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Rapid Regulation of an Anthranilate Synthase Aggregate by Hysteresis

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The anthranilate synthase aggregate from *Bacillus subtilis* is composed of two nonidentical subunits, denoted E and X, which are readily associated or dissociated. A complex of subunit E and X can utilize glutamine or ammonia as substrates in the formation of anthranilate. Partially purified subunit E is capable of using only ammonia as the amide donor in the anthranilate synthase reaction. The stability of the EX complex is strongly influenced by glutamine and by the concentrations of the subunits. Glutamine stabilizes the aggregate as a molecular species in which the velocity of the glutamine-reactive anthranilate synthase is a linear function of protein concentration. In the absence of glutamine the aggregate is readily dissociated following dilution of the extract; that is, velocity concaves upward as a function of increasing protein concentration. Reassociation of the EX complex is characterized by a velocity lag (or hysteretic response) before steady-state velocity for the glutamine-reactive anthranilate synthase is reached. We propose that association and dissociation of the anthranilate synthase aggregate may be physiologically significant and provide a control mechanism whereby repression or derepression causes disproportionate losses or gains in activity by virtue of protein-protein interactions between subunits E and X.

A number of amidotransferases are capable of utilizing either glutamine or ammonia as an amide donor. In some cases the activity with glutamine is dependent upon the aggregational state of the enzyme. One such enzyme is anthranilate synthase, the first enzyme in the tryptophan biosynthetic pathway. Anthranilate synthase from *Bacillus subtilis* is composed of two nonidentical subunits, E and X (13). Subunit E is the gene product of *trpE*, the first locus in the tryptophan operon, and can catalyze the formation of anthranilate using only ammonia as the amide donor. Subunit X is a low-molecular-weight protein that is coded by *trpX*, an extraoperonic gene involved in tryptophan biosynthesis (11). This subunit complexes with subunit E to form an anthranilate synthase aggregate that can use either glutamine or ammonia as the amide donor. Subunit X is also a component of *p*-aminobenzoate synthase (11). Recent results suggest that a similar dual-function protein may occur in *Acinetobacter calcoaceticus* (R. V. Sawula and I. P. Crawford, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 61, 1972) and perhaps in species of *Pseudomonas* (18).

The EX complex from *B. subtilis* is a readily associated and dissociated enzyme that loses catalytic activity with glutamine upon dissociation (11). The reassociation of the aggregate is characterized by a 2- to 4-min lag before steady-state velocity is reached (W. M. Holmes and J. F. Kane, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 137, 1972). Such slow kinetic responses in activity have been termed hysteresis (5). Thus, the anthranilate synthase complex from *B. subtilis* is a hysteretic enzyme whose kinetic properties vary significantly as a function of the aggregational state of the enzyme. The possible regulatory significance of the protein-protein interactions which characterize the EX complex are considered in this paper.

MATERIALS AND METHODS

Growth conditions. The *B. subtilis* culture isolates used in this study are all derivatives of strain 168 (1) and are described in Table 1. Cells were grown at 37 C in minimal glucose medium (10) containing

TABLE 1. *Strains of Bacillus subtilis*

Collection no.	Genotypic[a] deficiency	Description
NP 40		Hybrid strain prototroph (14)
NP 16	*aroB*	Aromatic amino acid auxotroph blocked in second enzyme of common pathway; grows on shikimate.
NP 100	*trpR2*	5-Methyltryptophan resistant; partial growth requirement for phenylalanine (12-14)
I-12	*trpR2 trpE8*	Prototroph with leaky block in subunit E of anthranilate synthase (13)
I-15	*trpR2 trpX7*	5-Methyltryptophan-resistant prototroph (13)

[a] *aroB*, genetic locus for 5-dehydroquinate synthase; *trpR*, mutation to constitutivity of enzyme synthesis in the tryptophan pathway; it is linked but not contiguous to the tryptophan gene cluster (17) and presumably codes for apo-repressor.

amino acid supplements as indicated.

Enzymological procedures. Extracts were prepared as previously described (12). Assay conditions have been described for anthranilate synthase, with ammonia (13) or glutamine (12) used as amide donors, and for tryptophan synthase B (11). Under some conditions there is a lag in the initial velocity of anthranilate synthase when glutamine is the substrate. Therefore, in all cases the rate of the reaction was determined after steady-state velocity was reached.

In complementation studies the source of subunit E was either a crude extract of isolate I-15 or a partially purified preparation obtained by gel filtration on Sephadex G-100 (Fig. 1). The activities with both preparations were comparable, as shown in Table 2. Similar data were obtained with either a crude extract containing subunit X (from isolate I-12) or partially purified subunit X.

Column chromatography. Gel filtration studies were carried out on a 2.5 by 80 cm Sephadex G-100 column equilibrated with 0.04 M potassium phosphate buffer, *p*H 7.2, containing 0.1 mM ethylenediaminetetraacetic acid, 6 mM β-mercaptoethanol, and 30% glycerol. The column was standardized with alkaline phosphatase (*Escherichia coli*), bovine serum albumin, and cytochrome C (horse heart). The flow rate was 12 ml/hr at 4 C, and 2.0-ml fractions were collected. Appropriate column fractions were pooled and then concentrated with Amicon membranes. Protein concentrations were estimated by the method of Lowry et al. (15).

Derepression studies. Isolate NP 16 has a leaky block in 5-dehydroquinate synthase, the second enzyme in the aromatic amino acid biosynthetic sequence. This mutant will grow on shikimate, an intermediate which bypasses the enzymatic lesion. Mutant NP 16 was grown in 1.5 liters of minimal glucose medium supplemented with 100 μg of shikimate per ml to an absorbance of 0.7 at 600 nm (1.8×10^8 cells/ml). The culture was centrifuged, and the cells were washed and resuspended in 1.5 liters of minimal glucose medium containing 0.1% casein hydrolysate. Samples were taken every 20 min for the next 2 hr, and extracts were prepared in a buffer lacking glutamine. During this 2-hr period, cell density doubled. The glutamine-reactive enzyme which is indicative of the amount of EX complex was determined directly with 500 μg of crude extract protein. The total amount of subunit E in 500 μg of protein was determined by assaying for the glutamine-reactive anthranilate synthase in the presence of saturating subunit X (a crude extract of I-12). Similarly, the total amount of subunit X in 500 μg of protein was determined by assaying for the glutamine-reactive anthranilate synthase in the presence of saturating subunit E (a crude extract of I-15).

Repression studies. To study repression, the prototroph, NP 40, was grown in 1.5 liters of minimal glucose medium containing 0.1% casein hydrolysate. At an absorbance of 0.35 at 600 nm (1.0×10^8 cells/ml), tryptophan was added to a final concentration of 25 μg/ml. A sample was taken immediately and every 20 min thereafter. The doubling time of this culture was 45 min. Extracts were prepared in a buffer lacking glutamine and assayed directly for the glutamine-reactive anthranilate synthase as well as the total amount of subunits E or X as described above.

Chemicals. All chemicals were of the highest commercial grade available. The glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON), was obtained from the Drug Research and Development, Chemotherapy Division, National Cancer Institute.

RESULTS

Aggregational properties of the EX complex. An EX complex can be eluted from a Sephadex G-100 column that is equilibrated with 10 mM glutamine (13). In the absence of glutamine, however, no EX complex is recovered (Fig. 1). Subunit E elutes as a single peak with an apparent molecular weight of 83,000 and is completely separated from subunit X, which has an apparent molecular weight of 16,000. Therefore, dilution of the anthranilate synthase aggregate in the absence of glutamine results in total dissociation. A similar phenomenon might explain the failure to detect glutamine-reactive anthranilate synthases in several species of fungi after sedimentation through surcose density gradients unless glutamine were present (9).

Partially purified subunits will readily associate to form the glutamine-reactive anthranilate synthase complex (Fig. 2). In this experiment the ratio of subunit E to subunit X which approximates the ratio in a crude extract of NP 100 was constant, but the total protein concentration was increased. There is a nonlinear relationship between the velocity and the pro-

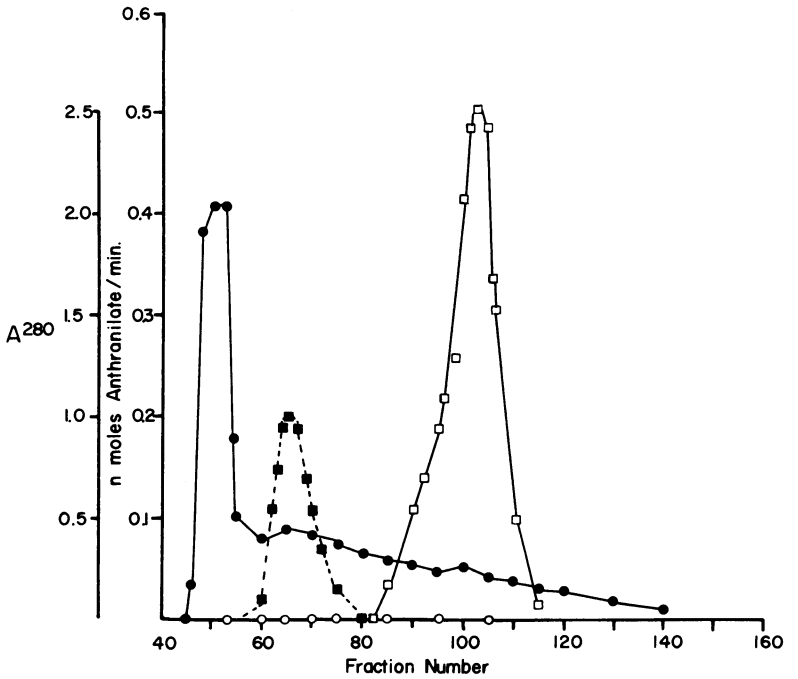

FIG. 1. *Gel filtration on Sephadex G-100 in the absence of glutamine. An extract of mutant NP 100 containing 50 mg of protein was layered on the top of the column. The glutamine-reactive anthranilate synthase (the EX complex) was determined on 0.1 ml of the eluate fractions* (○). *Subunit E was located by assaying 0.1 ml of the eluate fractions for the ammonia-reactive anthranilate synthase* (■). *The peak of subunit X was determined by assaying 0.1 ml of the eluate fraction for the glutamine-reactive anthranilate synthase in the presence of 500 μg of crude extract from isolate I-15* (□). *Eluate protein was estimated by absorbance at 280 nm* (A^{280}) (●).

TABLE 2. *Reconstitution of glutamine-reactive anthranilate synthase by using crude or partially purified subunit E and partially purified subunit X*

Protein concn[a] (μg/ml)			Velocity[b]
Crude E	Partially purified E	Partially purified X	
0	0	20	0
200		20	0.18
400		20	0.29
600		20	0.28
	50	20	0.15
	100	20	0.21
	200	20	0.25

[a] Crude E was supplied as a crude extract of mutant I-15 which had been passed through Sephadex G-25. Partially purified subunits E and X were obtained as shown in Fig. 1. The peak fractions were pooled and concentrated before being used in the assay.

[b] Velocity is expressed as nanomoles of anthranilate formed per minute.

tein concentration.

The reassociation of subunits E and X is characterized by a 2- to 4-min lag before steady-state velocity is reached. This type of slow response in enzyme activity has been termed hysteresis (5). The data in Fig. 3 illustrate the effect of glutamine on the hysteretic response of the glutamine-reactive anthranilate synthase. A sample of a crude extract of mutant NP 100 (Fig. 3A) was added to a reaction mixture (final volume, 0.9 ml) containing: (i) 60 μmoles of tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.55), 10 μmoles of $MgCl_2$, and 500 nmoles of potassium chorismate; or (ii) 60 μmoles of Tris buffer (*p*H 7.55), 10 μmoles of $MgCl_2$, and 20 μmoles of glutamine. The reaction mixtures were incubated for 2.5 min at 37 C. The reaction was started by adding 0.1 ml of glutamine (20 μmoles) or chorismate (500 nmoles), respectively. There is a noticeable lag before steady-state velocity is reached when the enzyme is diluted and incubated in the absence

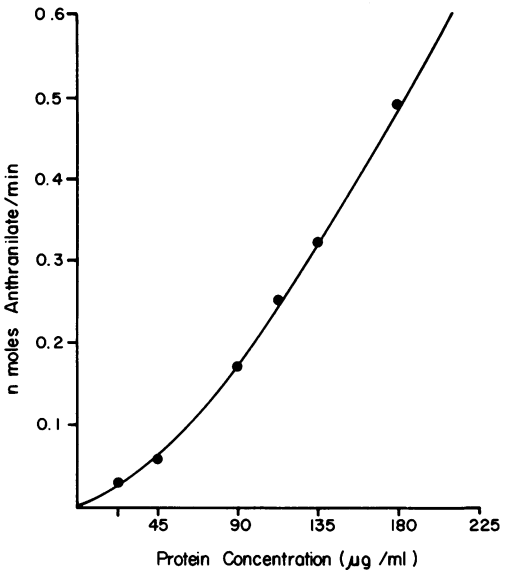

FIG. 2. *Activity of the glutamine-reactive anthranilate synthase as a function of the protein concentration of partially purified subunits. Subunits E and X were obtained as described in Fig. 1. The velocity of the first point, 0.03 nmoles/min, was obtained by adding 12.5 μg of protein containing partially purified subunit E to 10 μg of protein containing partially purified subunit X. The velocity is plotted as a function of the sum of the protein concentrations. The ratio of subunit E to subunit X was constant as the total protein concentration increased.*

of glutamine (Fig. 3A). This lag is completely abolished if glutamine is included in the incubation. A similar effect can be noted when partially purified components are used (Fig. 3B). Isolated subunits E and X are mixed together in the presence or absence of 20 μmoles of glutamine in a volume of 0.2 ml and incubated at 37 C for 2.5 min before starting the reaction by the addition of 0.8 ml of buffer containing Mg^{2+} and substrate(s). If the enzyme is incubated with glutamine, there is no detectable lag before steady-state velocity is reached; incubation in the absence of glutamine is characterized by a 2- to 4-min lag before steady-state velocity is reached. Chorismate or Mg^{2+}, or both, do not influence the kinetics of the lag with either crude enzyme or partially purified components.

These data clearly show that glutamine is required for the aggregation of subunits E and X; however, it does not distinguish between the following possibilities: (i) glutamine binds only to the EX aggregate; (ii) glutamine binds first to subunit X which then binds to subunit E. The data in Table 3 suggest that the first alternative is the more likely possibility. DON is an analogue of L-glutamine and binds irreversibly to a number of glutamine-requiring enzymes (16). Our data indicate that DON inhibits the glutamine-reactive activity of the EX complex and that this inhibition is irreversible since gel filtration with Sephadex G-25 does not restore activity (Holmes, *unpublished data*). When partially purified subunit X is preincubated with DON, this component is still capable of reconstituting an EX complex that is catalytically active with glutamine as a substrate. This indicates that DON does not bind to free subunit X. As expected, the glutamine analogue does not significantly influence the ammonia-reactive activities of free subunit E or the EX complex. When both subunits are preincubated together with DON, there is a significant inhibition of the glutamine-reactive anthranilate synthase reaction (Table 3).

Stability of subunit E and X. A possible explanation for the nonlinear relationship between protein concentration and reaction velocity is that subunit E or X or both are more labile when not associated as an EX complex. The stability of subunit E was examined in

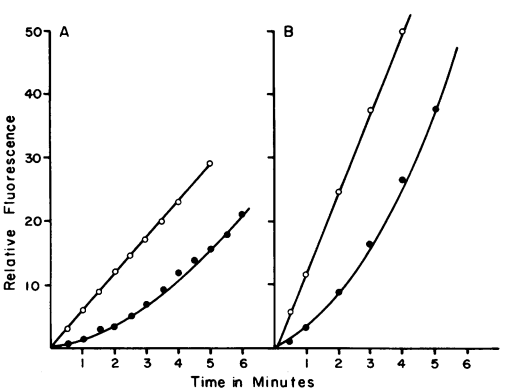

FIG. 3. *Effect of glutamine on the lag of the glutamine-reactive anthranilate synthase. These points were taken from a trace in order to reduce the ordinate and abscissa scales. A, A 50-μliter sample of a crude extract of mutant NP 100 (255 μg of protein) was preincubated at 37 C for 2.5 min in the presence (○) or absence (●) of 20 μmoles of glutamine. The reaction was initiated by the addition of 0.1 ml of glutamine (●) or chorismate (○). The relative fluorescence is plotted as a function of time after starting the reaction. B, 100 μg of protein containing partially purified subunit X was added to 20 μg of protein containing partially purified subunit E in the presence (○) or absence (●) of 20 μmoles of glutamine. After 2.5 min at 37 C, the reaction was initiated by the addition of buffer containing $MgCl_2$ and potassium chorismate plus (●) or minus (○) glutamine.*

TABLE 3. *Inhibition of anthranilate synthase activity by the glutamine analogue DON*[a]

Enzyme components[b]		% Inhibition	
During preincubation	After preincubation	Glutamine-reactive	Ammonia-reactive
E + DON	X	1	0
E + DON	None		0
X + DON	E	2	
E + X + DON	None	64	2

[a] DON, 6-Diazo-5-oxo-L-norleucine.

[b] Subunit E and X were prepared as in Fig. 1. In each experiment, 0.02 ml of a pooled, concentrated column eluate containing subunit E and/or 0.1 ml of a pooled, concentrated column eluate containing subunit X were preincubated at 25 C for 20 min with 50 nmoles of DON in a final volume of 0.2 ml. After preincubation, 0.8 ml of reaction mixture containing subunit E or X where indicated, 60 μmoles of L-glutamine or 50 μmoles of NH_4Cl_2, 600 nmoles of chorismic acid, 10 μmoles of $MgCl_2$, and 60 μmoles of Tris buffer (*p*H 7.5 or 8.5) was added to initiate the reaction. In the case of ammonia activity initial velocities were determined, whereas the glutamine activity represents the steady-state velocity that is achieved after the hysteretic lag. Under these conditions of extreme glutamine excess, DON did not significantly inhibit the glutamine activity of the non-preincubated control assays during the time required to obtain a reliable steady-state velocity. Furthermore, subunits E and X were stable to the conditions of preincubation and retained at least 95% of control activities: ammonia activity = 0.04 nmoles of anthranilate formed/min, glutamine activity = 0.11 nmoles of anthranilate formed/min.

crude extracts and partially purified preparations; the stability of subunit X was determined with a preparation of partially purified subunit. As shown in Table 4 subunit X is stable for 3 min at 37 C, whereas subunit E is not. We examined the effect of aggregation on the stability of E in the following experiments. Mutant I-15 synthesizes a defective subunit X and cannot utilize glutamine as an amide donor. It should be possible, therefore, to distinguish the aggregating effect of glutamine from its role as a substrate. In the absence of glutamine, preincubation at 37 C resulted in a significant loss of anthranilate synthase activity with ammonia as a substrate. When glutamine was present during the preincubation, essentially no activity was lost. This indicates that glutamine can bind to the EX aggregate from mutant I-15 and that subunit E is stabilized by aggregation. The data with isolate NP 100 provide further evidence for an increased stability of subunit E when it is complexed with X. Preincubation of enzyme from mutant NP 100 in the absence of glutamine results in an 80% loss in the activity with glutamine as substrate. If, however, glutamine is included in the preincubation mixture, there is only a 10% loss of activity.

Effect of protein concentration on the activity of the glutamine-reactive anthranilate synthase. Glutamine-reactive anthranilate synthase was assayed in crude extracts of NP 100 prepared in the presence or absence of

TABLE 4. *Stability of anthranilate synthase subunits E and X*

Protein concn[a] (μg/ml)		Preincubation[b] at 37 C (min)	Relative activity[c]	
Crude extract	Partially purified subunits		Ammonia	Glutamine
76 (NP 100)		0	1.0	1.0
76 (NP 100)		3	0.3	0.2
76 (NP 100)		3 + Glutamine		0.9
120 (I-15)		0	1.0	
120 (I-15)		3	0.4	
120 (I-15)		0 + Glutamine	1.0	
120 (I-15)		3 + Glutamine	0.9	
	50 (E)	0	1.0	
	50 (E)	3	0.5	
	50 (E)	3 + Glutamine	0.5	
	50 (X)	0		1.0
	50 (X)	3		1.0

[a] Crude extracts of isolates NP 100 and I-15 were prepared as previously described (12). Partially purified subunits E and X were prepared as described in Table 2.

[b] Preincubation was carried out as follows. For the ammonia activity, the enzyme was preincubated for the time indicated in 60 μmoles of Tris buffer (*p*H 8.55), 10 μmoles of $MgCl_2$, and 50 μmoles of NH_4Cl (and in some cases 20 μmoles of glutamine) in a volume of 0.9 ml. The reaction was started with 0.1 ml of chorismate (400 nmoles). For the glutamine activity the enzyme was preincubated for the time indicated in 60 μmoles of Tris buffer (*p*H 7.55), 10 μmoles $MgCl_2$, and in some cases 20 μmoles of glutamine in a volume of 0.9 ml. The reaction was started by the addition of 0.1 ml of chorismate (400 nmoles) and/or glutamine (20 μmoles). Where necessary (bottom two lines) subunit E was added (500 μg of crude extract protein from isolate I-15).

[c] The nanomoles of anthranilate produced per minute in the zero-time controls are as follows: NP100, 0.3 (ammonia) and 0.8 (glutamine); I-15, 0.3 (ammonia); subunit E, 1.8 (ammonia); subunit X, 0.73 (glutamine).

glutamine. In extracts prepared in the absence of glutamine, the velocity was not a linear function of the protein concentration (Fig. 4). Since a similar curve was obtained with partially purified subunits (Fig. 2), it is unlikely that the presence of an activator or inhibitor in the crude extract is responsible for the disproportional relationship of velocity to protein concentration. If the nonlinear response results from the dissociation of the EX complex upon dilution, one would predict a linear protein versus velocity relationship under conditions which stabilize the EX complex. The activity of an extract prepared in the presence of glutamine was, in fact, a linear function of protein concentration (Fig. 4). Furthermore, the nonlinear response obtained with an extract prepared in the absence of glutamine could be made linear by the addition of excess subunit E or X (Fig. 4), but not comparable concentrations of bovine serum albumin (Kane, *unpublished observations*).

The data in Fig. 5 further illustrate that the decreased activity of the glutamine-reactive enzyme in extracts prepared in the absence of glutamine correlates with a greater dissociation of the EX complex. If the enzyme is prepared in the absence of glutamine and samples are added to reaction mixtures containing increasing concentrations of subunit E or X, the activity of the glutamine-reactive anthranilate synthase increases up to 2.5-fold (Fig. 5A). If the enzyme prepared in the presence of glutamine is assayed in a similar fashion, stimula-

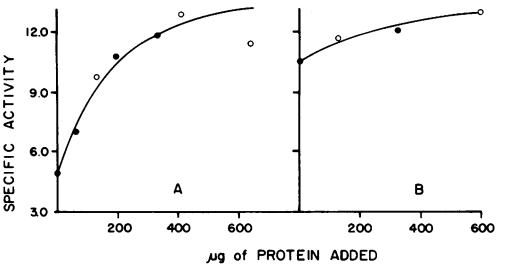

FIG. 5. *Stimulation of the glutamine-reactive anthranilate synthase by subunits E and X. A, An extract of mutant NP 100 was prepared in the absence of glutamine. Anthranilate synthase activity was determined by adding 200 μg of protein from isolate NP 100 to a reaction mixture containing the indicated amount of protein from either a crude extract of mutant I-15* (○) *or a crude extract of mutant I-12* (●). *Specific activity is expressed as units per milligram of NP 100 protein and is plotted against the quantity of added protein from I-12 or I-15. One unit is defined as 1 nmole of anthranilate produced per min at 37 C. B, An extract of mutant NP 100 was prepared in the presence of glutamine, and anthranilate synthase was assayed in the presence of added subunit E from isolate I-15* (○) *or subunit X from isolate I-12* (●) *as described above.*

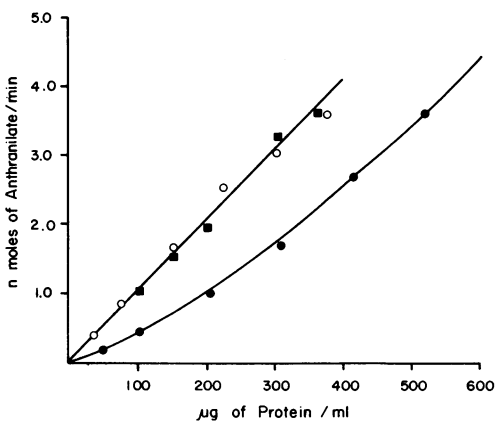

FIG. 4. *Activity of the glutamine-reactive anthranilate synthase as a function of protein concentration. An extract of mutant NP 100 was prepared in the presence* (○) *and absence* (●) *of glutamine. The activity of the glutamine-reactive enzyme was determined at various protein concentrations. The effect of adding excess subunit E (500 μg of crude extract protein from isolate I-15) or subunit X (500 μg of crude extract protein from isolate I-12) on the protein versus velocity curve for the extract prepared in the absence of glutamine is illustrated by the closed squares* (■).

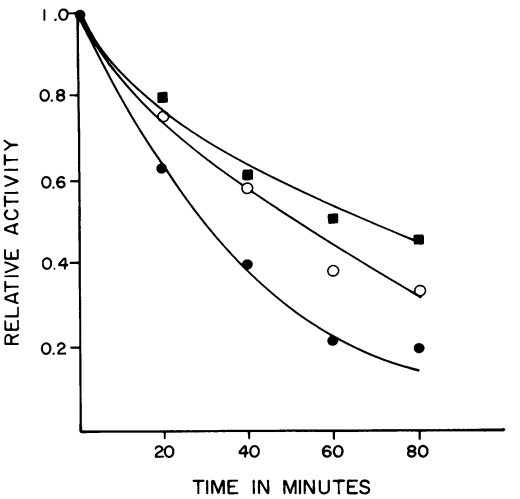

FIG. 6. *Relative activities of the anthranilate synthase subunits during repression. Relative activities are plotted as a function of the time after the addition of tryptophan to a culture of NP 40 growing in the presence of 0.1% casein hydrolysate. Symbols: EX complex* (●), *total subunit E* (○), *and total subunit X* (■). *A relative activity of 1.0 corresponds to the following specific activities: EX complex, 0.25; total subunit E, 0.40; total subunit X, 0.72.*

tion in activity is only 30% (Fig. 5B). The final specific activity, however, is the same.

Activity of the glutamine-reactive anthranilate synthase during repression and derepression. The activity of the EX complex was followed during repression in the prototroph NP 40. The data in Fig. 6 show that, under the conditions of repression of the tryptophan biosynthetic enzymes, the activity of the complex decreases at a faster rate than the decrease in either subunit. Since these extracts are prepared in the absence of glutamine, the activity of the glutamine-reactive anthranilate synthase is a function of the concentration of subunits E and X and reflects the degree of enzyme aggregation. Thus, under repressing conditions the relative decrease in the activity of the EX complex is greater than the decrease in either subunit.

The tryptophan biosynthetic enzymes in isolate NP 16, which has a defective 5-dehydroquinate synthase, were derepressed as described in Materials and Methods. In Fig. 7 the relative activities of the glutamine-reactive anthranilate synthase, subunits E and X, and tryptophan synthase B are plotted as a function of time after resuspension in casein hydrolysate. Again, the extracts were prepared in the absence of glutamine. The activity of the EX complex increases over 100-fold compared to a 30-fold increase in the total amount of subunit E and a 15-fold increase in subunit X. Tryptophan synthase B also increased 30-fold and is represented by the same symbols as subunit E (see Fig. 7). Thus, during derepression, the relative increase in the activity of the EX complex is greater than the increase in either subunit.

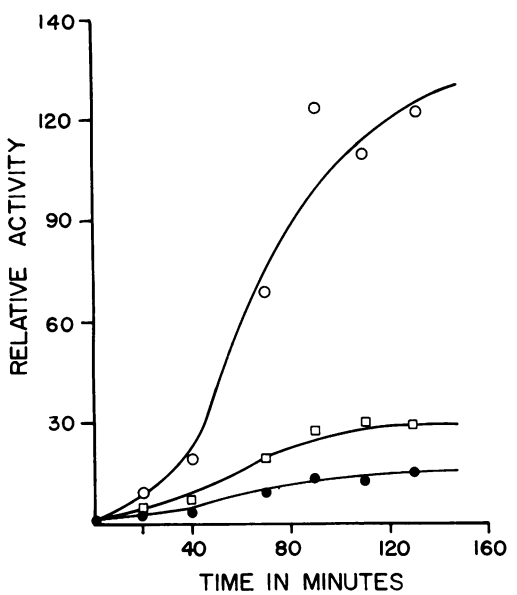

FIG. 7. *Derepression of the tryptophan biosynthetic enzymes in mutant NP 16. Relative activities of the EX complex (○), total subunit E (□), total subunit X (●), and tryptophan synthase B (□) are plotted as a function of the time in minutes after the cells were resuspended in casein hydrolysate. The relative activities of subunit E and tryptophan synthase B were the same and, for the purpose of clarity, are represented by the same symbols. A relative activity of 1.0 corresponds to the following specific activities: EX complex, 0.05; total subunit E, 0.24; total subunit X, 0.38.*

DISCUSSION

Effect of glutamine on the EX complex. In addition to the obvious role of glutamine as a substrate, this metabolite is important in maintaining the aggregational state of the glutamine-reactive anthranilate synthase. It appears that glutamine binds only to the EX aggregate for the following reasons. First, when partially purified subunits E and X are added to a reaction mixture containing glutamine, there is a 2- to 4-min lag before steady-state velocity is reached. Preincubation of subunits E or X with glutamine does not influence the kinetics of this lag. If, however, subunits E and X are preincubated together with glutamine, no lag is observed. Secondly, data with the glutamine analogue, DON, suggest that the binding site for glutamine is not available to the analogue in partially purified preparations of subunit X. Preincubation of subunit X with DON does not inactivate the activity of the complex with glutamine. When DON is preincubated with a mixture containing both subunits E and X, inhibition is observed. Whether the glutamine binding site is located on subunit X (but only becomes available when the EX complex is formed) or on both subunits E and X will require further experimentation with purified components.

Our present hypothesis can be summarized in the following equations:

$$E + X \rightleftharpoons EX \text{ (protein dependent)}$$

$$EX + \text{glutamine} \rightleftharpoons EX\ \text{glutamine}$$

This hypothesis provides the following explanation for the disproportional relationship of reaction velocity to protein concentration for the glutamine-reactive anthranilate synthase. The dilution of the enzyme into the reaction mixture results in dissociation of the EX complex,

and the degree of dissociation is dependent upon the concentration of subunits. At low protein concentrations almost complete dissociation of the EX complex results, and there is a 2- to 4-min lag before steady-state velocity is reached. This slow hysteretic response of the glutamine-reactive enzyme, therefore, represents the reassociation of subunits E and X after exposure to glutamine. If the extract is prepared in the presence of glutamine, there is no lag, and the stability and steady-state velocity of the EX complex are greater than in extracts prepared in the absence of glutamine. The differences in the steady-state velocity can be attributed to (i) the decreased rate of EX formation as a result of decreasing concentrations of subunits, or (ii) the instability of subunit E when it is not complexed with subunit X, or both (i) and (ii).

Possible physiological role of association and dissociation. It has been proposed that the association-dissociation reactions of an enzyme complex (6) may play an important role in metabolic regulation if (i) aggregation or disaggregation alters the kinetic or regulatory properties of the enzyme (or both), and (ii) the enzyme dissociates under physiological conditions. Our in vitro studies indicate that the anthranilate synthase complex in *B. subtilis* is a readily dissociable enzyme that loses catalytic activity with glutamine as a substrate upon dissociation. This enzyme complex is more readily disaggregated in the absence of glutamine than in the presence of glutamine. Available evidence indicates that in *B. subtilis* there is no detectable pool of glutamine (8), reflecting a stringent control over its synthesis. The activity of glutamine synthase is inhibited by glutamine (3, 4, 19), and the enzyme is repressed by the presence of 20 mM ammonium sulfate or 0.1% casein hydrolysate, or both, in the growth medium (3, 4, 19). Since all cultures were grown in the presence of 20 mM ammonium sulfate, glutamine synthase can be considered to be repressed. Although glutamine is critical in determining the level of the EX complex, in the absence of glutamine the concentration of the anthranilate synthase aggregate appears to be primarily the result of the concentration of subunits E and X. Hence, ordinary conditions of growth seem to correlate with low intracellular levels of glutamine, providing suitable threshold sensitivity for the association-dissociation phenomenon. If this is correct, then the role of association and dissociation may be a very significant aspect of in vivo regulation. During growth in the presence of tryptophan, the synthesis of subunits E and X will stop. In the absence of a large glutamine pool, this in vivo dilution will result in the dissociation of the EX aggregate, with the result that the activity of the glutamine-reactive anthranilate synthase will rapidly decrease. This is expected from the disproportional protein concentration versus reaction velocity relationships shown in Fig. 2 and 4. Similar observations have been reported with the glutamine-reactive anthranilate synthase from *Bacillus alvei* (2); that is, the activity of this enzyme from *B. alvei* was not a linear function of protein concentration, and there was a rapid decrease in the activity of the enzyme during repression (2).

Limitation of tryptophan will result in derepression of subunits E and X. This will increase the concentration of the EX complex which can bind glutamine. The binding of available glutamine to the EX complex would also tend to release the feedback control of glutamine synthase. Thus, these factors would combine to amplify the activity of the glutamine-reactive enzyme to a greater extent than the factor of increase in the level of subunits E or X (Fig. 7). This provides the potential for rapid synthesis of tryptophan in response to relatively small concentration changes, i.e., a threshold phenomenon. The hysteretic response may provide a buffer effect (5), resisting subtle or short-lived changes in the level of the controlling ligands. A slight decrease in the pool of tryptophan may cause some release from repression, but the system would respond slowly to this change as a result of the observed time lag before the catalytically functional EX-glutamine complex is formed. Similarly, these changes in the concentration of glutamine may not significantly increase tryptophan production because a lag will precede the formation of the EX-glutamine complex.

The mechanisms of repression and derepression may provide, in effect, an activity range that resembles the sensitivity of cooperative substrate interactions. In the case of the EX complex the molecular basis for the modulation of activity is protein-protein interactions between subunits E and X. Our in vitro studies indicate that the glutamine-reactive anthranilate synthase may be capable of responding rapidly and sensitively to changes in the level of tryptophan in vivo. It is interesting in this regard that no mutants of *B. subtilis* have been described which possess a feedback-resistant anthranilate synthase. All tryptophan analogue-resistant mutants isolated thus far are R^- constitutive and still possess a feedback-sensitive anthranilate synthase (7). It is possible that the association-dissociation reactions that

occur during repression or derepression are more efficient control mechanisms than feedback inhibition, a result consistent with previous data (7, 12). Thus, a feedback-resistant class of mutants may not excrete tryptophan because of the sensitivity of the repression control which is mediated through the interactions of subunits E and X.

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