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Metabolic Interlock

THE DUAL FUNCTION OF A FOLATE PATHWAY GENE AS AN EXTRA-OPERONIC GENE OF TRYPTOPHAN BIOSYNTHESIS*

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SUMMARY

A mutation denoted as *trpX* defines a previously unrecognized cistron whose gene product, subunit-X, has a catalytic role in two biochemical pathways. Experiments with strains carrying this mutation provided insight into the following gene-enzyme relationships in *Bacillus subtilis*.

1. The *trpX* locus codes for a subunit component (subunit-X) of the anthranilate synthase aggregate which appears to confer catalytic reactivity with glutamine.

2. Subunit-X is also a component of *p*-aminobenzoate synthase in the pathway of folate biosynthesis.

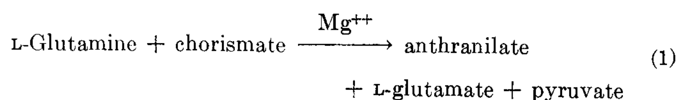
3. A mutation of *trpX* is not located in the previously described tryptophan operon, showing no linkage by DNA transformation or PBS1 transduction to the various genes located in or near the tryptophan gene cluster of *B. subtilis*. However, the synthesis of subunit-X is controlled by tryptophan. The synthesis of subunit-X varies coordinately with respect to the other enzymes of the tryptophan operon throughout a large fraction of the time course of the derepression interval; subunit-X is synthesized discoordinately, however, at both very low and very high enzyme levels (semicoordinate repression).

A mutant which synthesizes a faulty subunit-X requires a derepressed level of subunit-X in order to synthesize sufficient folate and tryptophan to support growth. Exogenous tryptophan inhibits growth in the mutant because the repression of the synthesis of an already deficient subunit-X by tryptophan results in folate starvation. The mutant enzyme cannot utilize glutamine, the normal physiological substrate, as a nitrogen source in the anthranilate synthase reaction. However, the mutant enzyme retains a substantial level of ammonia-reactive activity, a result which provides suggestive evidence that ammonia may serve *in vivo* as an amino donor for tryptophan synthesis.

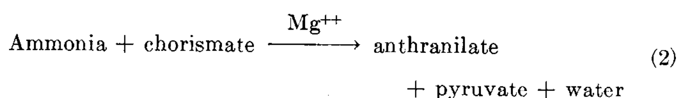
In *Bacillus subtilis* anthranilate synthase is a molecular complex composed of two nonidentical subunits (1). All tryptophan auxotrophs known to lack activity for anthranilate synthase display a modification in subunit-E, the gene product of *trpE*. We recently described another mutant class (*trpX*) which genetically defines another component of anthranilate synthase, subunit-X (1). Subunit-X was characterized as a small protein (molecular weight: approximately 16,000) which was stable to partial purification and whose synthesis was repressible by tryptophan. Crude extracts made from cultures of *trpX* and *trpE* mutants were able to complement one another, thereby reconstituting activity for anthranilate synthase (utilizing glutamine as a substrate reactant). No *trpX* mutants are known to have a phenotype of tryptophan auxotrophy. Apparently the altered protein complex for anthranilate synthase in *trpX* mutants can utilize ammonia directly in an amination reaction under ordinary physiological conditions of growth.

The original observations relating to subunit-X were made in a genetic background of constitutivity (*trpR*) for the synthesis of the tryptophan biosynthetic enzymes (1). It was of interest to characterize the *trpX* mutation by separating it from the *trpR* regulatory mutation. This was accomplished genetically by deoxyribonucleate-mediated transformation. The characteristics of this *trpX* isolate revealed the dual role of *trpX* in tryptophan and folate synthesis. Subunit-X is shown in this communication to be a common subunit for anthranilate synthase and for PABA[1] synthase. These reactions are shown in Fig. 1. Both catalytic steps utilize chorismate and glutamine as substrates. The reaction products, *p*-aminobenzoate and anthranilate(*o*-aminobenzoate), are chemically similar. Both reactions are complex, involving the loss of an enolpyruvyl side chain, the loss of a hydroxyl substituent, the addition of an amide group, and the formation of a fully aromatic ring.

Anthranilate synthase of *B. subtilis* can undergo two reactions *in vitro*.



$$\text{L-Glutamine} + \text{chorismate} \xrightarrow{Mg^{++}} \text{anthranilate} + \text{L-glutamate} + \text{pyruvate} \quad (1)$$



$$\text{Ammonia} + \text{chorismate} \xrightarrow{Mg^{++}} \text{anthranilate} + \text{pyruvate} + \text{water} \quad (2)$$

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[1] The abbreviations used are: PABA, *p*-aminobenzoate; PR transferase, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase.

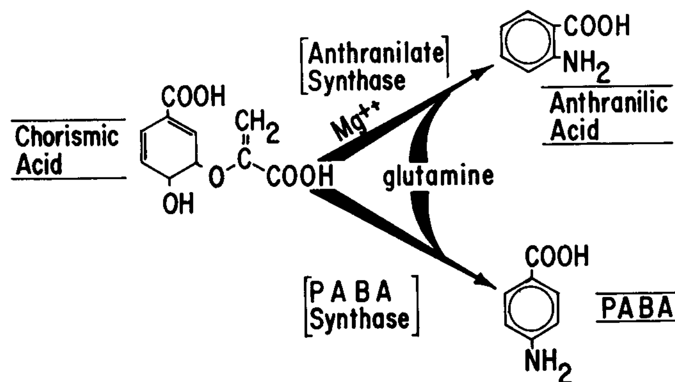
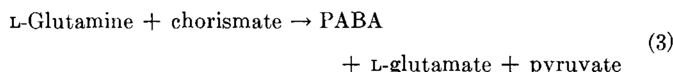


FIG. 1. Chorismate, a multifunctional metabolite serving as substrate for the biosynthesis of PABA and anthranilate. The corresponding end products are folate and tryptophan, respectively. Others of the multiple biochemical fates for chorismate, the major metabolic branch point of aromatic synthesis, were described in detail recently by Young *et al.* (2).

Reaction 3 is that of PABA synthase.



L-Glutamine + chorismate → PABA + L-glutamate + pyruvate (3)

It has not yet been determined in any system whether a fourth reaction occurs, analogous to Reaction 2 above, and catalyzed by PABA synthase (namely, the amination reaction with ammonia as a nitrogen source in the formation of PABA).

MATERIALS AND METHODS

Growth Conditions—The *B. subtilis* isolates used in this study, all derivatives of strain 168 (3), are described and referenced in Table I. Unless otherwise indicated, cultures were grown with vigorous rotary aeration at 37° in 200 ml of minimal medium (9) supplemented with glucose to a final concentration of 0.5%. Culture media were supplemented with any indicated amino acids to a final concentration of 50 μg per ml each.

Enzymological Procedures—Cells used in the assay of enzymes of tryptophan synthesis were suspended in 0.04 M potassium phosphate buffer, pH 7.8, containing 0.5 M KCl, 0.1 mM EDTA, 6 mM β-mercaptoethanol, and 30% glycerol. Extracts were prepared as previously described (5). The conditions for the assay of the activity of anthranilate synthase in *B. subtilis* using glutamine (5) or ammonia (1) as the nitrogen donor have been reported. The preparation of extracts and the assay conditions used in the measurement of PR transferase were previously described (5). The procedure for gel filtration using Sephadex G-100 has also been described (1). Tryptophan synthase B was assayed at 37° for 5 or 30 min, depending upon the level of activity, in a reaction mixture containing 88 nmoles of indole, 10 μmoles of L-serine, 25 nmoles of pyridoxal phosphate, 250 nmoles of EDTA, 500 μmoles of potassium phosphate buffer (pH 7.8), and 5 μmoles of KCl in a final reaction volume of 0.5 ml. Indole concentrations were determined by the method of Meduski and Zamenhof (10).

The activity of cytidine triphosphate synthetase was assayed according to the method of Long and Pardee (11). Cells were disrupted by treatment in the presence of 100 μg of lysozyme per ml and 10 μg of deoxyribonuclease per ml (20 min at 37°). The suspending buffer consisted of 0.04 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 2 mM L-glutamine, and 6 mM β-mercaptoethanol. Debris was sedimented by low speed centrifugation and the crude extract was passed through a Sephadex G-25 column. This crude preparation gave unsatisfactory results in the assay of cytidine triphosphate synthetase. Solid $(NH_4)_2SO_4$ (enzyme grade) was added slowly with gentle stirring in a 4° cold room to 50% of saturation at 4°. Stirring was continued for 20 min. The protein fraction precipitating at 0 to 50% of $(NH_4)_2SO_4$ saturation was collected by low speed centrifugation and resuspended in buffer at 4°. After 10 min on ice, a faint sediment was removed by centrifugation. This preparation was a satisfactory source of enzyme activity. Under conditions of proportionality with respect to reaction time and protein concentration, the specific activities of isolate I-12 and I-15 were 1.50 and 2.06 nmoles per min per mg of extract protein at 37°.

PABA synthase was assayed in extracts prepared in 0.05 M-*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate buffer, pH 8.0, containing 30% glycerol, 6 mM β-mercaptoethanol, and 0.1 mM EDTA. Cells were harvested in the late exponential phase of growth and lysed as previously described (5). The reaction mixture contained 4 mg of extract protein, 25 μmoles of L-glutamine, and 0.3 μmole of chorismate in a final volume of 0.4 ml. The enzyme activity was assayed for 30 min at 37° and the reaction was stopped with 0.1 ml of 1 N HCl. The PABA was extracted into 1.5 ml of ethyl acetate and measured fluorometri-

TABLE I
Strains of B. subtilis

Collection number	Genotypic deficiency[a]	Phenotypic description
NP 40		Hybrid strain prototroph.[b]
NP 42		Prototrophic derivative (4) of strain 168.
NP 100	*trpR2*	5-methyltryptophan-resistant; partial growth requirement for phenylalanine (4–7).
I-15	*trpR2 trpX7*	5-methyltryptophan-resistant; prototrophic (1).
I-12	*trpR2 trpE8*	Prototroph having leaky block in anthranilate synthase (1).
NP 102	*trpX7*	Prototroph; growth is inhibited by tryptophan; hypersensitive to 5-methyltryptophan; hypersensitive to sulfathiazole.
E-35	*tyrA1*	Tyrosine auxotroph blocked in prephenate dehydrogenase. Synonymous with SB 103 (8).
NP 19	*trpC2*	Tryptophan auxotroph (168) blocked in indoleglycerol phosphate synthetase (3).
E-78	*trpE1*	Tryptophan auxotroph blocked in anthranilate synthase (subunit-E).
C-4	*pabA4*	Auxotroph derived from strain 23 requiring either PABA or folate for growth.

[a] *trpR*: mutation to constitutivity of enzyme synthesis in the tryptophan pathway; it is linked but not contiguous to the tryptophan gene cluster (8) and presumably codes for the aporepressor entity. *trpX*: structural gene for subunit-X of anthranilate synthase; unlinked to cluster of other tryptophan structural genes; molecular weight, 16,000 (1).

[b] Hybrid of strains 168 and 23 carrying the gene for the chorismate mutase of strain 23 in the genetic background of strain 168 (4).

cally as described by Huang and Gibson (12). Isolate NP 102, prior to assay for PABA synthase, was grown in minimal medium to a turbidity at 600 nm of 0.5 at 37°. Tryptophan was then added to a final concentration of 25 μg per ml. The culture was harvested at a turbidity of 1.3 at 600 nm. Other strains were grown in the presence of tryptophan from the time of inoculation. Under these conditions the activity of anthranilate synthase was repressed, allowing the assay of PABA synthase (12).

Protein concentrations in extracts were determined by the method of Lowry *et al.* (13).

Derepression Studies—Isolate E-78 was grown in 1200 ml of minimal salts medium containing 0.5% glucose and 5 μg of L-tryptophan per ml at 37° to an A_{600} of 1.0. These cells were centrifuged, washed in 150 ml of minimal salts medium, centrifuged again, and resuspended in 1500 ml of minimal salts medium (at 37°) containing 0.5% glucose and 0.1% vitamin-free casein hydrolysate (acid-hydrolyzed; Calbiochem). A sample of 150 ml was taken prior to resuspension in casein hydrolysate in order to establish a zero point. Subsequently, 150-ml samples of culture incubating at 37° were removed every 15 min. The cells were immediately resuspended in buffer and lysed as previously described (5). The extracts were passed through Sephadex G-25 prior to enzyme assay.

The specific activity of the glutamine-reactive anthranilate synthetase in crude extracts varies disproportionately as a function of protein concentration (note Fig. 8). Valid comparisons of specific activity require the presence of excess subunit-X since conditions of proportionality are only obtained when saturating concentrations of subunit-X are used. Unless otherwise indicated, the specific activities cited in the text are determined by assay with excess subunit-X. Under these conditions the specific activities are constant and independent of protein concentration.

DNA Transformation—Genetic mapping studies were done by DNA transformation with limiting concentrations of donor DNA by methods previously described (9).

Genetic separation of *trpR2* and *trpX7* by DNA-mediated transformation was used to separate by genetic recombination the loci coding *trpR2* and *trpX7* in isolate I-15. Donor deoxyribonucleate was extracted from a culture of isolate I-15. A tyrosine auxotroph (E-35), carrying a mutation in the gene of prephenate dehydrogenase (which maps adjacent to the tryptophan cluster (8)), was used as a recipient in the cross. In contrast to ordinary mapping procedure, high concentrations of donor DNA (about 20 μg per ml) were used to cotransfer the unlinked donor markers *tyrA1*$^+$ and *trpX7* (*i.e.* by congression) to the recipient strain. Prototrophic transformants were recovered on minimal glucose media with independence of the tyrosine requirement serving as the selective marker. Subsequent replica plating results revealed a rare *tyr*$^+$ recombinant (1/300), recognizable by its 5-methyltryptophan hypersensitive phenotype. This recombinant received the unlinked *tyrA1*$^+$ and *trpX7* loci of the donor as independent recombinational events, retaining the *trpR*$^+$ allele of the recipient strain. The latter *trpX7* recombinant was numbered NP 102.

Tryptophan Excretion—Tryptophan excretion was determined by taking 2-ml samples at various times during growth. After determining turbidity at 600 nm, cell suspensions were clarified by centrifugation and 0.1 to 0.5 ml of supernatant was added to 0.5 M glycine buffer at pH 11 to give a final volume of 1 ml. The samples were assayed for tryptophan concentration in an Aminco-Bowman spectrophotofluorometer at an excitation wave length of 282 nm and an emission wave length of 355 nm (both uncorrected). All strains except isolate NP 100 were grown in an unsupplemented minimal salts-glucose medium (9). Phenylalanine at a concentration of 50 μg per ml was included in the medium for the growth of isolate NP 100. The presence of phenylalanine did not significantly influence the level of tryptophan excretion by the other strains.[2]

TABLE II

Characterization of anthranilate synthase in mutant strains

Collection number	Genotypic deficiency	Growth supplement[a]	Specific activity of anthranilate synthase[b]	
			Glutamine-reactive	Ammonia-reactive
NP 42			0.60	0.08
		Aro	<0.01	<0.01
NP 100	*trpR2*	Phe	11.60	2.59
		Aro	10.60	2.47
I-15	*trpR2 trpX7*		<0.01	2.50
		Aro	<0.01	2.31
NP 102	*trpX7*		<0.01	1.86
		Aro	<0.01	<0.01

[a] All strains were grown in minimal salts medium plus 0.5% glucose (9). Phe refers to supplementation of minimal medium with 50 μg of phenylalanine per ml. Aro refers to supplementation with 50 μg each of tryptophan, tyrosine, and phenylalanine per ml and 0.5 μg each of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 3,4-dihydroxybenzaldehyde per ml.

[b] Specific activity is defined as the nanomoles of anthranilate produced per min per mg of protein. The sensitivity of the assay is sufficient to measure specific activities as low as 0.01. The value, Δ activity to Δ protein, was used in the calculation of specific activities (see legend of Fig. 7).

PABA Excretion—Cultures to be examined for PABA excretion were grown in 10 ml of minimal glucose medium in 125-ml side arm flasks. Growth was monitored on a Spectronic 20 equipped with a red filter. At an A_{600} of 0.5 the cells were centrifuged. One milliliter of 1 N HCl was added to the supernatant. The PABA was extracted with 10 ml of ethyl acetate. Authentic PABA was used as a standard and its concentration was related to fluorescence measurements taken by using an excitation wave length of 282 nm and an emission wave length of 338 nm (both uncorrected). The PABA excretion is expressed as nmoles per 2 ml of ethyl acetate.

Chemicals—All chemicals were of the best reagent grade commercially available. The preparation and use of barium chorismate has been described (5). DL-5-Methyltryptophan and all amino acids were purchased from Calbiochem. 1,2,4-Triazole 3-alanine was donated by Dr. Robert Guthrie. All other biochemicals were obtained from Sigma.

RESULTS

trpX-Anthranilate Synthase Relationship—The comparative data in Table II illustrate the effect of the mutation at the *trpX* locus upon the catalytic properties of anthranilate synthase. The protein of wild type strains of *B. subtilis* is catalytically

[2] J. F. Kane, unpublished results.

reactive with either glutamine or ammonia as a substrate, hereafter designated as the glutamine-reactive or the ammonia-reactive activities, respectively. Reference prototroph NP 42 possesses a low specific activity for anthranilate synthase during growth in a minimal glucose medium, and this is further repressed beyond the level of assay sensitivity when growth occurs in media containing tryptophan. Constitutive regulatory mutants of the *trpR* type show the very substantial range of derepression which also characterizes a number of other microbial systems of tryptophan biosynthesis.

Mutations which lie in *trpE* result in a failure to synthesize a normal subunit-E in *B. subtilis*, uniformly abolishing both the glutamine and ammonia-reactive activities. In contrast, the mutation in *trpX* (I-15 and NP 102) differentially reduces the glutamine-reactive activity, leaving the ammonia-reactive activity nearly unaltered (Table II). The results obtained with isolate NP 102 show that although the synthesis of anthranilate synthase is clearly repressible in the presence of aromatic end products, it differs from the wild type parent strain in its physiological derepression during growth in minimal glucose media lacking tryptophan.

Prototrophic Phenotype of trpX-deficient Mutants—The growth data given in Fig. 2 show that isolate NP 102 is not auxotrophic in phenotype. It grows as well as reference prototroph NP 42 in a minimal salts-glucose medium. As a matter of fact, the presence of the *trpX* locus leads to the suppression of the partial phenylalanine auxotrophy of isolate NP 100. Mutant NP 100 is a regulatory mutant (*trpR*) which synthesizes tryptophan-specific enzymes consitutively. Accordingly, it excretes large quantities of tryptophan (5, 7). Since tryptophan is a potent inhibitor of prephenate dehydratase, the mutant requires phenylalanine in order to sustain optimal rates of growth (6). The *trpX7* locus in isolate I-15 suppresses the partial phenylalanine requirement characteristic of isolate NP 100 by decreasing the rate of anthranilate formation and ultimately of tryptophan. Experimental verification of the negative influence of the *trpX7* mutation upon tryptophan production is given in Fig. 3 where comparative data are shown for the four culture isolates used in the growth experiments of Fig. 2. Cultures of isolate NP 100 excrete large quantities of tryptophan. Suppression of the partial phenylalanine auxotrophy of isolate NP 100 by the presence of *trpX7* (I-15) correlated with a marked diminution of tryptophan excretion. Mutant I-15 still excreted about 3-fold more tryptophan than did reference prototroph NP 42 (Fig. 3, *inset*). Therefore, complete derepression of the ammonia-reactive anthranilate synthase activity more than compensated for the *trpX* defect. In the absence of the *trpR* allele, the presence of *trpX* (*i.e.* NP 102) abolished any detectable excretion of tryptophan in the growth supernatant. The range of tryptophan excretion, comparing mutants NP 100 and 102, varied by a factor of 250.

Tryptophan-sensitive Phenotype of trpX-deficient Mutants—The initial observation implicating the role of subunit-X in two biosynthetic pathways was made with the *trpX* mutant NP 102. Not only is mutant NP 102 nutritionally independent of tryptophan, but its growth is inhibited by tryptophan. Concentrations of tryptophan as low as 2 μg per ml produce a maximal and sustained inhibitory effect (Fig. 4). Lower concentrations of tryptophan are also effective but the duration of inhibition is transient, presumably due to the eventual metabolism of small amounts of tryptophan. The onset of inhibition in the presence of tryptophan is slow, becoming apparent after about one generation. The subsequent decrease in growth rate is progressive, and growth is eventually inhibited completely. This pattern of growth inhibition is suggestive of a mechanism involving repression by tryptophan of the synthesis of an enzyme required at derepressed levels for normal growth. Either PABA or folate completely reversed inhibition of growth by tryptophan, a result implicating interference by tryptophan with folate bio-

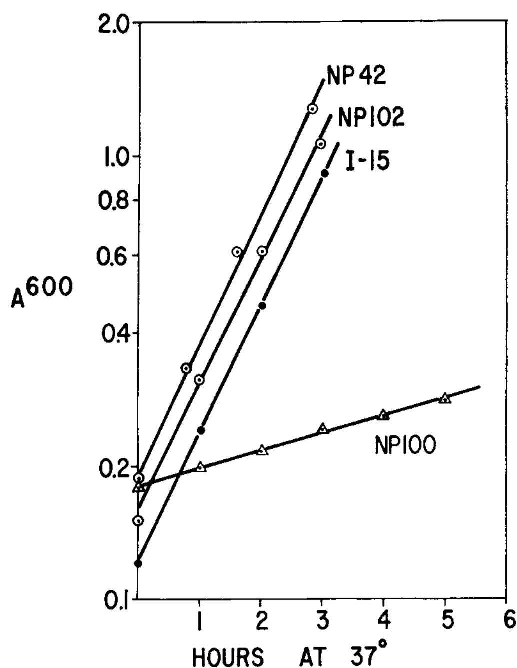


FIG. 2. Comparison of growth rates for isolates NP 42, NP 100, NP 102, and I-15. Cultures were grown in minimal medium containing 0.5% glucose at 37° with vigorous shaking. Samples were taken at hourly intervals for the measurement of turbidity at 600 nm with a Gilford 240 spectrophotometer.

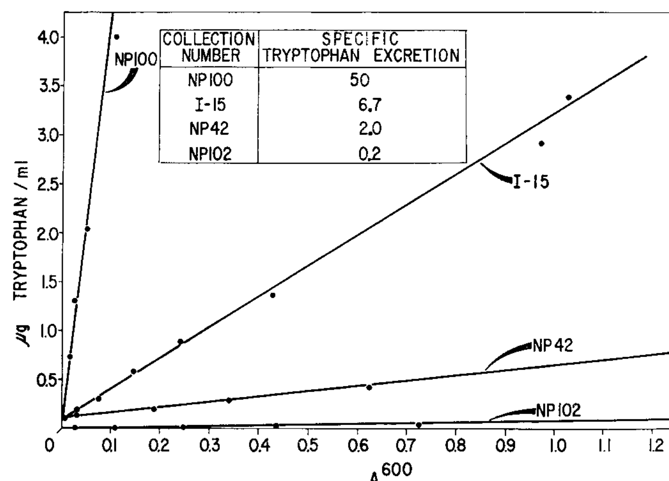


FIG. 3. Comparison of tryptophan excretion by isolates NP 42, NP 100, NP 102, and I-15. Cultures were grown as described in the text. Tryptophan concentrations were estimated by fluorometric measurements (see "Materials and Methods"). The concentration of tryptophan found in the culture medium is plotted as a function of cell density. The data in the *inset* are values of specific tryptophan excretion calculated for cultures of each strain. Specific tryptophan excretion is defined as micrograms of tryptophan per mg, dry weight, per hour.

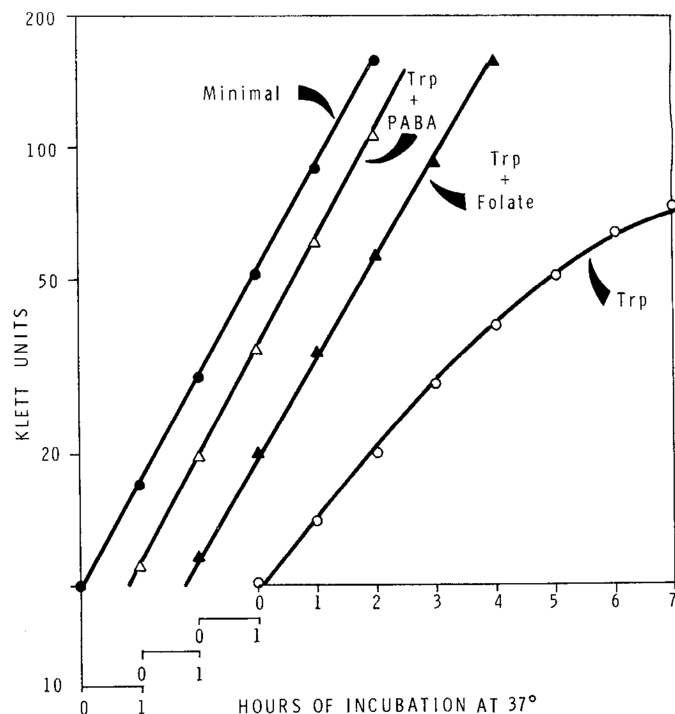


FIG. 4. Reversal of growth inhibition caused by tryptophan with PABA and folate in mutant NP 102. Isolate NP 102 was grown overnight in a minimal glucose medium. This culture was used as an inoculum into a fresh minimal glucose medium several hours before the experiment was begun. The various culture flasks were supplemented as follows: ●——●, none; ○——○, 20 μg per ml of L-tryptophan; △——△, 20 μg per ml of L-tryptophan plus 2.5 μg per ml of PABA; and 20 μg per ml of L-tryptophan plus 4.0 μg per ml of folate, ▲——▲. Turbidity was determined with a Klett-Summerson colorimeter (red filter). The time course and extent of inhibition by tryptophan were the same at 2 μg per ml as at 20 μg per ml.

synthesis in the mutant. Other aromatic compounds such as *p*-hydroxybenzoate, 2,3-dihydroxybenzoate, and 3,4-dihydroxybenzaldehyde which are formed from chorismate (2) did not reverse the inhibition of growth by tryptophan in the mutant.

Extra-Operon Location of trpX—The newly described mutation, *trpX7*, does not lie within the operon which contains all other known cistrons of tryptophan synthesis. The data given in Table III show that *trpX7* was not cotransformed with a cistron of the tryptophan gene cluster or to a nearby gene of tyrosine synthesis. Joint transfer of completely unlinked genes by DNA transformation (*i.e.* congression) occurs at frequencies of 0.01 or less in *B. subtilis* (8, 9). If *trpX7* were located in the tryptophan gene cluster, one would expect cotransfer with *trpC2* of well over 50%. The *trpX7* gene also shows no linkage with genes of the tryptophan operon by transduction analysis with phage PBSI.[3]

Semicoordinate Control of Subunit-X by Tryptophan—Although the foregoing data indicate that the *trpX* locus does not lie in the tryptophan cluster of structural genes, the synthesis of subunit-X is nevertheless controlled by tryptophan (1). Hence, it was of interest to determine whether the expression of the genetic locus for *trpX* was controlled coordinately with that of the other genes in the cluster. The results of this experiment are given in Fig. 5. A tryptophan auxotroph, E-78, lacking activity for anthranilate synthase because of a mutation in the *trpE* locus was used in a derepression experiment. The synthesis of subunit-X following derepression was determined by assaying glutamine-reactive anthranilate synthase in the presence of excess subunit-E (from crude extracts of isolate I-15). Mutant E-78 was derepressed according to the regimen given under "Materials and Methods." Extracts prepared from various samples were assayed for levels of subunit-X and tryptophan synthase B. Subunit-X is coordinately synthesized with tryptophan synthase B, except at low repressed enzyme levels or at high derepressed levels. Since tryptophan synthase B has been reported to be synthesized coordinately with the other enzymes of the pathway (14), it can be concluded that subunit-X is also

[3] B. S. Glatz and R. A. Jensen, unpublished results.

TABLE III

Genetic mapping of trpX7 in NP 102

Deoxyribonucleate transformation was carried out with limiting concentrations of DNA as described in Reference 9. Stock culture phenotypes are given in Table I. The selective medium for prototrophic recombinants in each cross was unsupplemented minimal glucose medium. Cotransfer of the unselected marker was scored by replica plating to solid media containing 50 μg of 5-methyltryptophan per ml. The hypersensitive 5-methyltryptophan phenotype corresponding to *trpX7* is designated m-trp^{HS}.

Donor DNA	Recipient	Recombinant class		Cotransfer frequency
		Selected	Unselected	
NP 102 (*trpX7*)	NP 19 (*trpC2*)	trp$^+$	m-trp^{HS}	
		390	3	0.008
NP 102 (*trpX7*)	E-35 (*tyrA1*)	tyr$^+$	m-trp^{HS}	
		519	4	0.008

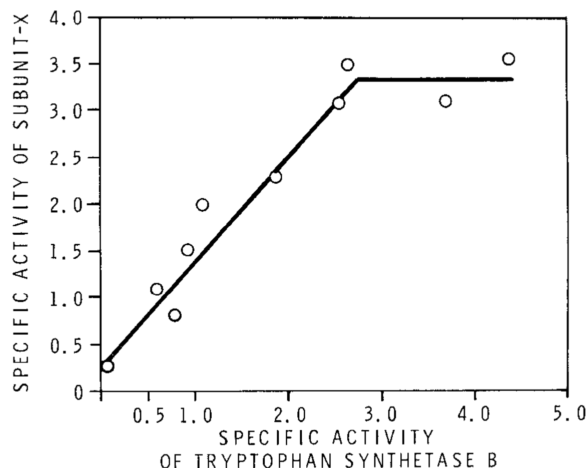


FIG. 5. Semicoordinate control of subunit-X. Isolate E-78 was grown in 1100 ml of minimal glucose medium supplemented with 50 μg of L-tryptophan per ml to an absorbance at 600 nm of 1.1. The cells were pelleted by centrifugation and washed in 150 ml of minimal glucose medium. One sample was removed and the remaining cell pellet was resuspended in 1500 ml of minimal glucose medium containing 0.1% casein hydrolysate. Cell samples of 100-ml volume were taken every 15 min thereafter. The cells were lysed immediately and the crude extracts were passed through a Sephadex G-25 column prior to enzyme assay. The activity of subunit-X (*ordinate*) is plotted as a function of the activity of tryptophan synthase B (*abscissa*).

TABLE IV

Correlation of analogue sensitivity with trpX mutation

Each value of k represents a value proportional to the slope of a growth curve plotted from turbidity measurements of cultures growing at 37° in the presence of the indicated concentration of 5-methyltryptophan. Strain descriptions are given in Table I.

Collection number	5-Methyltryptophan	k[a]
	μg/ml	
NP 42	0	0.53
	10	0.50
	20	0.48
NP 102	0	0.55
	10	0.20
	20	0.17

[a] k is the specific growth rate (15) expressed in hours^{-1}.

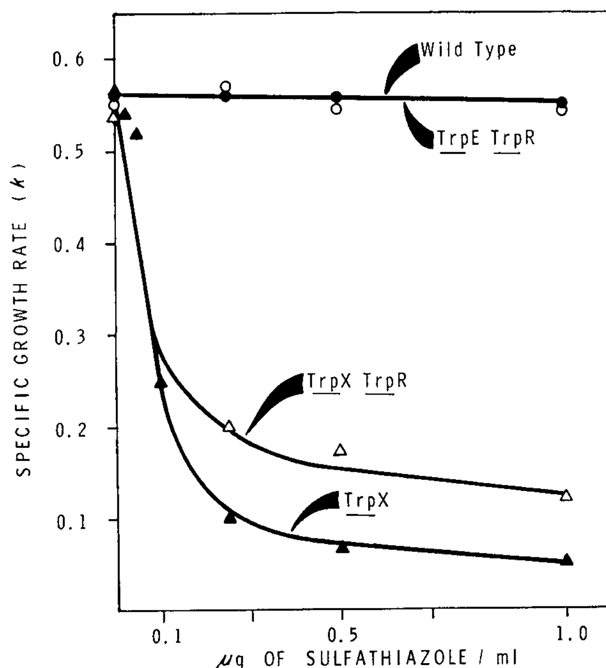


FIG. 6. Sulfathiazole hypersensitivity in *trp*X mutants. Cultures of NP 42, ○——○; I-12, ●——●; I-15, △——△; and NP 102, ▲——▲ were grown overnight in minimal glucose medium at 37° with shaking, diluted to an optical density at 600 nm of 0.02 in the same medium supplemented with the indicated final concentrations of sulfathiazole. Used for culture incubations were 125-ml culture flasks with side arms containing 10-ml culture volumes. The specific growth rate, k (15), expressed in reciprocal hours, is plotted as a function of the concentration of sulfathiazole present.

synthesized in semicoordination with the enzymes of the entire tryptophan operon.

Hypersensitivity of trpX-deficient Mutant to 5-methyltryptophan and Sulfathiazole—Table IV shows that the *trpX7* mutation confers hypersensitivity to 5-methyltryptophan, a result consistent with the decreased ability of mutant NP 102 to synthesize tryptophan. Concentrations of the analogue which produced only a slight inhibition of growth rate in wild type NP 42 were very effective in the inhibition of liquid cultures of mutant NP 102.

Since PABA effectively reversed inhibition of growth by tryptophan in NP 102, and since tryptophan repressed the synthesis of subunit-X (1; Fig. 5), participation of subunit-X in the synthesis of PABA from chorismate is implicated. Presumably, the derepressed level of the altered subunit-X is necessary for adequate folate biosynthesis. Qualitative support of these interpretations was obtained on solid media. Little or none of the feeding, which characterizes wild type strains, occurs when isolate NP 102 is tested against a PABA/folate-requiring confluent lawn. Isolate NP 102, grown in minimal glucose medium does not excrete significant amounts of PABA into the medium. It makes less than a wild type strain that was fully repressed for the synthesis of subunit-X by tryptophan. The specific PABA excretion defined as nanomoles of PABA extracted from 10 ml of culture supernatant into 10 ml of ethyl acetate was 0.4 for NP 102 compared to 1.9 for NP 42 grown in the presence of 50 μg per ml of L-tryptophan. The difference in the PABA levels of isolates NP 42 and NP 102 are qualitatively apparent on solid media. For example, isolate NP 42 feeds the inhibited background of growth of isolate NP 102 quite well on tryptophan-supplemented agar. Also isolate NP 42 feeds a PABA-requiring auxotroph (C-4) very well, whereas isolate NP 102 does not. Finally, the specific activities of PABA synthase were 4.0 and <0.1 nmoles of PABA per 30 min per mg of protein in crude extracts from isolates E-78 and NP 102, respectively.

If the *trpX7* mutation does result in the decreased synthesis of PABA as well as of tryptophan, one might anticipate a correlating hypersensitivity to PABA analogues similar to the hypersensitivity of isolate NP 102 to growth inhibition by tryptophan analogues. In confirmation of this expectation are the growth data given in Fig. 6. Isolates I-15 and NP 102 were inhibited by concentrations of the PABA analogue, sulfathiazole, which did not perturb the growth rates of strains carrying the wild type allele of *trpX*. Mutations in *trpE* or in *trpR* did not influence the antimetabolite effect of sulfathiazole in *trpX* genetic backgrounds.

The inhibition of growth of isolates NP 42 and I-12 by sulfathiazole, however, is dependent upon the size of the inoculum. There is about a 4-hour lag between the addition of the analogue and the onset of growth inhibition in isolates NP 42 and I-12, whereas in the *trpX* mutants it is about 1 hour. These lag times probably are proportional to the pool sizes for folic acid in the various test strains. In other systems sulfathiazole not only competitively inhibits the enzymic utilization of PABA by dihydropteroate synthase, but it may also be incorporated into a folic acid analogue which is catalytically inert (16, 17).

Relationship of Subunit-X and Glutamine-reactive Anthranilate Synthase—Apparently subunit-X is specifically involved in the utilization of glutamine as the nitrogen donor in the anthranilate synthase-catalyzed reaction. This is supported by the following observations. First, the *trpX* mutation in NP 102 and I-15 results in the loss of the glutamine-reactive anthranilate synthase activity but leaves the ammonia-reactive activity intact. Second, in crude extracts the velocity of the glutamine-reactive enzyme is not a linear function of protein concentration, whereas the ammonia-reactive activity is linear (Fig. 7). These observations are consistent with a differential effect of subunit-X upon the glutamine- and ammonia-reactive activities.

At high protein concentrations in crude extracts the specific activity of the glutamine-reactive anthranilate synthase is about 3-fold higher than the ammonia-reactive activity (1; Table II). When such an extract is passed through a Sephadex G-100

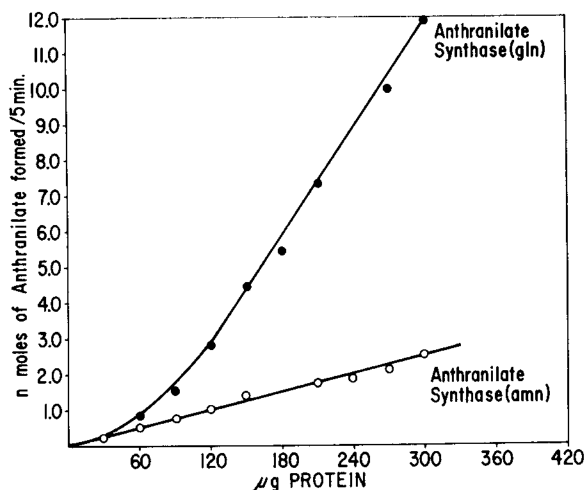


FIG. 7. The velocity of the anthranilate synthase reaction as a function of protein concentration. Anthranilate synthase, with either ammonia (*amn*) or glutamine (*gln*) as the nitrogen donor, was assayed for activity in crude extracts prepared from the constitutive mutant NP 100. Similar relationships were noted with an extract of the wild type organism except that the enzyme activities were lower. In the presence of excess subunit-X, the activity of anthranilate synthase (*gln*) is a linear function of protein concentration, *i.e.* a line parallel to that shown for anthranilate synthase (*gln*) and extrapolating through the origin. The value, Δ activity to Δ protein, on the linear portion of the biphasic curve below for anthranilate synthase (*gln*) is a valid expression of specific activity. It is exactly identical with the specific activity obtained in the presence of saturating concentrations of subunit-X.

column, one anthranilate synthase activity (molecular weight 96,000)[4] is eluted which has the same specific activity whether glutamine or ammonia is the nitrogen donor (Table V). When a rate relationship of velocity as a function of protein concentration is determined on this partially purified enzyme species, the glutamine-reactive activity is a linear function of the protein concentration (Fig. 8). When partially purified subunit-X (molecular weight 16,000) is added back to the partially purified enzyme, the glutamine-reactive anthranilate synthase activity is stimulated but not the ammonia-reactive anthranilate synthase (Table V). The magnitude of stimulation by subunit-X is protein-dependent (Fig. 8).

A similar observation was made with crude extracts of isolates I-15 and NP 102 which carry the *trpX7* mutation and therefore, lack activity for the glutamine-reactive anthranilate synthase. When active subunit-X is added to a reaction mixture containing extract from either mutant NP 102 or I-15, a striking activity for glutamine-reactive anthranilate synthase is observed (Table VI). This is the result of *in vitro* complementation between intact subunit-E of isolate NP 102 or I-15 and intact subunit-X of isolate I-12 (1).

The data in Figs. 9 and 10 illustrate the elution profiles of extracts from the isolates I-12 (*trpE* mutant) and I-15 (*trpX* mutant), respectively, from a Sephadex G-100 column. Isolate I-12 lacks detectable levels of anthranilate synthase with either nitrogen donor. When column eluate fractions of extract from isolate I-12 were assayed for glutamine-reactive enzyme in the

[4] Since the complex of anthranilate synthase as isolated is not saturated with subunit-X (Table V; Fig. 8), it seems likely that a "native" complex of higher molecular weight may exist *in vivo*.

TABLE V

Activity of partially purified anthranilate synthase

Sephadex G-100 eluate[a]	Nitrogen donor reactant	Specific activity of anthranilate synthase[b]
Component I	Ammonia	1.8
	Glutamine	1.8
Component I plus Component II	Ammonia	1.8
	Glutamine	5.2

[a] Component I is the 96,000 (our previous estimate was 84,000 (1)) molecular weight complex of anthranilate synthase and Component II is free subunit-X (1). In the mixing experiment equal volumes of Components I (25 µg of protein) and II (8 µg of protein) eluates were used.

[b] Specific activity is expressed as nanomoles of anthranilate formed per min per 0.2 ml of column eluate from peak tube.

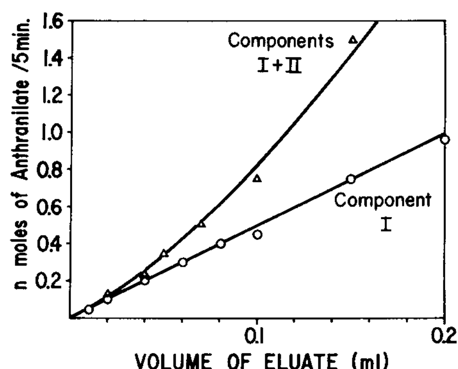


FIG. 8. The velocity of the partially purified anthranilate synthase as a function of protein concentration. The activity of the partially purified (Sephadex G-100) anthranilate synthase component I (119 µg of protein) of isolate NP 100 (1) was assayed in the presence of glutamine as a function of protein concentration. Under these conditions this activity is a linear function of the amount of column eluate added (○——○). When partially purified subunit-X (Component II) (38 µg of protein) was added to partially purified Component I, the glutamine-reactive activity shows the biphasic curve characteristic of the data obtained from crude extracts (△——△). The *abscissa* refers to the volume used in the reaction mixture of the pooled fractions of a Sephadex G-100 gel fractionation representing two-thirds of the peak profile of the 96,000 molecular weight species of anthranilate synthase, *i.e.* Component I.

presence of extract from either isolate NP 102 or I-15, two fractions of activity were recovered (Fig. 9) which correspond to the two components found with isolate NP 100 (1). In this experiment the complementation assay detects two peaks of subunit-X, by adding excess subunit-E (crude extract of mutant I-15). The low molecular weight peak of activity represents free subunit-X while the early eluting peak appears at the position of the anthranilate synthase complex (in this case, altered inactive subunit-E and subunit-X). (The trailing shoulder of the early eluting peak is probably PABA synthase and will be discussed in greater detail in a subsequent communication.)

In Fig. 10 the results of a similar experiment with crude extract of isolate I-15 are shown. The ammonia-reactive anthranilate synthase eluted as a single peak, having a molecular weight of approximately 96,000. Again this coincides in molecular weight position to the anthranilate synthase complex previously described (1). When active subunit-X was added to the frac-

TABLE VI

Role of subunit-X in anthranilate synthase reaction

The source of subunit-X was 500 μg of a crude extract of isolate I-12. Partially purified subunit-X gives the same complementation results.

Collection number	Specific activity, glutamine-reactive anthranilate synthase	
	−Subunit-X	+Subunit-X
NP 102	<0.01	2.02
I-15	<0.01	2.65

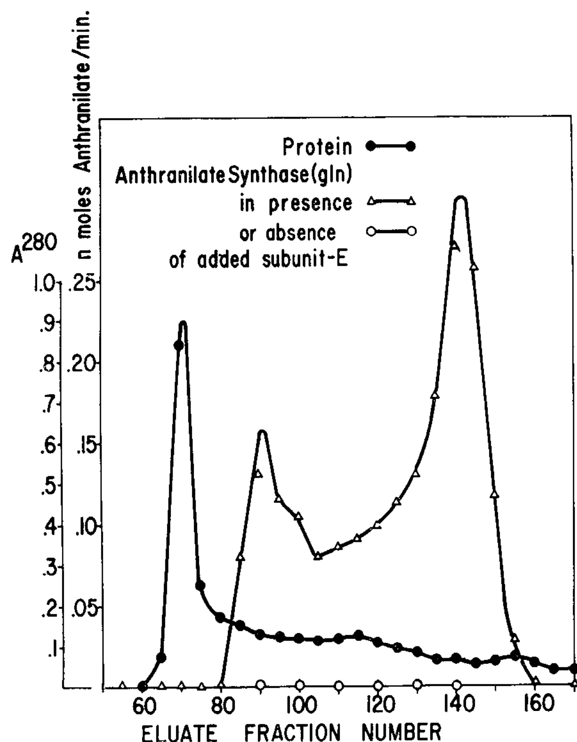


FIG. 9. The elution profile of anthranilate synthase with isolate I-12. Gel filtration and extract preparation were previously described (1). Neither ammonia- nor glutamine-reactive activities were found in eluate fractions. Glutamine-reactive anthranilate synthase activity (△——△) was found when the assay was done in the presence of 500 μg of protein from isolate I-15. Protein was estimated as absorbance at 280 nm (●——●).

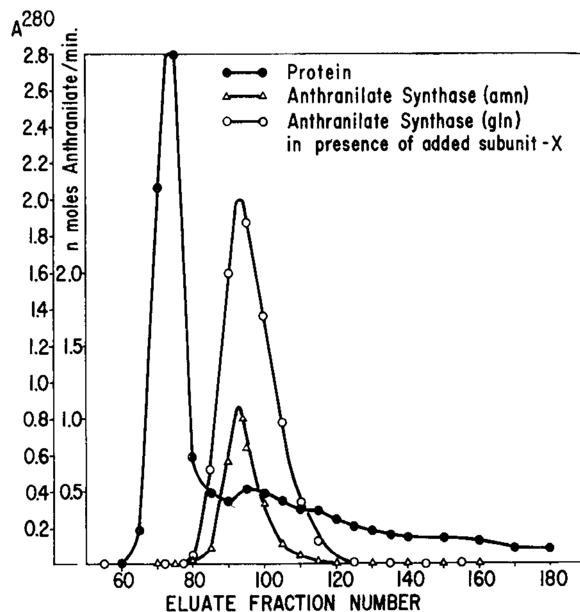


FIG. 10. The elution profile of anthranilate synthase with isolate I-15. Gel filtration and extract preparation were previously described (1). Anthranilate synthase with ammonia as the amide donor (△——△) was assayed as previously described. No enzyme activity was found when glutamine was used as the amide donor. In the presence of 500 μg of a crude extract from isolate I-12 (or of partially purified subunit-X) the glutamine-reactive enzyme activity was recovered (○——○) and it coincided with the ammonia-reactive activity. Protein concentrations (●——●) were estimated from the absorbance of eluate fractions at 280 nm. Similar results were obtained with an extract obtained from isolate NP 102.

tions (*i.e.* complementation), a single peak of glutamine-reactive activity was found which coincided exactly with the position of the ammonia-reactive activity. Similar results were achieved with extracts from isolate NP 102 obtained from cultures grown in minimal glucose medium. The results of Figs. 9 and 10 indicate that mutants I-12 and I-15 synthesize altered gene products, subunit-E and subunit-X, respectively.

The detection of both subunits in the 96,000 molecular weight complex in each mutant following *in vitro* reconstitution suggests that the mutant subunits can exchange with their normal counterparts efficiently in the complementation assay. All of these results suggest that subunit-X has two effects. It provides glutamine reactivity to the enzyme complex and it stimulates this glutamine reactivity. The interaction of additional subunit-X molecules with the 96,000 molecular weight complex differs from the interaction of subunit-X with subunit-E to form the complex. The association of extra subunit-X molecules with the complex is very dependent upon protein concentration while the association of subunits-X and -E as the complex is relatively stable.

DISCUSSION

Multifunctional Role of Subunit-X in Two Biochemical Pathways—Subunit-X is part of the anthranilate synthase aggregate in *B. subtilis* (1). Several lines of evidence are presented in this paper to show that subunit-X also is a component of PABA synthase. Both the anthranilate synthase and PABA synthase reactions utilize the same substrates, chorismate, and glutamine (see Fig. 1); the two reaction products differ only in the position of the amino substituent on the ring. Subunit-X undoubtedly functions in the binding of glutamine at the catalytic site. This glutamine specificity seems especially clear in the case of anthranilate synthase since (*a*) a *trpX* mutation abolished glutamine-reactive anthranilate synthase but left ammonia-reactive anthranilate synthase intact, and (*b*) the addition of partially purified subunit-X to extracts of *trpX* mutants reconstituted the glutamine-reactive anthranilate synthase activity.

In contrast to the ammonia-reactive activity, the glutamine-reactive activity is not a linear function or protein concentration in the presence of excess subunit-X. In partially purified preparations in which the excess of subunit-X present in crude extracts was removed, the glutamine-reactive activity was a linear function of protein concentration. Addition of partially purified subunit-X back to the 96,000 molecular weight activity restored the nonlinear protein *versus* velocity response that is characteristic of crude extracts. In the partially purified anthranilate synthase aggregate (molecular weight 96,000) the specific activity

of the ammonia-reactive enzyme is equivalent to the glutamine-reactive enzyme. This is not the case in crude extracts where the specific activity of the glutamine-reactive enzyme is about 3-fold greater than the ammonia-reactive enzyme. Addition of subunit-X to the partially purified 96,000 molecular weight Component I, however, stimulates the glutamine-reactive activity about 3-fold without altering the ammonia-reactive activity. This system bears some similarity to one described for phenylalanine hydroxylase in rat liver (18). A small protein component possessing no known activity of its own stimulates the hydroxylase, and this stimulation is protein-dependent.

PABA synthase has not yet been characterized as completely as has been anthranilate synthase. While it is qualitatively obvious the PABA synthase activity is nearly abolished in *trpX* mutants, it is not certain whether an ammonia-reactive PABA synthase remains (as is the case for anthranilate synthase) or whether the glutamine-reactive enzyme retains a very low residual specific activity. Partially purified subunit-X does not have any PABA synthase activity. Presumably some unidentified subunit of PABA synthase competes with subunit-E for association with subunit-X to form PABA synthase and anthranilate synthase, respectively. Since subunit-X probably has no catalytic activity of its own, it may function in the manner of so-called "specifier proteins" (*e.g.* α-lactalbumin associates with galactosyltransferase to convert it to a lactose synthetase (19); σ factors interact with RNA polymerase to specify recognition sites for transcription (20)).

B. subtilis is so far the only system for which a mutant subunit-X has been characterized. However, it now appears that a low molecular weight protein possessing a glutamine-binding site is a portion of the anthranilate synthase complex in a number of microorganisms known to possess two-component systems (21–23). Hwang and Zalkin (24) have recently described a low molecular weight "fragment" of PR transferase in *Salmonella typhimurium* which binds glutamine. In view of the suggestion that such a subunit might play a universal role in the action of many of the glutamine-ammonia-reactive enzymes in the cell, this possibility was considered in *B. subtilis*. Especially suggestive was the finding that subunit-X is indeed common to the function of both anthranilate synthase and PABA synthase. The following considerations led us to the conclusion that subunit-X does not play a general role in other glutamine-reactive activities of the cell. (*a*) Although tryptophan effectively represses the synthesis of the deficient subunit-X in a *trpX* mutant, the mutant has no requirements other than PABA (or folate) in the presence of tryptophan. (*b*) The *trpX* mutant is hypersensitive to growth inhibition by analogues of tryptophan and PABA. Hence, other pathways which might be stressed in the presence of the mutant subunit-X should also be hypersensitive to an analogue antimetabolite. The histidine analogue, 1,2,4-triazole 3-alanine, is an effective inhibitor of growth in *B. subtilis* (25), but no difference in analogue sensitivity was noted when *trpX* and *trpX*$^+$ strains were compared.[5] (*c*) Direct enzyme analysis of cytidine triphosphate synthetase, another glutamine-ammonia enzyme, did not show any catalytic differences when extracts of *trpX* and *trpX*$^+$ strains were compared.

Gene-Enzyme Relationships with Respect to Subunit-X in B. subtilis—The *trpX* locus is not linked to the tryptophan cluster of genes. Nevertheless, the expression of this locus is controlled coordinately with the expression of the tryptophan cluster of genes throughout most of the range of derepression. The relationship becomes discoordinate when either maximal repression or maximal derepression is approached. Accordingly, the range of expression for subunit-X is less than that of the gene products of the tryptophan operon. Repression control of subunit-X by tryptophan suggests its ancient origin as a tryptophan-specific protein. Subsequent translocation of the *trpX* gene from the tryptophan operon may have been of selective advantage in order to ensure that a residual nonrepressible level of subunit-X could form vitamin amounts of folate, even in the presence of tryptophan.

Hoch *et al.* (14) reported that while the glutamine-dependent anthranilate synthase increased coordinately with the other tryptophan biosynthetic enzymes during early derepression, its synthesis leveled off prematurely with respect to other enzymes of tryptophan synthesis. Their results may now be explained by noting that subunit-X, a component of glutamine-reactive anthranilate synthase, reaches maximal derepression at a time when the remaining tryptophan biosynthetic enzymes continue to be derepressed. These relationships resemble ones described for leucine-specific enzymes in *Neurospora crassa* (26), where the control was described as semicoordinate.

Lack of Tryptophan Auxotrophy in trpX Mutants—A mutational loss of active subunit-X did not result in a nutritional requirement for tryptophan during growth. This observation allows an explanation for the fact that the genetic locus, *trpX*, has not been previously recognized. Mutational loss of active subunit-X does not necessarily prevent the biosynthesis of tryptophan, presumably owing to the utilization of ammonia by the mutant enzyme. Therefore, a convenient auxotrophic phenotype is not correlated with a mutation in *trpX*. A similar situation may exist in several species of *Pseudomonas* (21, 27). In the latter system a small subunit of the anthranilate synthase aggregate is not yet represented by a corresponding cistron that is defined by a class of tryptophan auxotrophs. This subunit is necessary for catalytic reactivity of anthranilate synthase with glutamine as is the case for the *B. subtilis* enzyme.

Evidence that Ammonia Can Serve as Nitrogen Source in Vivo—A large and intriguing class of enzyme proteins can use either glutamine or ammonia as a source of nitrogen (28–35) *in vitro*. However, the adequacy *in vivo* of ammonia as a nitrogen donor in such reactions is equivocal. The generally poor affinity of such enzymes for ammonia and the high pH optimum in particular have raised doubts about the *in vivo* function of ammonia as a substrate reactant. Davis (33) described mutants of *N. crassa* which lacked a glutamine-reactive carbamyl phosphate synthetase but retained the ammonia-dependent activity. Nevertheless, these mutants required both arginine and uridine for growth indicating that ammonia cannot substitute very well for glutamine *in vivo*.

On the other hand, Gibson *et al.* (34) have shown that anthranilate synthase can generate anthranilate from chorismate and ammonia in whole cells in *Escherichia coli*. The multifunctional nature of the anthranilate synthase complex in *E. coli* (22) in which the glutamine-reactive anthranilate synthase apparently depends upon the integrity of an associated PR transferase is consistent with the fact that no prototrophs have been reported which lack the glutamine-reactive anthranilate synthase activity but retain both the ammonia-dependent activity and PR transferase. Since it was therefore necessary that the work in *E. coli* be carried out with tryptophan auxotrophs (blocked in activity for PR transferase) under nongrowing conditions, it could

[5] R. A. Jensen, unpublished observations.

not be shown to what extent the rate of anthranilate formation from ammonia was adequate to sustain a normal rate of growth.

In the mutant NP 102 (*trpX7*), the considerable *in vitro* potentiality to utilize ammonia as a nitrogen source for anthranilate biosynthesis may be realized *in vivo*. The mutant isolate grows as well as the parental strain in a minimal medium containing an inorganic source of nitrogen. This is apparently accomplished by the derepression of the ammonia-dependent anthranilate synthase. This derepression is the physiological consequence of a decreased rate of tryptophan synthesis. The results indicate that an almost completely derepressed ammonia-reactive anthranilate synthase is required to accomplish a level of catalysis equivalent to that of an almost completely repressed glutamine-reactive activity. When the mutant possessed an additional mutation resulting in the constitutive synthesis of the tryptophan biosynthetic enzymes, the fully derepressed activity of the ammonia-dependent anthranilate synthase was sufficient to contribute to an actual overproduction of tryptophan. It cannot be rigorously excluded, however, that a low, unstable reactivity with glutamine *in vivo* may exist in the subunit-X mutant.

Metabolic Interlock—It seems likely that as the regulatory specificities of particular pathways continue to be documented in the literature, an increasing emphasis will be tuned to the regulatory specificities which coordinate the metabolite flow rates of different pathways. We refer to such interpathway regulatory relationships as metabolic interlock. Such regulatory relationships exerted between different biochemical pathways can now be considered at four levels with respect to molecular mechanism: (i) interpathway regulatory interactions mediated by allosteric inhibitions or activations, (ii) interpathway interactions which affect the control of the levels (*i.e.* the synthesis) of pathway-specific enzymes, (iii) interpathway relationships involving subordinate enzymes, and (iv) interpathway regulatory relationships mediated by protein-protein interactions. Examples of interlock type (i) are perhaps the most familiar and were summarized in References 5, 6, and 25. Interlock type (ii) is represented by a regulatory element affecting enzyme levels in the tyrosine and histidine pathways of *B. subtilis* (36) as well as a relationship between the histidine pathway and the level of enzymes in the tryptophan biosynthetic pathway in *N. crassa* (37, 38). Subordinate enzymes mediate metabolic interlock type iii relationships. Subordinate enzymes are proteins which possess regulatory specificities which depend upon the function of other regulatory enzymes, *e.g.* chorismate mutase in *B. subtilis* (4); or they are proteins for which a *lack* of regulation is appropriate because of the indirect control exercised upon the unregulated enzyme via the control of some other enzyme, *e.g.* phosphoribosyl aminoimidazole carboxamide transformylase in *E. coli* (39). This communication describing the joint involvement of a single protein subunit in two biochemical pathways constitutes an example of an interlock type iv mechanism.

In addition to the foregoing account of various modes of regulatory interplay between specific pathways, there exist interlocking relationships in which the specific control of particular pathways is modulated by metabolites which reflect the general physiological state of the cell. Atkinson's account (40) of the "energy charge" effect on regulatory enzymes and the mediation of catabolite repression by adenosine 3′,5′-monophosphate (41) illustrate the modification of the specific control of a particular pathway in response to the over-all metabolic state of the cell. Regulatory relationships at this level can be mediated by any of the interlock mechanism types described above.

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