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Isolation and Characterization of Two Tryptophan Biosynthetic Enzymes, Indoleglycerol Phosphate Synthase and Phosphoribosyl Anthranilate Isomerase, from *Bacillus subtilis*

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Two of the enzymes responsible for tryptophan biosynthesis in *Bacillus subtilis* have been extensively purified. These proteins are indole-3-glycerol phosphate synthase and *N*-(5'-phosphoribosyl) anthranilate isomerase. By comparison to the non-differentiating enteric bacteria in which these two enzymes are fused into a single polypeptide, the isolation of the indoleglycerol phosphate synthase and phosphoribosyl anthranilate isomerase from *B. subtilis* has demonstrated that the two proteins are separate species in this organism. The two enzymes were clearly separable by anion-exchange chromatography without any significant loss of activity. Molecular weights were determined for both enzymes by gel filtration and sodium dodecyl sulfate-slab gel electrophoresis, and indicated that the indoleglycerol phosphate synthase is the slightly larger of the two proteins. The minimum molecular weight for indoleglycerol phosphate synthase was 23,500, and that for phosphoribosyl anthranilate isomerase was 21,800. Both enzymes have been examined as to conditions necessary to achieve maximal activity of their individual functions and to maintain that activity.

The organization and control of the tryptophan biosynthetic pathway have been extensively investigated in both procaryotic and eucaryotic species, including heterotrophic and autotrophic bacteria, algae, yeast, fungi, protozoa, and angiosperms. In terms of intermediary metabolism, the pathway is an essential one and comparative studies reveal details of evolutionary divergence and adaptation. There are five sequential steps involved in the conversion of chorismate to tryptophan. All organisms studied used the same reaction sequence. There are, however, marked differences in the number and organization of the components used to effect this sequence, as well as of the genetic loci which code for these enzymes. A comprehensive review of this subject has recently appeared (4). The exploration of the tryptophan pathway has been most effective where a reliable mapping system allows correlation between the genetic and enzymological data. *Bacillus subtilis* has such a well-defined chromosomal map (25). Thus, the present study of *B. subtilis* indole-3-glycerol phosphate (InGP) synthase (EC 4.1.1.48) and *N*-(5'-phosphoribosyl) anthranilate (PRA) isomerase was initiated to characterize the enzymes from a gram-positive, differentiating organism. InGP synthase has previously been purified from the gram-negative enteric bacteria *Escherichia coli*, *Salmonella typhimurium*, and *Aerobacter aerogenes* (7, 18); from the fungus *Neurospora crassa* (14); and from the alga *Euglena gracilis* (11). Thus, it has only been isolated and characterized from those organisms in which it is fused to PRA isomerase (and to the anthranilate synthase G subunit in *N. crassa* and to phosphoribosyltransferase and tryptophan synthase in *Euglena*). The present study clearly demonstrates that the two enzymes InGP synthase and PRA isomerase are distinct and separable polypeptides in *B. subtilis*.

MATERIALS AND METHODS

Bacteria and growth. The bacterial strain used in the isolation of InGP synthase and PRA isomerase was *B. subtilis* T3, a *trpB* mutant (1). Strain T3 was grown in Spizizen minimal medium (21) containing 0.5% glucose, 0.05% acid-hydrolyzed casein (pH 7.0), and 1 μg of tryptophan per ml. The cells were grown for 12 to 16 h at 37°C with vigorous aeration either in a 150-liter fermentation tank or in Fernbach flasks on a rotary shaker. The cells were harvested, washed twice with 5 volumes of 1 M KCl to remove proteases (19), and stored at −20°C until preparation of the cell-free extract.

Enzyme assays. PRA isomerase was assayed as described by Crawford and Gunsalus (5) with the

Pseudomonas putida strain S-21 to generate the PRA substrate. InGP synthase was assayed by the method of Smith and Yanofsky (20) modified by using, in a 1-ml reaction volume, the following assay mix: 0.3 M potassium phosphate buffer (pH 7.8) containing 0.24 M sucrose, and approximately 1.5 mM 1-(*O*-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP). One unit of activity is defined as the disappearance of 1 μmol of PRA per min or the appearance of 1 μmol of InGP per min for the PRA isomerase and InGP synthase assays, respectively. Protein concentrations were determined colorimetrically by the method of Lowry et al. (17).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was carried out by the system of Laemmli (16); the gels were stained by the procedure of Fairbanks et al. (9). The gels were 1.5 mm thick. Samples were prepared by exhaustive dialysis against water or 10 mM sodium phosphate (pH 7.0) before lyophilization. The dried samples were suspended in 10 mM sodium phosphate (pH 7.0) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol, and then boiled for 3 min before application to the gel. Gel standards were the following proteins: conalbumin (molecular weight, 77,000); human immunoglobulin G heavy chain (molecular weight, 50,000); ovalbumin (molecular weight, 43,000); human immunoglobulin G light chain (molecular weight, 22,500).

Sephadex chromatography. Molecular weight determinations were made with Sephadex G-100 columns (1.6 by 60 cm) equilibrated in 0.2 M potassium phosphate (pH 6.6) containing 0.5 M sucrose, 1 mM 2-mercaptoethanol, and 1 mM EDTA. The upward flow of the column was regulated at 9 ml/h. The void volume was determined with blue dextran. The column was calibrated by the method of Whitaker (22) by use of three proteins of known molecular weight: conalbumin (77,000), ovalbumin (43,000), and chymotrypsinogen A (25,700).

Reagents. CDRP was chemically synthesized from anthranilic acid and ribose-5-phosphate (sodium salt) (Sigma Chemical Co.) by the procedure of Creighton and Yanofsky (8) and was assayed by its enzymatic conversion to InGP. Acrylamide was purchased from J. T. Baker Co. and recrystallized before use. Microgranular DEAE-cellulose (Whatman DE-52) and *O*-(carboxymethyl)-cellulose (Whatman CM-52) were purchased from Reeve Angel, and hydroxylapatite was from Bio-Rad. Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann. All other chemicals and reagents used were reagent grade or the highest grade available.

RESULTS

Purification of InGP synthase. For the isolation of InGP synthase, a strain of *B. subtilis* (T3) that contained elevated levels of this enzyme was selected from the collection of tryptophan mutants of Anagnostopoulos and Crawford (1). All operations were carried out in potassium phosphate buffers containing 0.8 M sucrose and 1 mM 2-mercaptoethanol (buffer A) or in potassium phosphate buffers containing 0.8 M sucrose, 1 mM 2-mercaptoethanol, and 1 mM EDTA (buffer B). The temperature was maintained at 4°C unless otherwise noted.

Step 1: extraction of enzyme. An 80-g amount of *B. subtilis* T3 was suspended in 3 volumes of 0.1 M buffer A (pH 7.8) containing 5 mM magnesium sulfate. The mixture was stirred at room temperature with a rotary blade until the cells appeared evenly suspended. A 1-mg quantity of lysozyme per g of cells and 20 μg of DNase and RNase per g of cells were added to the mixture, and the cells were incubated in a 37°C water bath with occasional stirring for 30 min. Twenty micrograms of DNase per g of cells was again added to the mixture, and incubation continued for 15 min. The cell debris was removed by centrifugation for 30 min at 43,500 × *g* in the Sorvall RC5 centrifuge.

Step 2: ammonium sulfate fractionation. The crude extract was cooled to 0 to 5°C in an ice-rock salt mixture. Solid, enzyme grade ammonium sulfate was added slowly to the crude extract to 50% saturation. The suspension was then centrifuged for 30 min at 13,200 × *g*. The precipitate was discarded. The supernatant was saturated with ammonium sulfate and then centrifuged for 30 min at 13,200 × *g*. The precipitate was suspended in a minimum volume (25 ml) of 0.1 M buffer B (pH 6.6) and dialyzed overnight against 80 volumes of this buffer.

Step 3: DEAE-cellulose chromatography I. The dialyzate was loaded onto a Whatman DE-52 column (4 by 22 cm) which had been equilibrated with the dialysis buffer. The column was washed extensively with this buffer. The protein adsorbed to the column was eluted with a linear salt gradient (1,700 ml; 0 to 0.3 M NaCl) in the wash buffer. Fractions of 10 ml were collected. InGP synthase was adsorbed to the DE-52 column and eluted at a concentration of NaCl of approximately 0.1 M (Fig. 1). There was no loss of InGP synthase activity at this step, but only those fractions with a specific activity of 1.0 or greater were pooled and dialyzed overnight against 50 volumes of 0.1 M buffer B (pH 6.6) containing 0.04 M NaCl.

Step 4: DEAE-cellulose chromatography II. The dialyzate was adsorbed onto a second DE-52 column (1.5 by 16 cm) which had been equilibrated in the dialysis buffer. After extensively washing the column, the adsorbed protein was eluted with a linear salt gradient (300 ml; 0.04 to 0.2 M NaCl) in the wash buffer. Fractions of 2 ml were collected. Again, there was no loss of activity; the three peak tubes, with specific activities greater than 3.5, contain 47% of this activity. The overall purification scheme is pre-

sented in Table 1.

Purification of PRA isomerase. Fortuitously, the enzyme PRA isomerase could be copurified with InGP synthase from *B. subtilis* T3 through the first two steps of the InGP synthase isolation scheme. The buffers and temperature conditions were the same as those described for the InGP synthase purification.

Step 3: DEAE-cellulose chromatography. The third step of the InGP synthase purification clearly separated the two enzyme activities. The PRA isomerase was not adsorbed to the DE-52 column, as seen in Fig. 1, and eluted as a single sharp peak of activity on the trailing edge of the flow-through protein fraction. Fractions with a specific activity of 0.5 or greater were pooled and dialyzed overnight against 50 volumes of 0.01 M buffer B (pH 6.5).

Step 4: *O*-(carboxymethyl)-cellulose chromatography. The dialyzate was loaded onto a Whatman CM-52 column (1.5 by 10 cm) which had been equilibrated in the dialysis buffer. The column was washed extensively with this buffer. The protein adsorbed to the column was eluted with a linear salt gradient (180 ml; 0 to 0.3 M NaCl) in the wash buffer. Fractions of 3 ml were collected. The PRA isomerase was weakly adsorbed, eluting at an NaCl concentration of approximately 0.02 M NaCl (Fig. 2). The overall purification scheme is presented in Table 1. At this stage of the purification, the InGP synthase had been purified 50-fold, and the PRA isomerase had been purified 250-fold. Yet the PRA isomerase preparation still contained other contaminating proteins.

Step 5: hydroxylapatite chromatography. The peak tubes from several PRA isomerase purifications were pooled and dialyzed overnight against 40 volumes of 0.01 M potassium phosphate (pH 6.5) containing 15% glycerol, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethyl-

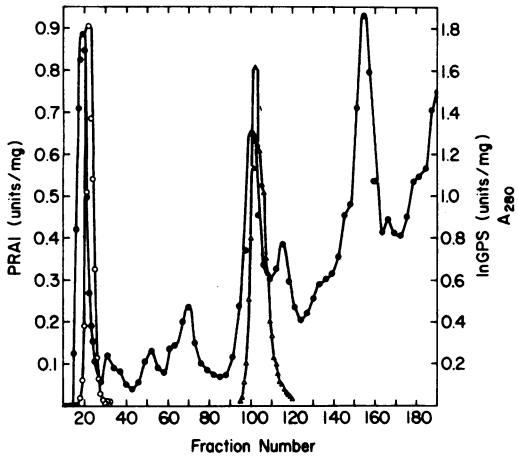

FIG. 1. *DEAE-cellulose chromatography of B. subtilis InGP synthase and PRA isomerase. A 750-mg amount of protein was applied to a DE-52 column (4 by 22 cm) equilibrated in 0.01 M potassium phosphate (pH 6.6) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.8 M sucrose. The linear gradient of 0 to 0.3 M NaCl was started at tube 26. Symbols: ●, absorbance at 280 nm (A_{280}); ○ PRA isomerase activity; △, InGP synthase activity.*

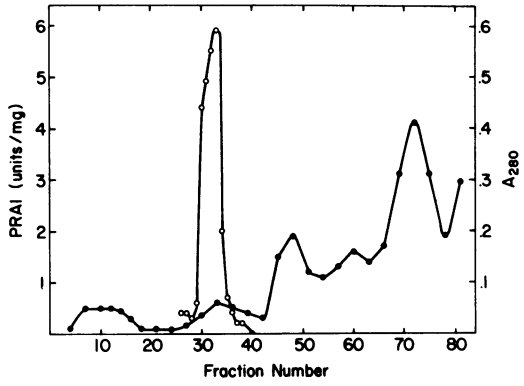

FIG. 2. *O-(carboxymethyl)-cellulose chromatography of B. subtilis PRA isomerase. A 28-mg amount of protein was applied to a CM-52 column (1.5 by 10 cm) equilibrated in 0.01 M potassium phosphate (pH 6.6) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.8 M sucrose. The linear gradient of 0 to 0.3 M NaCl was started at tube 19. Symbols: ●, absorbance at 280 nm (A_{280}); ○, PRA isomerase.*

TABLE 1. *Purification of InGP synthase and PRA isomerase from B. subtilis*

Procedure	Volume (ml)	Total protein (mg)	InGP synthase			PRA isomerase		
			Sp act (U/mg)	Total activity (U)	Yield (%)	Sp act (U/mg)	Total activity (U)	Yield (%)
1. Crude extract	234	1921	0.072	138	100	0.020	37.5	100
2. 50% saturation ammonium sulfate precipitation	45	750	0.097	73	53	0.026	19.8	54
3. DEAE-cellulose chromatography I	57	23	1.50	34.6	25			
	35	28				0.82	23.3	62
4. DEAE-cellulose chromatography II	9	4.4	3.70	16.1	12			
5. CM-cellulose chromatography	12	1.9				5.15	9.79	26

sulfonylfluoride, and 0.1 M NaCl. The dialyzate was adsorbed onto a Bio-Rad HT column (1.5 by 5 cm) which had been equilibrated with the dialysis buffer. The column was washed in succession with 1.5 column bed volumes (13 ml) of the same buffer containing potassium phosphate at concentrations of 0.05, 0.10, and 0.15 M. Fractions of 1.5 ml were collected. The PRA isomerase first eluted at the end of the 0.10 M wash and throughout the 0.15 M wash. The plateau of peak activity had an average specific activity of 6 U/mg.

Stabilization of InGP synthase and PRA isomerase. Previous studies on the extraction of InGP synthase activity had indicated that InGP synthase was stabilized by sucrose at an optimal concentration of 0.8 M but only in the presence of a potassium phosphate buffer (12). Glycerol was not used because it interfered with the InGP synthase assay. The purified InGP synthase was also tested for its sucrose requirements. Samples were diluted into 0.1 M potassium phosphate (pH 6.6), containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and varying amounts of sucrose (0.1 to 0.8 M). The samples were held at 4°C and periodically tested for activity in the usual manner (Fig. 3). After 15 days at this temperature, 80% of the InGP synthase activity was retained in the presence of 0.8 M sucrose. Below this concentration there was a precipitous drop in InGP synthase activity with a first-order relationship between the residual activity and the sucrose concentration. Sucrose was not nearly as effective in maintaining the PRA isomerase activity. There was a 20% loss of activity after 7 days at 4°C in the presence of 0.8 M sucrose, and a 65% loss by 15 days. For this enzyme, the high osmolarity additive glycerol was the most effective in maintaining activity. From days 6 through 15 at 4°C, an average of 76% of the original activity was retained when 10 to 15% glycerol (vol/vol) was added to the buffer. Based on these results, PRA isomerase is now equilibrated in buffers containing 15% glycerol after its separation from InGP synthase by the DEAE-cellulose chromatography.

Assay conditions. Although the InGP synthase assay was patterned on the one devised for *E. coli* in measuring InGP production, the reaction conditions were maximized for the *B. subtilis* enzyme. A monovalent cation (potassium, sodium, or ammonium) was required at a concentration of 0.2 M or greater, necessitating the use of potassium phosphate buffer (pH 7.8). Sucrose was routinely included at 0.24 M for its slight stimulatory effect, although a much lower concentration was required than for the long-term stabilization of InGP synthase. The apparent K_m for the substrate CDRP was 59 μM, a 10-fold-higher value than that determined for the *Escherichia coli* enzyme (7, 10). Thus, the high CDRP substrate concentration in routine assays precluded the use of a spectrophotofluorimetric assay for the *B. subtilis* InGP synthase (10) because the product InGP fluorescence is strongly quenched at the CDRP concentrations. Optimal assay conditions for the *B. subtilis* PRA isomerase were essentially the same as described for the *Pseudomonas putida* enzyme (5).

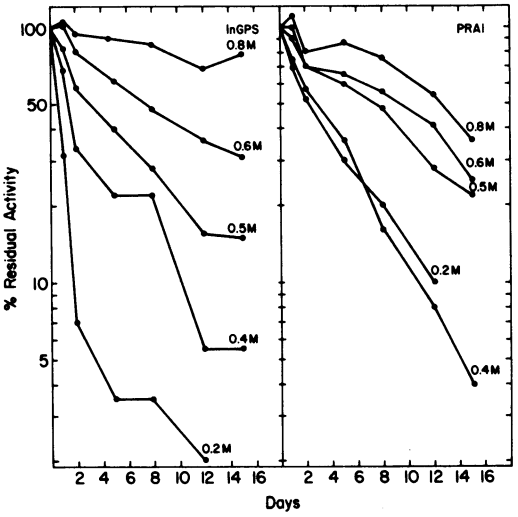

FIG. 3. *Sucrose stabilization of InGP synthase and PRA isomerase. InGP synthase (35 μg) was diluted into a 0.4-ml final volume of 0.1 M potassium phosphate (pH 6.6) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and varying amounts of sucrose (as indicated on the graph). The samples were held at 4°C, and the residual activity is expressed as the percentage of activity of the original sample in 0.8 M sucrose. PRA isomerase (16 μg) was measured in an identical protocol.*

Molecular weight determinations. The minimum molecular weights for the two proteins were determined by sodium dodecyl sulfate-slab gel electrophoresis. The average molecular weight of InGP synthase obtained from several determinations was 23,500 ± 580. The value obtained for PRA isomerase was 21,800 ± 660. Since the calculated difference in molecular weight for the two proteins was quite small, both proteins were simultaneously subjected to sodium dodecyl sulfate-slab gel electrophoresis to verify this difference under identical experimental conditions. The results are seen in Fig. 4. The minimum molecular weight for InGP synthase is clearly larger than that of PRA isomerase.

The two proteins were also subjected to gel filtration on a Sephadex G-100 column containing 0.5 M sucrose to determine their native molecular weight (Fig. 5). By using several dif-

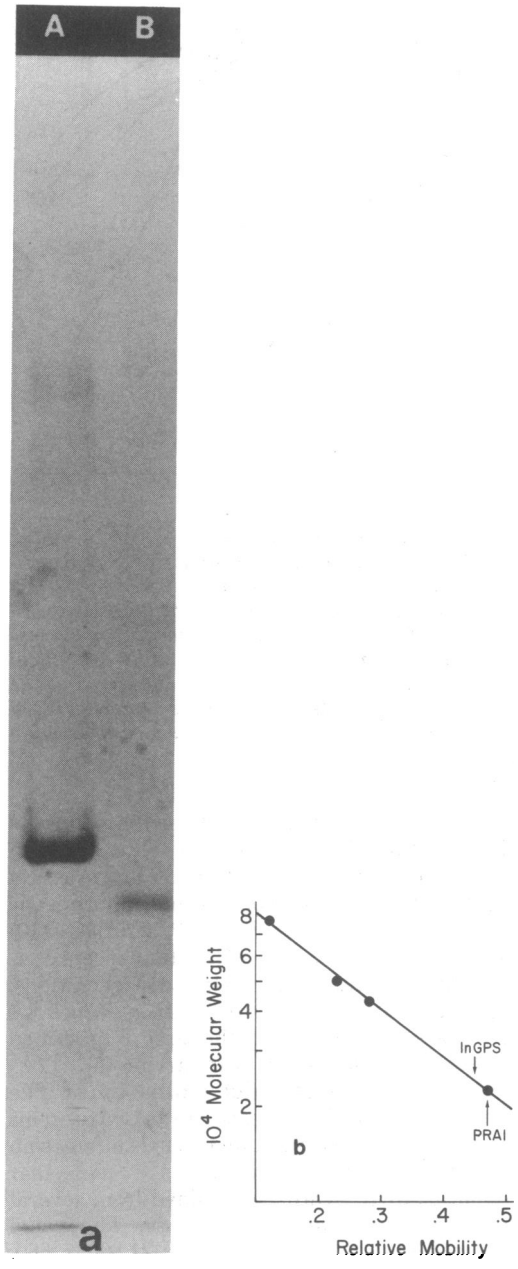

FIG. 4. *Minimum molecular weight determination of PRA isomerase and InGP synthase by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. (a) Left lane (A) InGP synthase (8 μg); right lane (B) PRA isomerase (13 μg). The gel concentration was 12.5%. (b) The molecular weight standards (●) used were: conalbumin, 77,000; IgG heavy chain, 50,000; ovalbumin, 43,000; IgG light chain, 22,500.*

ferent samples of purified enzyme, the molecular weight of PRA isomerase was determined to be 26,300 ± 770, and that of InGP synthase was found to be 31,100 ± 1,100. These values are in very close agreement with the molecular weights determined previously for these two enzymes with a crude extract of *B. subtilis* T16 (12). Again, the InGP synthase is clearly larger than and separable from PRA isomerase.

DISCUSSION

Many of the problems initially associated with the characterization of the tryptophan biosynthetic enzymes in *B. subtilis* (12, 23) appear to be attributable to the general lability of these proteins during isolation. However, use of the high osmolarity buffer additives glycerol or sucrose has now facilitated the substantial purification of six of the seven polypeptides required for tryptophan biosynthesis in this organism, including InGP synthase and PRA isomerase as discussed in this paper. The first attempt to determine whether these two activities resided on separate polypeptides made use of relatively crude extracts of *B. subtilis*. Gel filtration experiments indicated slightly different molecular weights as had been originally indicated for the *E. coli*-fused activities (2). Similar experiments with two other species of *Bacillus*, *B. pumilus* and *B. alvei*, indicated virtually identical molecular weights for the two enzymes (13), necessitating the substantial purification of the two

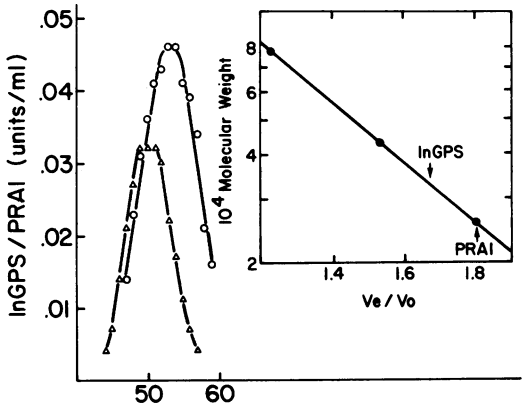

FIG. 5. *Molecular weight determination of PRA isomerase and InGP synthase by Sephadex G-100 chromatography. The column (1.6 by 60 cm) was equilibrated in 0.2 M potassium phosphate (pH 6.6) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.5 M sucrose. Symbols: ○, PRA isomerase activity; △, InGP synthase activity. Inset: Whitaker plot of molecular weight versus the ratio of elution volume to void volume. The void volume was determined with blue dextran; the molecular weight standards used were: conalbumin (77,000); ovalbumin (43,000); chymotrypsinogen A (25,700).*

enzyme activities to resolve the question. This purification clearly indicated that the two enzyme activities were separable by demonstrating: (i) a basic difference in the net charge of the two proteins as judged by their adsorption properties upon ion-exchange chromatography; (ii) significant differences in their minimum molecular weights as determined by sodium dodecyl sulfate-gel electrophoresis; and (iii) no appreciable loss of either activity upon the physical separation of the two polypeptides. It is interesting to note that the sum of the subunit molecular weights of the two *B. subtilis* enzymes is identical to that of the single polypeptide catalyzing these reactions in *E. coli* and that the activity levels of the purified enzymes are comparable (7). Moreover, the orientation of the contiguous *trpC* and *trpF* genes in *B. subtilis* (3) is the same as for the independent InGP synthase and PRA isomerase domains of the *E. coli trpC* gene (24). It is plausible that the fused InGP synthase-PRA isomerase from *E. coli* and the respective independent species from *B. subtilis* might share two common progenitors based on their observed similarities. There is no obvious catalytic advantage to the fused enzyme in the enteric bacteria since the active sites reside in separate domains, with CDRP as a free intermediate (6, 15). By analogy to this situation, we postulated that complex formation between the *B. subtilis* enzymes might occur without any obvious effect on activity. Efforts to provide evidence of such a physical association with the gel filtration columns have met with negative results to date (S.O. Hoch, unpublished data).

There does not appear to be an obvious progression in the tryptophan biosynthetic pathway from independent protein entities through multienzyme complexes to multifunctional polypeptides as one moves up the evolutionary scale (4, 15). But diversity exists, as is apparent just from a comparison of the properties of the PRA isomerase and InGP synthase from different organisms. Now the isolation of the *B. subtilis* InGP synthase will also enable us to explore the apparent evolution of the enzyme within a single genus, albeit a large and heterogeneous one, as a study of the tryptophan enzymes in three *Bacillus* species indicated a greater than 100-fold divergence in median activity level of the InGP synthase among the three species (13).

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