ENZYMES OF THE TRYPTOPHAN OPERON OF BACILLUS SUBTILIS

S.O. Hoch, C. Anagnostopoulos and I. P. Crawford

Scripps Clinic and Research Foundation
La Jolla, California 92037
Centre De Genetique Moleculaire, C.N.R.S.
Gif-sur-Yvette, France

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Methods for stabilizing the enzymes of the tryptophan synthetic pathway in extracts of Bacillus subtilis have been developed and are described. Examination of fourteen tryptophan auxotrophs showed only one enzymatic activity to be missing in each case. The size of the first four enzymes of the pathway was estimated by gel filtration. Synthesis of the enzymes of the pathway during derepression appears coordinate, as is the case in other bacteria where the structural genes for the pathway enzymes are clustered.

The structural genes for all the enzymes of the tryptophan synthetic pathway have been mapped in a cluster on the Bacillus subtilis chromosome (Anagnostopoulos and Crawford, 1961, 1967; Carlton, 1967). The gene order in this cluster is very similar to that found in the tryptophan operons of Escherichia coli (Yanofsky and Lennox, 1959) and Salmonella typhimurium (Baurle and Margolin, 1966; Blume and Balbinder, 1966).

Although B. subtilis is an organism well suited for detailed genetic studies, proof that a gene cluster represents an operon, in the sense of being transcribed as a unit onto a single polycistronic message, usually requires enzymatic as well as genetic analysis. With that in mind, a number of years ago we undertook to examine extracts of B. subtilis tryptophan auxotrophs for the enzymatic activities controlled by the tryptophan gene cluster (c.f. Anagnostopoulos and Crawford, 1961, 1967). Two of the six enzyme activities measurable in other organisms were always found to be absent or evanescent in extracts of B. subtilis, though they were demonstrable in whole cells. (Whitt and Carlton, 1968, report similar findings.) The unstable enzymes were those catalyzing the second and fourth steps of the pathway, phosphoribosyl transferase (PRT) and indoleglycerol phosphate synthase (InGPS). The present communication describes a method for stabilizing

these activities. Using this method, we have examined whether B. subtilis contains enzyme aggregates similar to those reported in this pathway in other microbes (c.f. DeMoss, 1965), and whether the tryptophan gene cluster in this organism behaves as a typical operon.

Methods. The mutants of B. subtilis used in this study have been described and mapped previously (Anagnostopoulos and Crawford, 1967). T12 has been reclassified as a class 3 mutant on the basis of anthranilate accumulation and the absence of PRAI activity. T7 was isolated at the same time as the others in the series, but was not reported earlier. It is a class 3 mutant which maps between T12 and class 4 (CDR accumulating) mutants.

All enzyme assays were performed with cell-free extracts. Cells were grown for 11-12 hours on a rotary shaker at 37 C in 300 ml of a minimal salts medium (Spizizen, 1958) supplemented with 0.5% glucose, 0.05% casein hydrolysate, and 0.5µg of L-tryptophan per ml. Cells were harvested by centrifugation at 16,000 x g, washed once in 0.1M KPO_4, pH 7.5 plus 10% glycerol (v/v) if anthranilate had been accumulated, and resuspended in 6 ml 0.1M KPO_4, pH 7.8, containing 40% glycerol (v/v), 0.01M L-glutamine and 4mM $MgSO_4$ or containing 0.8M sucrose. Lysozyme (2 mg) and DNAse (20µg) were added to the suspensions at 37 C for 30 min, followed by centrifugation at 43,000 x g for 30 min.

The anthranilate synthase (AS) and PRT assays were those used for E. coli (Ito and Crawford, 1965) except that assays were carried out in 1 ml reaction volumes containing 10 µmoles of the Tris-HCl buffer for the PRT assay and 25 µmoles of KPO_4, pH 7.5 plus 50 nanomoles of EDTA for the AS assay. Phosphoribosylanthranilate isomerase (PRAI) was assayed as described by Crawford and Gunsalus (1966). InGPS was assayed using the method of Smith and Yanofsky (1962), modified by using 60 µmoles KPO_4, pH 7.8, 240 µmoles sucrose, 2 µmoles hydroxylamine pH 7.0 and 1.8 µmoles of 1-(0-carboxyphenylamino)-1-deoxyribulose-5-P (CDRP) in a reaction volume of 1 ml. The B reaction of tryptophan synthase (TS-B) was assayed by following indole disappearance in a 1 ml reaction volume containing 100 µmoles KPO_4, pH 7.8, 30 µmoles of L-serine, 40 nanomoles of pyridoxal phosphate, 1 mmole of KCl and 400 nanomoles of indole. Indole was determined by the method of Smith and Yanofsky (1962). One unit of activity is defined as the disappearance of 1 nanomole of substrate or the appearance of 1 nanomole of product per minute. Specific activity is defined as units per mg of protein, assayed by the method of Lowry et al. (1951).

Molecular weight determinations were made using a Sephadex G-100 column (2.5 x 26 cm) equilibrated in 0.1 M KPO_4, pH 7.5, plus 30% glycerol (v/v), 0.01 M glutamine and 1mM NaN_3. The cell-free extracts were concentrated before application to the column by the addition of 3 volumes of cold saturated NH_4SO_4, pH 7.5, with stirring over a 20 minute period. After centrifugation at 43,000 x g for 30 minutes, the precipitate was resuspended in the buffer described. This procedure generally resulted in a 2-3 fold purification of the five enzymes. The upward flow of the column was regulated at 9 ml per hour. The void volume was determined with blue dextran. The column was calibrated with 3 proteins of known molecular weight, bovine serum albumin, trypsin and ovalbumin. The elution volumes of the standards were plotted according to the method of Whitaker (1963) in determining the approximate molecular weights of the tryptophan enzymes.

Results. We felt the enzymes under investigation had to be stabilized in cell-free extracts to make significant comparisons of activity levels possible. Although AS has been reported to be relatively stable (Whitt and Carlton, 1968 a,b) it slowly inactivated in our glycerol-containing extracts unless 0.01 M glutamine was present. Moreover, the glutamine seemed necessary to obtain a constant initial reaction rate during our continuous fluorimetric assay for the reaction. PRT was stabilized in cell-free extracts by glycerol. Phosphoribosyl pyrophosphate, one of the substrates, must be continuously present after the enzyme is diluted out of the glycerol into the reaction mixture. In its absence, PRT loses 74% of its activity within 30 seconds after dilution, and 94% within one minute. InGPS is stabilized by sucrose at an optimal concentration of 0.8 M but only in the presence of KPO_4 buffer as well.

TABLE 1. Summary of tryptophan synthetic activities in cell-free extracts.

		Specific Activities				
Class	Mutant	AS	PRT	PRAI	InGPS	TS-B
1	T8	0	4.4	43	0.2	7.8
	T24	0	0.5	49	33.0	6.2
2	T10	3.3	0	29	1.3	14.2
	T22	10.3	0	69	1.1	11.5
3	T7	8.8	4.8	0	0.05	12.5
	T11	1.6	2.0	0	0.5	6.9
	T12	2.8	3.4	0	1.0	8.6
	T19	5.5	6.0	0	0.07	13.7
4	T2	1.2	2.0	132	0	13.7
	T5	1.4	8.3	193	0	11.7
5a*	T4	2.8	7.5	72	1.2	0
	T16	6.7	6.2	63	0.5	11.0
5b	T3	0.9	1.8	67	66.0	0
	T6	1.3	3.8	80	0.5	0

*The absence of TS-A activity (InGP conversion to indole) in these mutants was shown earlier (Anagnostopoulos and Crawford, 1967). TS-B was assayed without the addition of exogenous component A.

TS-B is stabilized by either glycerol or sucrose, and remains active when diluted out of these solutions into a reaction mixture containing 1.0 M KCL (Schwartz and Bonner, 1964).

Extracts from several of each of the mutant classes were tested for the tryptophan synthetic enzymes. In every case, only one enzymatic activity was absent. Representative results are shown in Table 1. Two of the class 3 mutants, T7 and T19, exhibited significantly decreased InGPS levels, while two other mutants, T3 and T24, showed inordinately high levels. The low enzyme levels may be attributable to antipolar effects, since both T7 and T19 (as well as T11 in the same class) are nonsense mutants by genetic criteria (C. Anagnostopoulos, unpublished results). We have no explanation at present for the high enzyme levels in strains T24 and T3. The absence of any of the pleiotropism shown by Whitt and Carlton (1967) may be a result of the

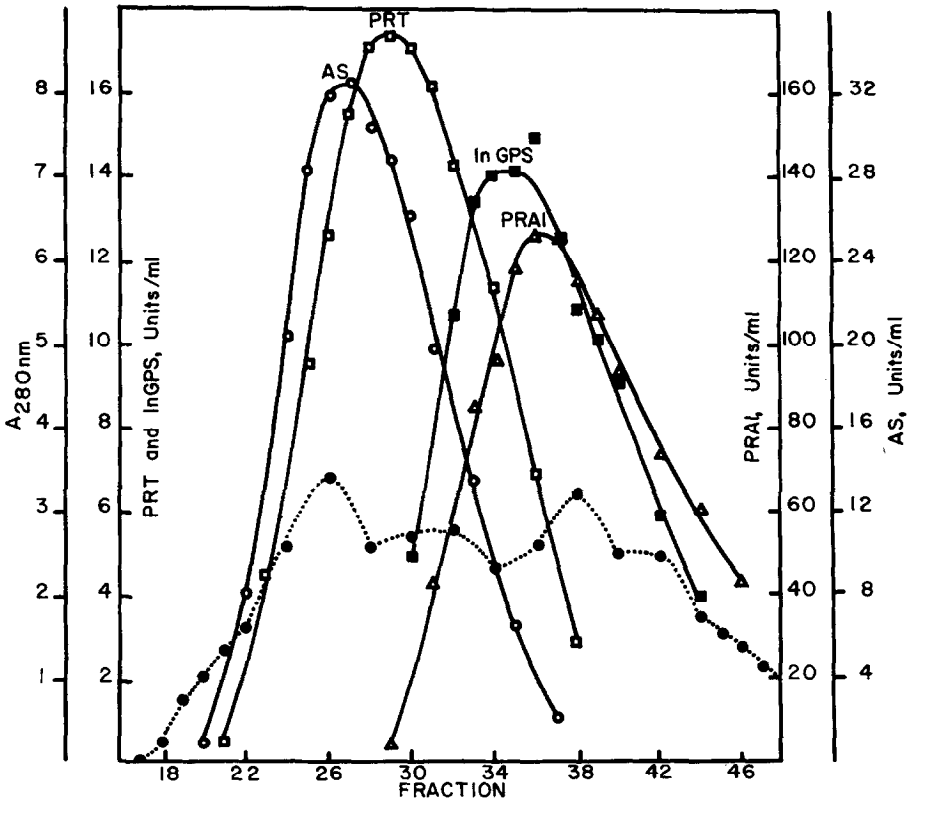

Figure 1. Gel filtration of a concentrated extract of B. subtilis T16 using a Sephadex G-100 column as described in Methods. The recovery of each activity was 56% or greater, based on the activity of a portion of the T16 extract held at 4 C for the same time period without being passed through the column.

differences in assay and extraction techniques. We have observed rapid loss of PRT and InGPS even in whole cell suspensions when the bacteria are held at 0-4 C.

Figure 1 shows the result of gel filtration of a concentrated extract of strain T16. The experiment was repeated several times using different tryptophan mutants and the average molecular weights observed were AS, 67,000; PRT, 57,000; PRAI, 27,000; InGPS, 30,500. The PRAI and InGPS peaks were clearly separable using extracts from either Class 3, 5a or 5b mutants, suggesting that these two reactions are not catalyzed by a single polypeptide chain as is the case in E. coli.

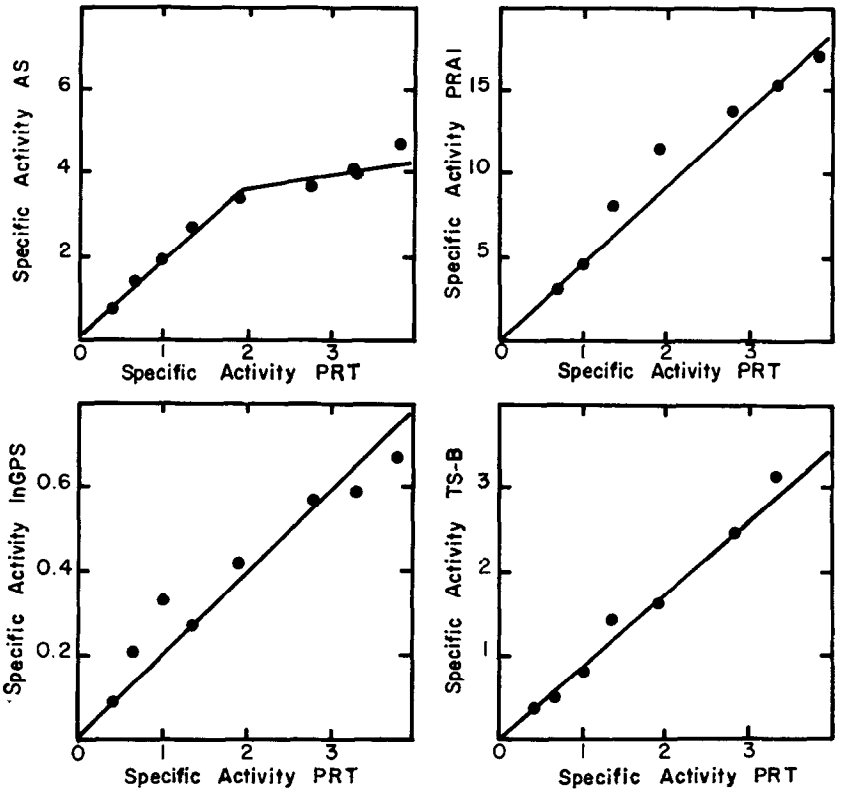

Figure 2. Coordinate variation of AS, InGPS, PRAI and TS-B with respect to PRT. Different enzyme levels were obtained as follows: Strain T16 PR was grown in minimal medium plus 0.5% glucose, 0.05% casein hydrolysate and 3 μg/ml tryptophan for 3.5 hours at 37C on a rotary shaker. The exponentially growing culture was harvested and washed with minimal medium. The washed cells were transferred to prewarmed minimal medium as above without tryptophan. At intervals during the next 2 hours samples were removed and harvested immediately in the cold but without allowing the samples to stand in an ice bath. The cells were resuspended in the 40% glycerol buffer described in Methods, extracted and assayed.

B. subtilis T16 PR, a bradytrophic revertant of T16, was grown under repressing conditions (excess tryptophan), then resuspended in medium lacking tryptophan. Samples were removed at intervals during a two hour period. The ratios of the specific activities of the tryptophan synthetic enzymes relative to PRT for one experiment have been plotted in Figure 2 according to the method of Ames and Garry (1958). The ratio for each pair of enzymes remains constant except in the case of AS. After 60 min the specific activity of AS relative to PRT decreases sharply. As B. subtilis is being grown under semistarvation for an amino acid, tryptophan, this decrease in the activity of the initial enzyme of the pathway may reflect the decision of the organism to sporulate. Other possibilities, notably inhibition or destabilization of the AS by some metabolite, have not yet been ruled out, however. In any event, the results of this derepression study strongly indicate the coordinate production of the tryptophan synthetic enzymes for at least 60 minutes after derepression.

Discussion. We were not able to demonstrate the existence of any enzyme aggregates among the first four enzymes of the tryptophan pathway in B. subtilis. An aggregate weak enough to be dissociated by passage over Sephadex would not have been revealed in our study, of course, but such an association can not be required for enzymatic activity or we would have seen low recoveries of the enzymes involved. The situation encountered in B. subtilis is analogous to that found in Chromobacterium violaceum (Wegman and Crawford, 1968) and Pseudomonas putida (Enatsu and Crawford, 1968). Also as in these two organisms, the proteins catalyzing the PRAI and InGPS reactions are physically separable, in contrast to the enteric bacteria where a single polypeptide chain catalyzes both activities. The presence in B. subtilis of two distinct enzymes to catalyze the PRAI and InGPS reactions is further substantiated by the normal InGPS activity levels present in mutant T11. This strain lacks PRAI as a result of a nonsense mutation. (C. Anagnostopoulos, unpublished results).

Enzymological similarities in various organisms do not always imply an identical genetic organization. The trp genes of P. putida have already been shown to be dispersed into three linkage groups having different regulatory modes within each group (Chakrabarty, Gunsalus and Gunsalus, 1968). In B. subtilis, on the other hand, a single linkage group is present, and the data presented in Figure 2 suggest that the production of five of the enzymes is coordinate, in the first hour of derepression at least, as though the trp cluster is an operon transcribed onto a single, polycistronic messenger RNA molecule.

In our first experiments we were unable to show that the production of PRT and InGPS was coordinate with the other enzymes. As these two activities were known to be quite labile, we searched for a physiological explanation and found that these enzymes are rapidly inactivated, even inside the cells, when cultures are chilled and held for a time before being centrifuged and resuspended in glycerol-containing buffer. We suggest that the relative decrease in AS production after 60 minutes may also have a physiological explanation. This is supported somewhat by our observation that the decrease is dependent on the time following transfer to the derepression medium rather than the actual AS enzyme levels; in a separate experiment we achieved higher AS levels during the 60 minute period of coordinate production.

Our results do not, of course, firmly establish that transcription of the trp genes invariably involves a complete polycistronic message. Additional genetic and enzymological experiments involving regulator gene and operator mutations, as well as structural gene nonsense mutations having polar and antipolar effects, should be performed.

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