 3 ml of Medium 3³ supplemented with 300 mg/liter of phenylalanine and 150 mg/liter of tyrosine at 30°C for 72 hr. Among them, 53 strains accumulated more than 0.85 g/liter of anthranilate. These mutants cultured in the same medium for 24 hr, were treated with 0.6 mM cetyltrimethylammonium bromide (CTAB) in

0.1 M potassium phosphate buffer, pH 7.5, at 30°C for 10 min. Then, the cells were centrifuged, resuspended in the same buffer and examined for AS activity. Among them, 8 strains including strains No. 27-1 and No. 33-2 were found to have derepressed levels of AS.

Mutants having AS resistant to the feedback inhibition were induced and selected by resistance to 4-methyltryptophan as follows: Phenylalanine-producing strain No. 237, a reisolate of strain No. 239,¹²⁾ grown for 16 hr in Medium 30¹²⁾ supplemented with 1 g/liter of Casamino acids, was harvested and washed once with 10 ml of 0.1 M sodium phosphate buffer, pH 7.0. It was treated with 2000 µg/ml of NG at 30°C for 30 min to give 9.8% survivors. The surviving cells were spread on Medium 30 agar plates containing 1 g/liter of Casamino acids and 200 to 700 mg/liter of DL-4-methyltryptophan (5.5×10^6 to 5.5×10^4 cells/plate). Colonies appearing during 9 days' incubation were picked up as 4-methyltryptophan-resistant mutants. About 1000 of these strains were cultured in test tubes containing 3 ml of Medium 31¹²⁾ at 30°C for 72 hr. The mutants (85 strains) which accumulated more than 150 mg/liter of tryptophan, were treated with CTAB as described above and examined for AS activities. Among them, 30 strains were found to have AS resistant to the feedback inhibition (less than 60% inhibition by 1 mM tryptophan), one strain to have about a 4-fold higher level of AS than the parent strain, and 2 strains to have AS resistant to both the feedback inhibition and the repression.

Enzyme preparation. Cells of *B. flavum* No. 2247 cultured in 500 ml-flasks containing 50 ml of Medium 30 supplemented with 100 mg/liter of DL-7-methyltryptophan at 30°C for 24 hr were harvested, washed twice with 0.2% KCl and suspended with 0.1 M Tris-HCl buffer, pH 7.5. Then, the cells were sonically disrupted at 10 kc for 20 min. The cell-free extract obtained by centrifugation at $32,000 \times g$ for 30 min was placed on DEAE-cellulose column (2.5 × 21 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The column was washed with the same buffer and further with 30 ml of the same buffer containing 0.1 M KCl. PRT was eluted using a KCl concentration gradient consisting 400 ml of the same buffer containing 0.1 M KCl and containing 0.4 M KCl. Fractions of 8.0 ml were collected. The fractions containing PRT activity were pooled and concentrated with an ultrafiltration apparatus (Amicon PM-10). After gel-filtration on a Sephadex G-50 column equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, they were used for studies on PRT properties.

Cell-free extracts used in Table V were prepared by the following procedures. The sonicate supernatant solution was obtained as described above, but using the buffer containing 40% glycerol. Then, it was gel-filtered with a Sephadex G-50 column (1.5 × 10 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 30% glycerol. The filtrates were used as the crude enzyme extract.

Cell-free extracts of *B. subtilis* mutants used in Fig. 8

were also prepared in the same manner as described above, except that the cells were washed with 0.05 M Tris-HCl buffer, pH 7.5, and sonically disrupted at 10 kc for 10 min.

Assay methods. PRT-, AS-, indole-3-glycerol phosphate synthase- and tryptophan synthase-activities and protein were measured by the methods described previously.²⁾ The standard assay system for PRT contained 25 mM Tris-HCl buffer, pH 7.8, 5 µM anthranilic acid, 0.25 mM PRPP, 2 mM MgCl₂ and the enzyme in a total volume of 3.0 ml.

Tryptophan,³⁾ anthranilate,¹²⁾ 1-(*o*-carboxyphenylamino)-1-deoxyribulose,¹²⁾ indole-3-glycerol¹²⁾ and indole²⁾ accumulated in the culture medium were determined by the methods described previously.

RESULTS

Accumulation of anthranilate and tryptophan in phenylalanine tyrosine double auxotroph No. 10-1 and its derepressed mutants

As shown in Table I, phenylalanine tyrosine double auxotroph No. 10-1, which lacked chorismate mutase,¹⁵⁾ accumulated anthranilate in the culture medium, depending on the amount of tyrosine added. Accumulation of 1-(*o*-carboxyphenylamino)-1-deoxyribulose was also detected by paper chromatography. As described above, it seemed that anthranilate was accumulated by competitive overcoming of the feedback inhibition of AS by a high concentration of chorismate, which occurred under the tyrosine-limiting conditions.¹⁶⁾ Then, mutants having normally sensitive AS to the feedback inhibition but a genetically derepressed level of it were derived from strain No. 10-1 (Table II). Representative strains of these mutants, No. 27-1 and No. 33-2, also showed 5- to 12-fold increased levels of the other three enzymes in the tryptophan-pathway. Furthermore, syntheses of these four enzymes were not affected by the addition of an excess amount of tryptophan to the medium, which repressed them in the wild-type strains.²⁾ In strain No. 10-1, these enzyme levels were similar to those in wild strain No. 2247, where the enzyme syntheses had been fully repressed by tryptophan synthesized *de novo*, and therefore, were not affected by an excess amount of tryptophan.^{2,4)} We reported previously⁴⁾ that the repression of phosphoribosylanthranilate isomerase and tryptophan

TABLE I. ACCUMULATION OF TRYPTOPHAN AND INTERMEDIATES IN THE TRYPTOPHAN-PATHWAY IN PHENYLALANINE TYROSINE DOUBLE AUXOTROPHS

Strains were cultured at 30°C for 72 hr in Medium 3³) supplemented with 300 mg/liter of phenylalanine and various concentrations of tyrosine. Accumulation was determined by the method described in MATERIALS AND METHODS. The growth was indicated by the absorbancy at 562 nm after 26-fold dilution with 1 N HCl. For strains No. 27-1 and No. 33-2, results of the cultivation which showed the maximum accumulation are only shown.

Strain	L-Tyr added (mg/liter)	Growth ($A_{562\text{nm}}$)	Accumulation (mg/liter)				
			Ant ^a	CdR ^a	InG ^a	Indole	Trp ^a
No. 10-1	25	0.635	160	+	—	<5	6
	50	0.750	410	+	—	<5	15
	75	0.860	580	+	—	<5	38
	100	0.900	925	+	—	<5	51
	125	0.900	<10	±	—	<5	<5
	150	0.900	<10	—	—	<5	<5
No. 27-1	150	0.930	990	+	—	<5	45
No. 33-2	150	0.845	1050	+	—	<5	69

^a Abbreviations: Ant, anthranilate; CdR, 1-(*o*-carboxyphenylamino)-1-deoxyribulose; InG, indole-3-glycerol Trp, tryptophan.

TABLE II. ALTERED REGULATION OF TRYPTOPHAN BIOSYNTHESIS IN 5-FLUOROTRYPTOPHAN-RESISTANT MUTANTS, No. 27-1 AND No. 33-2

After cultivation at 30°C for 24 hr in Medium 5²) supplemented with 3 g/liter of Casamino acids, 200 mg/liter of phenylalanine and 200 mg/liter of tyrosine, crude enzyme extracts were prepared by the methods described in MATERIALS AND METHODS.

Strain	Trp concn. for 50% inhibition of AS (mM)	Trp addition ^a	Specific activity (nmol/min/mg protein)			
			AS ^b	PRT ^b	InGPS ^b	TS-B ^b
No. 27-1	0.003	—	13	3.2	17	16
		+	14	3.7	17	16
No. 33-2	0.003	—	12	1.9	16	19
		+	11	3.0	18	18
No. 10-1	0.002	—	1.4	0.26	2.0	2.9
		+	1.1	0.36	0.94	2.4

^a Tryptophan at 500 mg/liter was supplemented to the medium (indicated by +) or not (indicated by —).

^b Abbreviations are as in Fig. 1.

synthase-A (not indicated in the table) was coordinate with that of indole-3-glycerol phosphate synthase and tryptophan synthase-B, respectively. Therefore, it was concluded that in these mutants, synthesis of all the enzymes of the tryptophan-pathway was genetically derepressed. When these derepressed mutants were cultured, as shown in Table I, they showed almost the same patterns of accumulation as did the parent strain. That is, removal

of the repression control did not cause conversion of anthranilate to tryptophan. The results strongly suggest that a regulatory mechanism other than the repression is presented in the pathway after anthranilate.

General properties of anthranilate phosphoribosyltransferase

The most probable regulatory mechanism suggested in the preceding section is feedback

inhibition by tryptophan of PRT, the second-step enzyme in the tryptophan-pathway, which uses anthranilate as a substrate. However, the previous experiments showed that PRT of *B. flavum* was unstable and its specific activity was very low in the wild strain (0.66 nmol/min/mg protein)⁴⁾ and even in tryptophan auxotrophs cultured under limitation of tryptophan which repressed PRT synthesis. Therefore, for the enzymatic study, it was necessary to increase the specific activity and to establish reliable assay conditions. 7-Methyltryptophan was found to inhibit AS activity and, therefore, to derepress PRT synthesis in the wild strain. Addition of 100 mg/liter of DL-7-methyltryptophan to the culture medium increased the specific activity 280-fold to 74 nmol/min/mg protein. Then, PRT was extracted from the cells thus obtained, and further purified by DEAE-cellulose column chromatography with a 63% yield. PRT was eluted from the column with 0.22 M KCl and completely separated from AS, which was eluted with 0.29 M KCl. The specific activity of the partially purified preparation was 1400-fold higher than that of the enzyme extract from cells grown in minimal medium (Medium 30).

PRT of *B. flavum* as well as those of some other bacteria^{11,17)} have been considered as yet to be unstable from the following results. The activity could not be detected when it was extracted in the absence of glycerol and even when it was done in its presence, the activities were scattered.²⁾ However, by reexamining the stability, PRT was found to be stable even in the absence of glycerol, the effect of which was stimulation rather than stabilization of the activity (Table III). Moreover, the relationship between the activity and concentration of the enzyme was not linear and markedly deviated from the straight line that connected the plots at higher concentrations with the zero point, as shown in Fig. 2. The result suggests that the PRT molecule dissociates into less active components at lower concentrations of the enzyme. Since glycerol not only stimulated the activity but also decreased the deviation, its

TABLE III. EFFECT OF VARIOUS ADDITIONS ON ANTHRANILATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY

The activity was assayed under the standard conditions but in the presence of the additions indicated, using 0.12 mg protein of the partially purified enzyme. The activities were expressed as percentages of that without addition (0.94 nmol/ml/min).

Addition	Concentration	Relative activity (%)
None	—	100
Glycerol	10%	259
Sucrose	10%	231
Polyethyleneglycol	10%	255
Ethyleneglycol	10%	108
1,3-Propanediol	10%	20
NH ₄ Cl	0.1 M	247
LiCl	0.1 M	249
NaCl	0.1 M	356
KCl	0.1 M	373
RbCl	0.1 M	293
CsCl	0.1 M	153
MgCl ₂	0.1 M	25
CaCl ₂	0.1 M	11
(NH ₄) ₂ SO ₄	0.1 M	259
Na ₂ SO ₄	0.1 M	428
K ₂ SO ₄	0.1 M	447

FIG. 2. Relationship between Anthranilate Phosphoribosyltransferase Activity and Concentration of the Enzyme.

The activity was assayed in the standard medium (○) or in that supplemented with 10% glycerol (△), 0.1 M KCl (●), or 10% glycerol and 0.1 M KCl (□).

FIG. 3. Effects of Glycerol and KCl Concentrations on Anthranilate Phosphoribosyltransferase Activity.

0.14 mg protein of the partially purified preparation was used. In (A), the activity was assayed at various concentrations of glycerol in the presence (Δ) and absence (○) of 0.1 M KCl. In (B), the activity was assayed at various concentrations of KCl in the presence (Δ) and absence (○) of 10% glycerol. The activities were expressed as percentages to that without addition (0.59 nmol/ml/min).

FIG. 4. Double Reciprocal Plots of Anthranilate Phosphoribosyltransferase Reaction Rate against the Substrate Concentration in the Presence and Absence of Glycerol or KCl.

The activity was assayed under the standard conditions except for the substrate concentration (○) and the addition of 0.1 M KCl (Δ) or 20% glycerol (□). The concentration of PRPP in (A) was 0.25 mM and that of anthranilate in (B) was 5 μM. 0.12 mg protein of the partially purified preparation of PRT was used.

stimulatory effect became greater at the low enzyme concentrations. Therefore, the activity would be difficult to measure at lower enzyme concentrations in the absence of glycerol. Moreover, when the enzyme was extracted in the presence of glycerol but the activity was measured in the absence of glycerol, the specific activity measured was scattered, because it depended on the amounts of both the enzyme and glycerol accompanied by the enzyme.

Table III also shows that PRT activity is stimulated not only by glycerol but also by polyethyleneglycol and sucrose. Furthermore, the activity was found to be strongly stimulated by monovalent inorganic cations such as potassium, sodium and ammonium ions. KCl also decreased the deviation from linearity, similar to as did glycerol, as shown in Fig. 2. Moreover, the simultaneous presence of 0.1 M KCl and 10% glycerol gave a complete linear relationship between the activity and concentration.

As shown in Fig. 3, glycerol and KCl showed the maximum effects at 10% and 0.1 M, respectively. The simultaneous presence of them further stimulated the activity, more than did single additions, suggesting different mechanisms for their effects.

Figure 4 shows double reciprocal plots of PRT reaction rate against the substrate concentration in the presence and absence of glycerol or KCl. Glycerol increased the enzyme affinity for PRPP (decrease from 624 to 93 μM in apparent Michaelis constant). On the contrary, KCl did not affect the affinity for both the substrates but increased the maximum activity. These results also indicate a difference in the mechanism for the stimulation by glycerol and KCl. The apparent K_m for anthranilate was estimated to be 0.42 μM from Fig. 4(A).

PRT required magnesium ions for its activity and the effect was saturated at 2 mM.

The optimum pH was 8.5 with Tris-HCl buffer and, in the presence of 0.1 M KCl, was also 8.5 with both *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid-NaOH and Tris-HCl buffers.

Inhibition of anthranilate phosphoribosyltransferase activity by tryptophan

As shown in Fig. 5, PRT from *B. flavum* was inhibited 33% by 2 mM tryptophan. Moreover, addition of glycerol but not KCl stimulated the inhibition, which reached its maximum, 84%, in the presence of 20% glycerol. Figure 6 shows the effect of tryptophan concentration on the activity in the presence

of 20% glycerol. Tryptophan inhibited the activity much more strongly than that of tryptophan synthase,¹²⁾ the final step-enzyme in the tryptophan-pathway but much less than that of AS,³⁾ the first step-enzyme. The concentrations of tryptophan giving 50% inhibition of PRT, tryptophan synthase and AS were estimated to be 0.15, 7.7¹²⁾ and 0.002

FIG. 5. Effect of Glycerol and KCl on the Inhibition of Anthranilate Phosphoribosyltransferase by Tryptophan.

0.1 mg protein of the partially purified preparation was used. Degrees of inhibition by 2 mM tryptophan were determined at various concentrations of glycerol or KCl. The activity without tryptophan, glycerol and KCl was 0.73 nmol/ml/min. ○, glycerol; △, KCl.

FIG. 6. Effect of Tryptophan Concentration on Anthranilate Phosphoribosyltransferase Activity.

0.14 mg protein of the partially purified preparation was used. The activity was assayed in the standard medium supplemented with 20% glycerol and various concentrations of tryptophan. Activities were expressed as percentages to that without tryptophan (2.1 nmol/ml/min). Results with respect to AS and tryptophan synthase were quoted from the references.^{3,12)} △, PRT activity; ○, AS activity; □, tryptophan synthase activity.

mm,³⁾ respectively. Addition of AS isolated by DEAE-cellulose column chromatography to the reaction mixture (the relative amount to PRT was almost the same as in the cell-free extract) did not affect the inhibition or the activity of PRT.

Figure 7 shows double reciprocal plots of PRT reaction rate against the substrate concentration in the presence and absence of tryptophan. In the presence (Fig. 7(A,B)) and absence (Fig. 7(C,D)) of glycerol, tryptophan was shown to inhibit the activity non-competitively with respect to both the substrates. The inhibitor constants for tryptophan, k_{is} , calculated from Fig. 7 were 0.26 and 4.3 mM in the presence and absence of 20% glycerol, respectively, whereas K_i of AS for tryptophan was 0.0048 mM.¹⁶⁾

Owing to the highly sensitive PRT assay method, the enzyme concentrations used in the

FIG. 7. Double Reciprocal Plots of Anthranilate Phosphoribosyltransferase Reaction Rate against the Substrate Concentration in the Presence and Absence of Tryptophan.

The partially purified preparation, 0.73 (A), 0.12 (B, C) or 0.20 mg (D) of protein, was used. The activity was assayed under the standard conditions except for the substrate concentration in the presence (A, B) and absence (C, D) of 20% glycerol. The concentrations of PRPP in (A) and (C) were 0.25 and 1.0 mM, respectively. The concentration of anthranilate in (B) and (D) was 5 μM. The concentrations of tryptophan were 0 (○), 0.2 (▲) and 5 mM (△).

above experiments were those at which the enzyme considerably dissociates (Fig. 2). On the other hand, the intracellular concentration in the derepressed cells which could actively synthesize tryptophan was calculated to be more than 10.6 mg/3 ml at the same measure as in Fig. 2, the concentration at which the enzyme did not dissociate. As glycerol prevented the enzyme from dissociating (Fig. 2), properties of PRT *in vivo* seem to be reflected by those in the presence of glycerol better than

those in its absence.

Table IV shows the effects of amino acids and tryptophan analogs on PRT activity and on AS activity for comparison. PRT was inhibited by L-tryptophan but not by D-tryptophan, phenylalanine, tyrosine or histidine at all. Furthermore, phenylalanine and tyrosine did not affect the inhibition by tryptophan. Among tryptophan analogs tested, those with substituents in the indole-ring inhibited the enzyme, as did L-tryptophan, whereas some of those with substituent in the alanine moiety (α -methyltryptophan, D-tryptophan and tryptophan hydroxamate) did not. On the other hand, AS activity was strongly inhibited by some of the latter analogs but not by 6-methyl-, 5-hydroxy- or 5-methoxy-tryptophan. Thus, the inhibition pattern of PRT by tryptophan analogs was clearly different from that of AS.

TABLE IV. EFFECTS OF SOME AMINO ACIDS AND TRYPTOPHAN ANALOGS ON ANTHRANILATE PHOSPHORIBOSYLTRANSFERASE AND ANTHRANILATE SYNTHASE ACTIVITIES

The partially purified preparation of PRT, 0.15 mg of protein, was used and a cell-free extract from wild strain No. 2247 was used for the AS assay after gel-filtration on a Sephadex G-50 column (1.5 $\times$ 10 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The PRT activity was assayed in the standard medium supplemented with 20% glycerol and 2 mM addition, and that of AS was assayed in the standard medium with 0.1 mM addition. Activities were expressed as percentages to those without addition (the activities of PRT and AS were 2.2 and 0.19 nmol/ml/min, respectively).

Addition	Relative activity (%)	
	PRT	AS
None	100	100
L-Tryptophan	16	0.2
D-Tryptophan	96	98
L-Phenylalanine	95	100
L-Tyrosine	106	99
L-Histidine	121	99
DL-4-Methyltryptophan	9.1	7.4
DL-5-Methyltryptophan	9.4	14
DL-6-Methyltryptophan	14	87
DL-7-Methyltryptophan	17	0.7
DL-5-Fluorotryptophan	17	0.3
DL-6-Fluorotryptophan	39	26
DL-5-Hydroxytryptophan	29	87
DL-5-Methoxytryptophan	14	95
DL-7-Azatriptophan	55	90
DL-N-Acetyltryptophan	47	97
N-t-Butoxycarbonyl-L-tryptophan	63	60
N-Carbobenzoxyl-L-tryptophan	29	97
DL- α -Methyltryptophan	93	9.6
DL-Tryptophan hydroxamate	88	0.9
Indole-3-propionic acid	69	94
L-3-(2'-Naphtyl)alanine	72	58

Mutational desensitization of anthranilate phosphoribosyltransferase to the feedback inhibition

5-Fluorotryptophan-resistant tryptophan-producing mutants derived previously^{3,12)} (strains No. 187, No. 404, No. 695 and No. 833) were examined for the inhibition by tryptophan of PRT. Table V shows that PRT as well as AS^{3,12)} was resistant to the feedback inhibition by tryptophan in all these mutants. In contrast, both the two enzymes in strain No. 33-2 showed normal sensitivity to the feedback inhibition. Furthermore, the feedback inhibition of PRT was also removed in strain No. 251 which accumulated anthranilate similarly to strain No. 33-2 but had AS resistant to the feedback inhibition. These results show that the feedback inhibition of PRT is removed by mutations concomitantly with that of AS. In order to confirm this, we further induced mutants having normal repression control but having AS resistant to the feedback inhibition. For this purpose, tryptophan analogs which inhibited AS but did not repress the tryptophan enzymes, differently from 5-fluorotryptophan, were selected by similar methods to those described previously.⁴⁾

TABLE V. SENSITIVITIES TO THE FEEDBACK INHIBITION OF ANTHRANILATE PHOSPHORIBOSYLTRANSFERASE AND ANTHRANILATE SYNTHASE IN VARIOUS MUTANTS

Strains were cultured at 30°C for 24 hr; strains No. 187 and No. 237 in Medium 31,¹²⁾ and strains No. 404, M4-90, M4-141, M4-223, M4-394, M4-424, No. 251 and No. 2247 in Medium 30¹²⁾ supplemented with 700, 200, 300, 100, 200, 100 and 100 mg/liter of DL-7-methyltryptophan, respectively, the amounts of which were optimum for the enzyme derepression with the respective strains. Strains No. 695 and No. 833 were cultured in Medium 5 supplemented with 3 g/liter of Casamino acids and strain No. 33-2 in the same medium further supplemented with phenylalanine and tyrosine (200 mg/liter each). Crude enzyme extracts were prepared by the method described in MATERIALS AND METHODS and activities were assayed with various concentrations of tryptophan. The concentration of tryptophan giving 50% inhibition was graphically estimated. PRT activity was measured in the standard medium (Exp. I) and in that supplemented with 20% glycerol and 0.2 M KCl (Exp. II).

Exp.	Strain	Character	Trp concn. for 50% inhibition (mM)	
			PRT	AS
I	No. 187	5FT ^r , Tryptophan-producing	14	2.4 ¹²⁾
	No. 404	5FT ^r , Tryptophan-producing	27	1.8 ³⁾
	M4-90	4MT ^r	8.9	4.8
	M4-141	4MT ^r	> 50	4.8
	M4-223	4MT ^r	7.5	3.8
	M4-394	4MT ^r	16	4.0
	M4-424	4MT ^r	17	3.6
	No. 237	Phenylalanine-producing	2.2	0.004
	No. 251	5FT ^r , Anthranilate-producing	11	1.7 ³⁾
II	No. 2247	Wild	2.0	0.002 ³⁾
	No. 695	5FT ^r , Tryptophan-producing	9.3	3.9 ³⁾
	No. 833	5FT ^r , Tryptophan-producing	12	27 ³⁾
	No. 33-2	5FT ^r , Anthranilate-producing	0.5	0.003
	No. 2247	Wild	0.5	0.002 ³⁾

Then, 4-methyltryptophan, a representative of the analogs thus obtained was used for selecting these mutants based on its resistance. Table V shows that in these 4-methyltryptophan-resistant mutants (M4-90, M4-141, M4-223, M4-394 and M4-424), mutational removal of the feedback inhibition of PRT also occurs concomitantly with that of AS.

Effect of tryptophan on anthranilate phosphoribosyltransferase of Bacillus subtilis

PRT of *B. subtilis* which also did not form a complex with AS as did that of *B. flavum*, was examined for the tryptophan inhibition, using tryptophan auxotroph TD-9¹³⁾, and 5-fluorotryptophan-resistant tryptophan-producing mutant FT-39,¹⁴⁾ having normally feedback-inhibited but genetically derepressed AS, both derived from *B. subtilis* K. As shown in Fig.

8, PRT of both *B. subtilis* strains was much weakly inhibited than that of the *B. flavum* wild strain. The tryptophan concentration giving 50% inhibition of PRT was estimated to be about 20 mM in strain FT-39, which was a 130-fold higher concentration than that in the *B. flavum* wild strain, but similar to that for desensitized PRT in a tryptophan-producing mutant of *B. flavum*, No. 187.

DISCUSSION

As suggested by the experiments on the accumulation of anthranilate in mutants with derepressed tryptophan enzymes, PRT, the second-step enzyme of the tryptophan pathway, was found to be feedback-inhibited by tryptophan, in addition to AS, the first-step

FIG. 8. Effect of Tryptophan on Anthranilate Phosphoribosyltransferase Activity of *Bacillus subtilis*.

After mutants of *B. subtilis*, FT-39 and TD-9, were cultured at 30°C for 24 hr in Medium 6¹⁴) and in that supplemented with 20 mg/liter of tryptophan, respectively, crude enzyme extracts were prepared by the method described in MATERIALS AND METHODS. Crude enzyme extracts of *B. flavum* No. 2247 and No. 187 were prepared as described in Table V, but using 0.1 M Tris-HCl buffer, pH 7.5, for the sonication and 0.05 M the same buffer for the gel-filtration. The activity was assayed in the standard medium supplemented with 20% glycerol and was expressed as the percentage to that without tryptophan (the activities in strains FT-39, TD-9, No. 2247 and No. 187 were 1.2, 0.5, 1.2 and 0.8 nmol/ml/min, respectively). ●, strain TD-9; ○, strain FT-39; □, strain No. 2247; △, strain No. 187.

enzyme. The inhibition of PRT was stronger than that of tryptophan synthase, the final step enzyme, but weaker than that of AS. That is, the tryptophan concentration giving 50% inhibition of PRT was 1/50 of that of tryptophan synthase¹²⁾ and 75-fold of that of AS.³⁾ Therefore, it seems likely that the intracellular concentration of tryptophan is regulated by the feedback inhibition of AS under normal conditions and is less than 0.01 mM, the concentration required for almost complete inhibition of AS.¹⁾ Therefore, the feedback inhibition of PRT does not seem to operate under normal conditions. However, when the concentration of chorismate, an intermediate, was increased through removal of the synergistic inhibition by phenylalanine plus tyrosine of DAHP synthase⁵⁾ under tyrosine-limitation¹⁶⁾ as occurred in phenylalanine tyrosine auxotrophic mutant No. 10-1, the intracellular concentration of tryptophan would be increased because the inhibition

of AS by tryptophan was competitive with respect to chorismate.¹⁾ Under these conditions, the feedback inhibition of PRT may operate and moreover, this inhibition is not overcome by an increased concentration of anthranilate, because of its non-competitiveness toward anthranilate. Therefore, the inhibition of PRT by tryptophan is probably able to prevent conversion of anthranilate to tryptophan. In fact, strain No. 33-2 produced a large amount of anthranilate in addition to a small amount of tryptophan. From these results, it can be concluded that overproduction of tryptophan in *B. flavum* is prevented by the following three regulatory mechanisms; the feedback inhibition of AS, the repression of the tryptophan enzymes and the feedback inhibition of PRT. This was further confirmed by the result that PRT was also resistant to the feedback inhibition in the previously described mutants, which had feedback resistant AS and produced anthranilate or tryptophan depending on the presence or absence of the repression control,^{3,12)} respectively.

The feedback inhibition of PRT in *B. flavum* is probably much stronger than that in other microorganisms. In *B. subtilis* K, the inhibition by tryptophan of the wild-type PRT was very weak and was almost the same as that of the feedback resistant PRT in *B. flavum* mutants. Furthermore, derepressed mutants of *B. subtilis*, FT-39, having the wild-type PRT overproduce tryptophan but not anthranilate.¹⁴⁾ The result is in contrast to that derepressed mutants of *B. flavum* accumulate anthranilate but not tryptophan.

Moreover, in *Escherichia coli*^{7,8)} and *Salmonella typhimurium*⁹⁾, PRT forms a complex with AS and is inhibited by tryptophan only in the complex form, up to about 60% at maximum. Therefore, it does not seem that the inhibition can prevent conversion of anthranilate to tryptophan.

In contrast to the *E. coli* enzyme, the *B. flavum* enzyme does not form a complex with AS as shown in the previous experiments with column chromatography.²⁾ The inhibition of

PRT was not affected by the addition of AS. Moreover, the specificity of the inhibition by tryptophan analogs was greatly different from that of AS. These results indicate that in *B. flavum*, the inhibition of PRT is independent of that of AS, in contrast to *E. coli*.

On the contrary, the inhibition of both the enzymes was simultaneously removed in all mutants tested. Therefore, it is possible that the two enzymes have an identical regulatory subunit.

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