JOURNAL OF BACTERIOLOGY, Jan. 1968, p. 107-112

Vol. 95, No. 1
Printed in U.S.A.

Enzymes of the Tryptophan Synthetic Pathway in *Pseudomonas putida*

TOSHIO ENATSU[1] AND IRVING P. CRAWFORD

Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92038

Received for publication 5 October 1967

The first four enzymatic activities of the tryptophan synthetic pathway in *Pseudomonas putida* were found on separate molecules. Gel filtration and density gradient centrifugation experiments did not disclose any associations or aggregations among them. These findings contrast with the situation found in the enteric bacteria, where the first two activities are found in an aggregate and the third and fourth are catalyzed by a single enzyme. Tryptophan synthetase, the last enzyme of the pathway, consists of two dissociable components. The affinity of these components is less in *P. putida* than is the case in *Escherichia coli*.

Genetic and enzymological studies have shown that *Pseudomonas putida* regulates the production of enzymes of the tryptophan pathway in a manner quite unlike that of the enteric bacteria (4; A. M. Chakrabarty, I. P. Crawford, S. Sikes, C. F. Gunsalus, and I. C. Gunsalus, *in preparation*). *P. putida* possesses six structural genes, whereas *Escherichia coli* and *Salmonella typhimurium* have only five. The *Pseudomonas trp* genes are found in three chromosomal locations, whereas the genes of the coliforms are clustered in a typical operon (13, 17). The enteric bacteria elaborate their tryptophan synthetic enzymes coordinately, in response to the availability of tryptophan to the cell (9). *P. putida* regulates the first, second, and fourth enzymes in a fashion similar to that of the coliforms. The production of the third enzyme varies little, however, and the two subunits of the last enzyme, tryptophan synthetase (TS), are induced by indoleglycerol phosphate, not repressed by tryptophan (4). The chromosomal clustering of the *Pseudomonas* genes corresponds to their mode of regulation (A. M. Chakrabarty et al., *in preparation*).

The present study was undertaken to determine the approximate molecular size and state of aggregation of the enzymes of the tryptophan pathway in *P. putida*. DeMoss (7) pointed out that the activities of the tryptophan pathway are variously assorted in the enteric bacteria, yeasts, and fungi. In *E. coli*, the first and second activities, anthranilate synthetase (AS) and phosphoribosyl transferase (PRT), are catalyzed by an aggregate formed from the products of the first two genes in the operon (10). The third and fourth activities, phosphoribosyl anthranilate isomerase (PRAI) and indoleglycerol phosphate synthetase (InGPS), are catalyzed by an enzyme consisting of a single polypeptide chain (6). The A and B activities of TS (indole formation from indoleglycerol phosphate, tryptophan formation from indole and serine) are catalyzed by a complex enzyme formed from the products of the last two genes in the operon. In *Saccharomyces cerevisiae*, the first and fourth activities appear in an aggregate, whereas in *Neurospora crassa* the first, third, and fourth activities appear in an aggregate; all the *trp* genes of these organisms are unlinked (7). The tryptophan genes and enzymes of bacterial species outside the enteric group have not received intensive study. It is known, however, that in *Bacillus subtilis* the genes of the pathway are disposed as in *E. coli* (1, 3), whereas the tryptophan synthetic enzymes of *Aeromonas formicans* are assembled and regulated like those of an enteric organism (5)

In this paper, we show that the enzymatic activities of the tryptophan pathway in *P. putida* are assorted in a unique fashion.

MATERIALS AND METHODS

Organisms. The parent strain used in this study, *P. putida* C1S, was derived from the terpene oxidizer C1B (2), from which it differs in colony type (translucent rather than opaque) and phage sensitivity. The tryptophan auxotrophs used, which were identified by replica plating after treatment with the mutagen *N*-methyl-*N*-nitrosoguanidine, were described in the previous paper (4). The organisms were maintained by monthly transfer on slants of L-agar (11).

Culture conditions. Cultures in 1 liter of medium E

[1] Present address: Department of Fermentation Technology, Osaka University, Osaka, Japan.

(14) were grown on a rotary shaker at 30 C in 2-liter DeLong flasks with baffles (Bellco Glass, Inc., Vineland, N.J.).

Enzyme preparation. Mutant cultures grown on limiting L-tryptophan (5 mg/liter) were incubated for 1 to 3 hr after exhaustion of the supplement. Cells were sedimented by centrifugation at 4 C, washed once with 0.1 M potassium phosphate, *p*H 7.0, and then suspended in three times their wet weight of the buffer. Succeeding operations were performed at 0 to 4 C. After disruption with a Branson sonic probe, by use of two 30-sec exposures, cell debris was removed by centrifugation for 30 min at 40,000 × *g*. For each 10 ml of extract, 0.5 ml of 5% protamine sulfate (Nutritional Biochemicals Corp., Cleveland, Ohio) dissolved in warm water and neutralized with NaOH, was added slowly with stirring. The mixture was allowed to stand for 10 min and then was centrifuged for 10 min at 40,000 × *g*. For each 10 ml of protamine supernatant fluids 1.44 g of powdered ammonium sulfate was added with stirring. After 10 min, the precipitate was removed by centrifugation and discarded. The second addition of ammonium sulfate consisted of 1.69 g of the salt per 10 ml of the original protamine supernatant fluid. This mixture was allowed to stand for 10 min, and then the precipitate was collected by centrifugation at 40,000 × *g* and was suspended in 1 to 2 ml of 0.1 M potassium phosphate, *p*H 7.0. The suspension (usually 4 to 10 ml) was dialyzed overnight, with one change of buffer, against 500 ml of 0.1 M potassium phosphate, *p*H 7.0. The dialyzed enzyme preparation was stored at 4 C.

Enzyme assay and substrates. The AS, PRT, and InGPS assays were those used in studies of *E. coli* (9). PRAI and TS-A and -B activities were assayed as described previously (4). Protein concentration was determined by the method of Lowry et al. (12), with bovine serum albumin as the standard. (One unit of any enzyme activity was defined as the consumption or production of 0.1 μmole of substrate or product per 20 min at 37 C.)

Gel filtration. Most of the experiments were done with a column of Sephadex G-100 (2.5 cm in diameter and approximately 40 cm in height) operated in the ascending mode at 5 C. A constant flow of 30 ml/hr of 0.1 M potassium phosphate, *p*H 7.0, was maintained. Occasional introduction of buffer supplemented with 1 mM sodium azide allowed operation over long periods. Samples of 1 ml, containing 10 to 100 mg of protein, were introduced into the buffer stream. Several known proteins, as well as blue dextran (Pharmacia), served as molecular weight standards according to Whitaker's (16) method.

Sucrose density gradient centrifugation. A linear density gradient was formed from 2.6 ml each of 20 and 5% sucrose solutions in 0.1 M potassium phosphate, *p*H 7.0. The sample (0.28 ml) was layered on top of the gradient. Centrifugation was performed at 48,000 rev/min in the SW 50L rotor (Spinco) for 11 hr at 3.7 C. Fractions were collected after puncturing the bottom of the tube.

RESULTS

Preliminary experiment with Sephadex G-150. Figure 1 shows the result of gel filtration of a dialyzed enzyme preparation from strain S21 (a mutant having AS, PRT, and InGPS, but lacking PRAI) on a Sephadex G-150 column. One ml of the dialyzed preparation (*see* Materials and Methods), containing approximately 50 mg of protein, was placed on the column. The three enzyme activities eluted separately. The AS and PRT peaks were quite symmetrical, but the InGPS peak in this experiment was a little irregular. In other experiments, however, the distribution of InGPS showed a completely symmetrical shape. All three enzymes eluted in positions corresponding to molecular weights below 100,000, so the succeeding experiments were performed with a column of Sephadex G-100 (*see* Materials and Methods).

Enzyme activities eluted from Sephadex G-100. The column was calibrated with ribonuclease, trypsin, chymotrypsin, and bovine serum albumin as standards. Figure 2 shows an example of an elution pattern obtained when a preparation from strain S1, a mutant lacking AS, was passed through this column. In the case of strains S11 and S31, mutants lacking PRT and InGPS, respectively, the distribution of enzyme activities also showed separate peaks with symmetrical shapes. No associations among the four enzymes

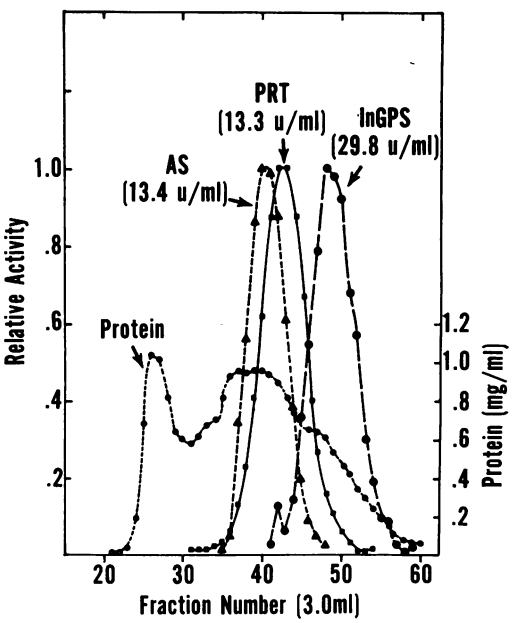

FIG. 1. *Sephadex G-150 gel filtration of an enzyme preparation from strain S21. The column, operated in the descending mode with 0.1 M potassium phosphate, pH 7.0, as eluent, measured 2.5 cm × 32 cm. The flow rate was 15 ml/hr. The activities shown in the figure have been normalized to give a uniform peak height; the activity of the peak fraction is in parentheses below the enzyme designation.*

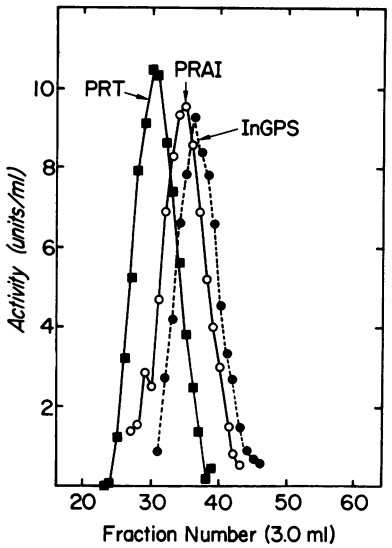

FIG. 2. *Sephadex G-100 gel filtration of an enzyme preparation from strain S1. The column was operated as described in Materials and Methods.*

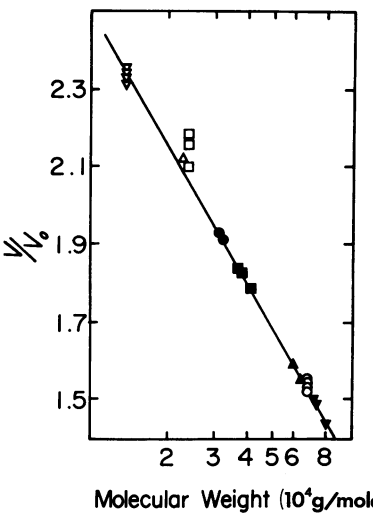

FIG. 3. *Relationship between elution volume (V/V_0) and the molecular weight of known and unknown proteins. The data are plotted according to Whitaker (16). Symbols: ○, bovine serum albumin; □, trypsin; △, chymotrypsin; ▽, pancreatic ribonuclease; ▼, AS; ▲, PRT; ■, PRAI; ●, InGPS.*

were observed. (The small irregularity in PRAI elution in fraction 29 is probably an experimental error, since it was not observed in other experiments.) The recovery of PRT, PRAI, and InGPS activities in the fractions varied between slightly less than 60 and greater than 80% of the amount placed on the column. The best recoveries were 81% for PRT, 82% for PRAI, and 85% for InGPS. Unfortunately, the recovery of AS activity was very poor, amounting to slightly more than 20% in the best case. AS was also the least stable of the enzyme activities when stored at 4 C.

Figure 3 shows the relationship between the molecular weights and elution volumes of standard and unknown proteins. From these data, molecular weights were assigned to the first four enzymes of the pathway (Table 1).

Sucrose density centrifugation. A sucrose density gradient experiment was performed to see if any associations between the early enzymes could be detected. The enzyme preparation was obtained from mutant S21. Fractions comprising 13 drops were collected and assayed. The distribution pattern of enzymes obtained is shown in Fig. 4. Again, no clear evidence of association was found. Recoveries in this experiment were 34% for AS, 64% for PRT, and 79% for InGPS.

Gel filtration of tryptophan synthetase. Two double mutants were used in studies of TS. Both contained the S1 mutation affecting AS. Strain S1i4 is a constitutive overproducer of TS, showing high levels of A and B activities under all growth conditions; strain S1i1 is a double mutant which

TABLE 1. *Molecular weights of tryptophan synthetic enzymes obtained by gel filtration on Sephadex G-100*

Strain	Molecular weight (10^4 g/mole)			
	AS	PRT	PRAI	InGPS
S1	—	6.0	3.8	3.2
S11	7.1	—	4.1	3.1
S21	8.0	6.5	—	3.2
S31	7.3	6.7	3.7	—
Avg	7.5	6.4	3.9	3.2

lacks TS-A activity but has high levels of TS-B activity, when grown in the presence of anthranilate (4). When extracts of either mutant were passed through the G-100 column, all TS activity disappeared. With strain S1i4, both activities were recovered quantitatively when fractions having molecular weights of about 80,000 and 30,000 were recombined. Figure 5 shows the separation of the two components, each being assayed in an excess of the other. In this case, pyridoxal phosphate and β-mercaptoethanol were added to the elution buffer to encourage any association. Analogous to the TS components in enteric bacteria, the larger of the *P. putida* components hereafter will be called the B component and the smaller, the A component.

An extract from strain S1i1 gave an identical separation when TS-B (indole to tryptophan)

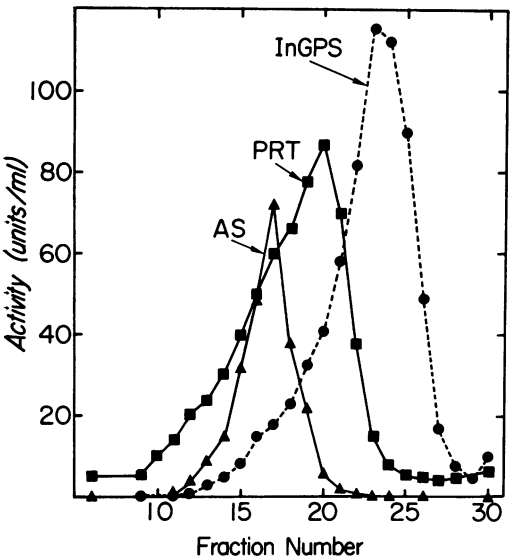

FIG. 4. *Sucrose gradient centrifugation of an enzyme preparation from strain S21. The experimental technique is described in Materials and Methods.*

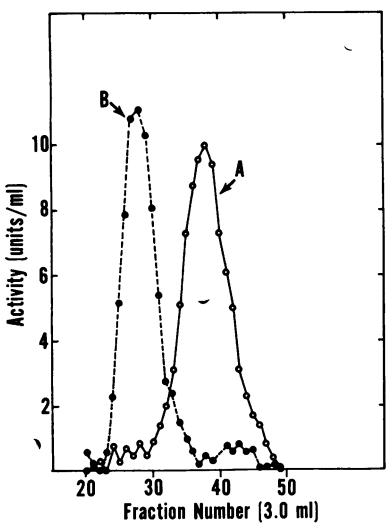

FIG. 5. *Sephadex G-100 gel filtration of an enzyme preparation from strain S1i4. The column was operated as described in Materials and Methods, with the exception that the elution buffer contained 38 μM pyridoxal phosphate and 1 mM β-mercaptoethanol as supplements. Activity was measured in the TS-B reaction.*

activity was measured, but no TS-A (indoleglycerol-phosphate to indole) activity was found. By mixing fractions from S1i4 and S1i1, it was shown that the A component of the S1i1 extract was the defective one. It appears, therefore, that the A component of the *P. putida* enzyme bears

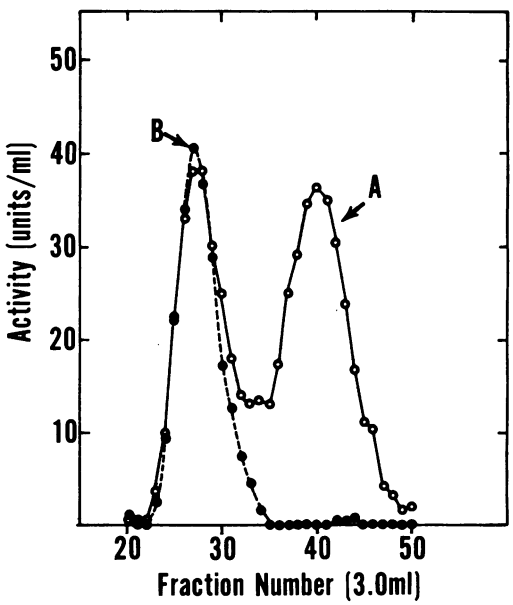

FIG. 6. *Sephadex G-100 gel filtration of an enzyme preparation from Escherichia coli strain T3 (lacking AS). A twofold excess of A component activity was present in the fraction added to the column. In other respects, the experimental procedure was identical to that of Fig. 5.*

the primarly responsibility for the TS-A reaction, as it does in *E. coli*.

An *E. coli* extract was passed through the same column under conditions similar to those illustrated in Fig. 5 to compare its behavior during filtration on G-100. Figure 6 presents the elution pattern obtained. Very little dissocation occurred, as is shown by the appearance of all the B component in combination with A component in the first peak.

DISCUSSION

We were unable to detect any associations among the products of the six *trp* genes of *P. putida* by use of the relatively gentle techniques of gel filtration and density gradient centrifugation. In view of the disparate regulatory behavior of PRAI and InGPS, it was not surprising to find these two activities residing on different molecules, but we expected to find some aggregation of the AS, PRT, or InGPS enzymes. The latter two activities, in particular, have been shown to be elaborated in strict coordination (4). The poor recovery of AS in these experiments is unexplained and may have resulted from dissociation as well as inactivation. Under conditions of derepression, however, *P. putida* increases its levels of AS more than twice as much as it does its levels of PRT and InGPS. Whether or not

weak aggregates may be formed between any of these molecules is still a subject for additional study.

P. putida TS was completely resolved into its A and B components by a single passage through Sephadex G-100. Under the experimental conditions chosen, the affinity between subunits was much weaker in *P. putida* than in *E. coli*. Incidentally, enzymatic experiments have been unable to show any intergeneric recognition of the separated *P. putida* and *E. coli* components. A more complete study of *P. putida* TS, involving purified components, will be the subject of a subsequent report. In the context of this paper, however, if the TS subunits had retained their activity after dissociation (i.e., the A subunits catalyzing the TS-A reaction and the B subunits catalyzing the TS-B reaction), we would not have detected the occurrence of a TS complex in *P. putida* by the methods used. The PRT subunit of the AS-PRT complex in *E. coli* apparently retains its activity when dissociated (10). If both of the subunits of a hypothetical complex of the early enzymes of *P. putida* retained their activity, and if the binding forces were no greater than those holding the TS subunits together, then we could expect results like those found in this study. Additional experiments, using the same techniques in the presence of substrates and possible metal ion ligands, or studies with even gentler techniques will be necessary before the existence of aggregates among the early enzymes of *P. putida* can be ruled out.

The present study has demonstrated the existence of a minimum of six distinct gene products in the pathway of tryptophan synthesis in *P. putida*. This finding agrees with the results of genetic analysis of the pathway (A. M. Chakrabarty et al., *in preparation*) and confirms the differences in gene number, position, and function between *P. putida* and the other bacterial genera studied. The studies of Holloway et al. (8, 15) indicate that *P. aeruginosa* probably possesses the same arrangement of *trp* genes and enzymes as *P. putida*. In recent work done in this laboratory (J. Wegman and I. P. Crawford, *in preparation*), *Chromobacterium violaceum* has been found to have the four early enzymes of the tryptophan pathway on separable molecules whose size is not greatly different from their counterparts in *P. putida*. In this organism, however, the TS activities coincide during gel filtration on Sephadex (the TS complex, if there is one, is only about half the size of that in *E. coli*). Unfortunately, the lack of repression or induction of synthesis of any of the enzymes of the pathway in *C. violaceum* makes further comparison with the *Pseudomonas* pattern impossible. Nevertheless, we feel that we have shown that bacteria have at least one strikingly different alternative to the enteric bacterial pattern of genes and enzymes for the tryptophan pathway. Studies of this pathway in other bacterial groups may be of considerable comparative and taxonomic interest.

ACKNOWLEDGMENT

This investigation was supported by grant GB-4267 from the National Science Foundation.

LITERATURE CITED

1. ANAGNOSTOPOULOS, C., AND I. P. CRAWFORD. 1967. Le groupe des genes regissant la biosynthese du tryptophane chez *B. subtilis*. Compt. Rend. **265**:93–96.
2. BRADSHAW, W. H., H. E. CONRAD, E. J. COREY, I. C. GUNSALUS, AND D. LEDNICER. 1959. Microbiological degradation of (+)-camphor. J. Am. Chem. Soc. **81**:5507.
3. CARLTON, B. C. 1967. Transformation mapping of the genes controlling tryptophan biosynthesis in *Bacillus subtilis*. J. Bacteriol. **94**:660–665.
4. CRAWFORD, I. P., AND I. C. GUNSALUS. 1966. Inducibility of tryptophan synthetase in *Pseudomonas putida*. Proc. Natl. Acad. Sci. U.S. **56**: 717–724.
5. CRAWFORD, I. P., S. SIKES, AND D. K. MELHORN. 1967. The natural relationships of *Aeromonas formicans*. Arch. Mikrobiol. **59**:72–81.
6. CREIGHTON, T. E., AND C. YANOFSKY. 1966. Indole-3-glycerol phosphate synthetase of *Escherichia coli*, an enzyme of the tryptophan operon. J. Biol. Chem. **241**:4616–4624.
7. DEMOSS, J. A. 1965. Biochemical diversity in the tryptophan pathway. Biochem. Biophys. Res. Commun. **18**:850–857.
8. FARGIE, B., AND B. W. HOLLOWAY. 1965. Absence of clustering of functionally related genes in *Pseudomonas aeruginosa*. Genet. Res. **6**:284–299.
9. ITO, J., AND I. P. CRAWFORD. 1965. Regulation of the enzymes of the tryptophan pathway in *Escherichia coli*. Genetics **52**:1303–1316.
10. ITO, J., AND C. YANOFSKY. 1966. The nature of the anthranilic acid synthetase complex of *Escherichia coli*. J. Biol. Chem. **241**:4112–4114.
11. LENNOX, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. MATSUSHIRO, A., K. SATO, J. ITO, S. KIDA, AND F. IMAMOTO. 1965. On the transcription of the tryptophan operon in *Escherichia coli*. I. The tryptophan operator. J. Mol. Biol. **11**:54–63.
14. VOGEL, H. J., AND D. M. BONNER. 1956. Acetylornithinase of *Escherichia coli*: partial purifica-

tion and some properties. J. Biol. Chem. **218:** 97–106.

15. WALTHO, J. A., AND B. W. HOLLOWAY. 1966. Suppression of fluorophenylalanine resistance by mutation to streptomycin resistance in *Pseudomonas aeruginosa*. J. Bacteriol. **92:**35–42.
16. WHITAKER, J. R. 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem., **35:**1950–1953.
17. YANOFSKY, C., AND E. S. LENNOX. 1959. Transduction and recombination study of linkage relationships among the genes controlling tryptophan synthesis in *Escherichia coli*. Virology **8:**425–447.