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# ANTHRANILATE SYNTHETASE-ANTHRANILATE 5-PHOSPHORIBOSYLPYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE FROM *SALMONELLA TYPHIMURIUM*

## INACTIVATION OF GLUTAMINE-DEPENDENT ANTHRANILATE SYNTHETASE BY AGAROSE-BOUND ANTHRANILATE

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## Summary

Exposure of the anthranilate synthetase-anthranilate phosphoribosyltransferase enzyme complex (chorismate pyruvate-lyase (amino-accepting) EC 4.1.3.27 and *N*-(5′-phosphoribosyl)-anthranilate pyrophosphate phosphoribosyltransferase, EC 2.4.2.18) from *Salmonella typhimurium* to agarose-bound anthranilate led to the slow inactivation of glutamine-dependent anthranilate synthetase activity, an activity dependent on protein-protein interaction in the enzyme complex. Region I of phosphoribosyltransferase, the location of the enzyme complex glutaminase activity, is the site of alteration. Phosphoribosyltransferase and $NH_3$-dependent anthranilate synthetase activities and tryptophan regulation of phosphoribosyltransferase were unaffected by the anthranilate matrix. The molecular weight (280 000) and subunit molecular weight (62 000) of the enzyme complex eluted from an anthranilate matrix were identical to those of enzyme complex purified by classical methodology. The enzyme complex could be partially protected against inactivation by storing in 0.1 M L-glutamine or 30% glycerol and completely protected by storing in 50% glycerol at −18° C. Evidence is presented that the anthranilate matrix acts as a hydrophobic matrix and may be binding to and altering a hydrophobic region in the enzyme complex. The anthranilate matrix provides a convenient tool for altering a specific region of an enzyme complex without covalent modification. At the same time, the results demonstrate that affinity matrices are not necessarily innocuous but may subject macromolecules to an adverse environment not previously recognized.

## Introduction

In *Salmonella typhimurium*, the bifunctional enzyme complex anthranilate synthetase (EC 4.1.3.27)-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (EC 2.4.2.18) catalyzes the first two steps unique to tryptophan biosynthesis [1]. The tetrameric complex is composed of two monomers each of anthranilate synthetase and phosphoribosyltransferase [2], the respective products of the *trp* A and *trp* B genes of the tryptophan operon. Anthranilate synthetase catalyzes the reaction shown in Eqn 1.

$$\text{chorismate} + NH_3 \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{pyruvate} + H_2O \quad (1)$$

Association of anthranilate synthetase with phosphoribosyltransferase allows either $NH_3$ or L-glutamine to be utilized as a substrate. The reaction with L-glutamine is shown in Eqn 2.

$$\text{Chorismate} + \text{L-glutamine} \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{pyruvate} + \text{L-glutamate} + H^+ \quad (2)$$

Associated or unassociated phosphoribosyltransferase catalyzes the reaction shown in Eqn 3.

$$\text{anthranilate} + \text{5-phosphoribosyl 1-pyrophosphate} \xrightarrow{Mg^{2+}} \textit{N}\text{-(5'-phosphoribosyl)-anthranilate} + PP_i \quad (3)$$

Anthranilate synthetase [3,4] and phosphoribosyltransferase in the complex [5] are subject to feedback inhibition by L-tryptophan. A single L-tryptophan binding site has been detected on the anthranilate synthetase subunit whether associated [2] or unassociated [4] with phosphoribosyltransferase. It is believed that L-tryptophan binds to the anthranilate synthetase subunit and elicits a conformational change in both anthranilate synthetase and phosphoribosyltransferase, causing an inhibition of all enzyme complex activities [6].

The glutamine binding site for the anthranilate synthetase reaction is located on the phosphoribosyltransferase subunit [7], a single polypeptide chain serving two enzymatic functions in the enzyme complex. Only the amino-terminal 40% of phosphoribosyltransferase (region I) is necessary for glutamine-dependent anthranilate synthetase activity [8]. A mechanism has been proposed [7] in which region 1 acts as a glutamine amido-transferase supplying the $\delta$-amide of glutamine for the amination of chorismate. The carboxy-terminal 60% of phosphoribosyltransferase (region II) is responsible for the phosphoribosyltransferase activity in the enzyme complex [8].

The present study is concerned with the structural alteration of a particular region in the enzyme complex which became evident during attempts to design a rapid and simple purification procedure for the enzyme complex using affinity chromatography. Marcus and Balbinder [9] have described the synthesis of various anthranilate-agarose matrices. In the present study, anthranilate linked to succinylamidohexamethylimino-agarose via its free amino group caused the inactivation of the glutaminase activity while not affecting $NH_3$-

dependent anthranilate synthetase activity, phosphoribosyltransferase activity or L-tryptophan inhibition of phosphoribosyltransferase.

## Methods

*Growth of cells and preparation of enzyme complex*

The enzyme complex was obtained from *S. typhimurium trp* E2 (ATCC 25566). Cultures were grown in 2800-ml Fernbach flasks containing 1 l of minimal salts [10] supplemented with 0.2% glucose, 0.005% acid-hydrolysed casein and 2 μg per ml of indole. Growth was started with 1 ml of a 22-h nutrient broth culture. The cultures were incubated at 37° C, with shaking, for 22 h. A yield of 2 g wet weight of cells per 1 l of medium, derepressed for the tryptophan enzymes, was routinely achieved. All of the remaining procedures were performed between 0 and 4° C except where noted. The cells were harvested by centrifugation, washed twice in 0.05 M potassium phosphate (pH 7.4) and resuspended in four volumes of Buffer 1 (0.05 M potassium phosphate, pH 7.4, containing 0.1 mM EDTA and 0.4 mM 2-mercaptoethanol). The cells were disrupted by sonication using a Branson S75 Sonifier. The cells were sonicated for 1 min at full power for every 2.9 g wet weight of cells in order to yield maximum enzyme complex activities. The enzyme complex was partially purified by the procedure of Henderson et al. [11]. The last step used of this procedure was a high speed spin separating the soluble from a membrane-bound form of the enzyme complex [11,12]. The ultracentrifuge supernatant, containing the soluble form, served as the starting material for the affinity chromatography work and the further purification of the enzyme complex, and will be referred to as the normal enzyme complex.

*Preparation and analysis of agarose matrices*

Sepharose 2B beads were activated by CNBr (250 mg per ml of packed gel volume) according to the method of Cuatrecasas [13]. The synthesis of aminohexamethylimino-agarose (HMD-agarose), succinylamidohexamethylimino-agarose (succinyl-HMD-agarose) and anthranilate linked via its free amino group to succinyl-HMD agarose (matrix A) using a carbodiimide-catalyzed reaction have been described [9]. Anthranilate was also linked through its free amino group to succinyl-HMD-agarose by an alternative procedure based upon the method of Cuatrecasas and Parikh [14] using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate as a catalyst. The dried activated gel was washed with one volume of 0.1 M potassium phosphate, pH 7.0, saturated with anthranilate, suspended in four volumes of this solution and then shaken for 20 h at 0—4° C. The final derivative (matrix B) was washed with 200 volumes of distilled water.

The amount of anthranilate bound per ml of packed gel volume was estimated using the method of Failla and Santi [15]. Matrix A or B, 0.1 ml, was solubilized by incubating in 9.9 ml of 1 M NaOH containing 0.1% $NaBH_4$ for 2 h at 75° C with shaking. A standard curve was generated by treating 0—0.2 mM anthranilate in the same manner. The amount of anthranilate in the various solutions was measured using a Farrand spectrofluorimeter equipped with a thermostated sample chamber maintained at 66° C (excitation 325 nm; emis-

sion 400 nm). Succinyl-HMD-agarose did not interfere with the standard curve measurements.

*Analytical procedures*

Anthranilate synthetase activity was measured using either L-glutamine or $NH_3((NH_4)_2SO_4)$ as the amino donor. L-Glutamine-dependent anthranilate synthetase activity was assayed using the method of Henderson et al. [11]. $NH_3$-dependent anthranilate synthetase assays were performed using a procedure modified from that of Zalkin and Kling [3]. Each reaction mixture contained 0.3 mM chorismate, 50 mM $(NH_4)_2SO_4$, 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 50 mM freshly prepared triethanolamine · HCl, pH 8.9, and a limiting amount of enzyme. Phosphoribosyltransferase activity was assayed according to Henderson et al. [5] except that 2-mercaptoethanol was substituted for dithiothreitol A 3-ml volume was used in all of the above assay procedures. 1 unit of anthranilate synthetase or phosphoribosyltransferase activity is defined as the quantity of enzyme catalyzing the production or disappearance respectively of 1 nmol of anthranilate per min at 25° C. Assays were performed with a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter equipped with a thermostated sample compartment (excitation 320 nm; emission 400 nm).

Phosphoribosyltransferase region I glutaminase activity was measured spectrophotometrically by coupling to glutamate dehydrogenase [16,17]. The reaction mixture contained in 1 ml: 0.24 mM NADH, 50 mM α-ketoglutarate, 100 mM potassium phosphate, pH 7.4, 5 mM L-glutamine, 5 mM EDTA, 118 μM chorismate, 24 μM 2-mercaptoethanol, 0.35 mg glutamate dehydrogenase and a limiting amount of enzyme. All enzymes were dialyzed for 20 h before use against 1000 volumes of Buffer 1 with a change of buffer after 10 h. Control assays were run in the absence of L-glutamine to correct for background activity. The velocities determined with the assay mixture minus chorismate or including 0.5 mM L-tryptophan (all enzyme complex glutaminase activity inhibited) were averaged and subtracted from the velocity determined with the complete assay mixture. The corrected velocity, determined for two enzyme complex concentrations, was used as a measure of phosphoribosyltransferase region I activity. The rate of NADH oxidation was followed at 340 nm ($\epsilon$ = 6.22 mM) using a Gilford 240 spectrophotometer with a thermostated sample compartment maintained at 25° C. 1 unit of glutaminase activity is defined as the quantity of enzyme catalyzing the production of 1 nmol of $NH_3$ per min at 25° C.

Protein was determined either by a microbiuret procedure [18] or by the method of Warburg and Christian [19]. Specific activity refers to units per mg of protein.

*Column procedures*

Large columns of matrix A or B with bed volumes of 34 ml were packed in 2.5 × 15 cm plastic syringes. Ultracentrifuge supernatant was applied in 5—6-ml batches and then the flow stopped for 20 min. This process was repeated until all the sample had entered the column. The column was then washed with 500 ml of Buffer 1. The enzyme complex was eluted with either 0.05 M triethanolamine · HCl, pH 9.5, containing 1 mM 2-mercaptoethanol and

20% glycerol, or 0.05 M carbonate/bicarbonate, pH 9.5, containing 1 mM 2-mercaptoethanol. Elution fractions were collected in vessels containing enough 1 M potassium phosphate, pH 7.4, so that the final concentration of potassium phosphate would be 0.1 M. Protein in the pooled elution effluent was precipitated using 50% saturated $(NH_4)_2SO_4$, stirred for 45 min and then centrifuged at 24 000 × *g* for 15 min. The pellet was dissolved in Buffer 2 (Buffer 1 but with 0.1 M potassium phosphate) or the buffer indicated. No further treatment of the matrices was required between use.

Gel filtration was performed on a 1.6 × 90 cm column of Biogel A5m arranged for upward elution. A pressure head of 60 cm was used resulting in a flow rate of 15.8 ml per h. The column was washed with Buffer 2 and stored between use in the same buffer containing 0.02% $NaN_3$. The void volume was determined using Blue Dextran 2000 and the column was calibrated with the following proteins: urease (mol.wt 480 000), glutamate dehydrogenase (mol.wt 320 000), pyruvate kinase (mol.wt 237 000), aldolase (mol.wt 160 000) and bovine serum albumin dimer (mol.wt 136 000) and monomer (mol.wt 68 000). Fractions of 3 ml were taken.

Anion-exchange chromatography was done with either DEAE-cellulose or DEAE-Biogel A. The resins were equilibrated with Buffer 2 containing 30% glycerol by monitoring the conductivity (with a CDM2e Radiometer conductivity meter) and pH (with a Radiometer PHM62 pH meter) of the suspension. Small columns (1.5 × 26 cm or 1.6 × 34 cm) were developed with a 300-ml linear gradient of 0—0.3 M $NH_4Cl$ in equilibration buffer and 3-ml fractions were collected. Large columns (2.5 × 41 cm) were developed with a 400-ml linear gradient of 0—0.3 M $NH_4Cl$ in equilibration buffer and 6-ml fractions were collected.

*Purification of altered enzyme complex*

Protein, in Buffer 2, eluted from a column of freshly synthesized matrix B was gel filtered on a column of Biogel A5m. Fractions of high phosphoribosyltransferase specific activity were pooled and diluted 1 : 1 with 60% glycerol containing 0.1 mM EDTA and 0.4 mM 2-mercaptoethanol and then applied to a 1.5 × 26 cm column of DEAE-cellulose. The enzyme complex eluted as a single peak. Fractions of high phosphoribosyltransferase specific activity were pooled and $(NH_4)_2SO_4$ was added to 50% saturation. After stirring for 45 min, the suspension was centrifuged at 24 000 × *g* for 15 min and the pellet dissolved in Buffer 2 containing 50% glycerol and stored at −18° C.

*Purification of normal enzyme complex*

Ultracentrifuge supernatant, derived from 16 l of growth media, was dialyzed against 130 volumes of Buffer 2 containing 30% glycerol for 12 h. The dialyzed solution was applied to a 2.5 × 41 cm DEAE-cellulose column. Fractions of peak phosphoribosyltransferase specific activity were pooled and concentrated with $(NH_4)_2SO_4$ added to 50% saturation. After the suspension was stirred for 45 min, it was centrifuged at 24 000 × *g* for 15 min and the pellet dissolved in Buffer 2. The solution was then gel filtered on a column of Biogel A5m. Fractions of peak glutamine-dependent anthranilate synthetase specific activity were pooled and diluted 1 : 1 with 60% glycerol containing 0.1 mM

EDTA and 0.4 mM 2-mercaptoethanol. This solution was then applied to a 1.6 × 34 cm column of DEAE-Biogel A. The enzyme complex was eluted in a single protein peak. Fractions of high glutamine-dependent anthranilate synthetase specific activity were pooled and concentrated using 60% saturated $(NH_4)_2SO_4$. The suspension was stirred for 1 h and then centrifuged at 24 000 × *g* for 15 min. The pellet was dissolved in Buffer 2 containing 50% glycerol and stored at −18° C.

*Electrophoresis*

Standard gel electrophoresis was done according to Davis [20] on 5% acrylamide with a Tris/glycine, pH 8.3, reservoir buffer. Protein in the gels was stained using Coomassie brilliant blue. Sodium dodecyl sulfate electrophoresis was performed according to the method of Weber and Osborn [21]. After completion of the runs the gels were removed from the tubes, exhaustively treated with 1.6 M acetic acid in 45% methanol to remove sodium dodecylsulfate (Chan, S., personal communication) and then stained as described by Weber and Osborn [21]. The gels were destained with the methanol/acetic acid mixture. The distances of migration of intensities were measured using a linear transport attachment on a Gilford 240 spectrophotometer.

*Chemicals*

The following materials were obtained from Sigma Chemical Co.: sodium dodecyl sulfate, 5-phosphoribosyl 1-pyrophosphate, L-glutamine, barium chorismate, ovalbumin, bovine serum albumin, bovine liver glutamate dehydrogenase, jack bean urease, rabbit skeletal muscle pyruvate kinase and rabbit muscle aldolase. All proteins were crystalline preparations of the highest purity available. Barium chorismate was converted to its potassium salt before use. Enzyme grade $(NH_4)_2SO_4$ came from Mann Research Labs; anthranilate (once recrystallized), succinic anhydride, acrylamide and bis-acrylamide from Eastman Chemical Co.; *N,N,N',N'*-tetramethylethylenediamine from Matheson, Coleman and Bell; and $(NH_4)_2S_2O_8$ from E-C Apparatus Corp. *N*-Hydroxysuccinimide, CNBr, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate and hexamethylenediamine were products of Aldrich Chemical Corp. DEAE-cellulose (DE-52) came from Reeve-Angel Inc.; DEAE-Biogel A and Biogel A5m from Bio-Rad Labs; Sepharose 2B and Blue Dextran 2000 from Pharmacia Fine Chemicals. All other chemicals were of reagent grade.

## Results

*Affinity matrices*

Three different synthetic preparations of matrix A yielded slurries with 5, 4, and 3 μmol of anthranilate bound per ml of packed gel volume. The one synthetic preparation of matrix B had 9 μmol of anthranilate bound per ml of packed gel volume. Matrices A and B behaved similarly in their affinity for the enzyme complex and contaminating protein.

Enzyme complex bound tightly to both matrices A and B (Fig. 1). Active enzyme complex could be eluted from an anthranilate matrix with either a triethanolamine · HCl or a carbonate/bicarbonate buffer of pH 9.5 or low ionic

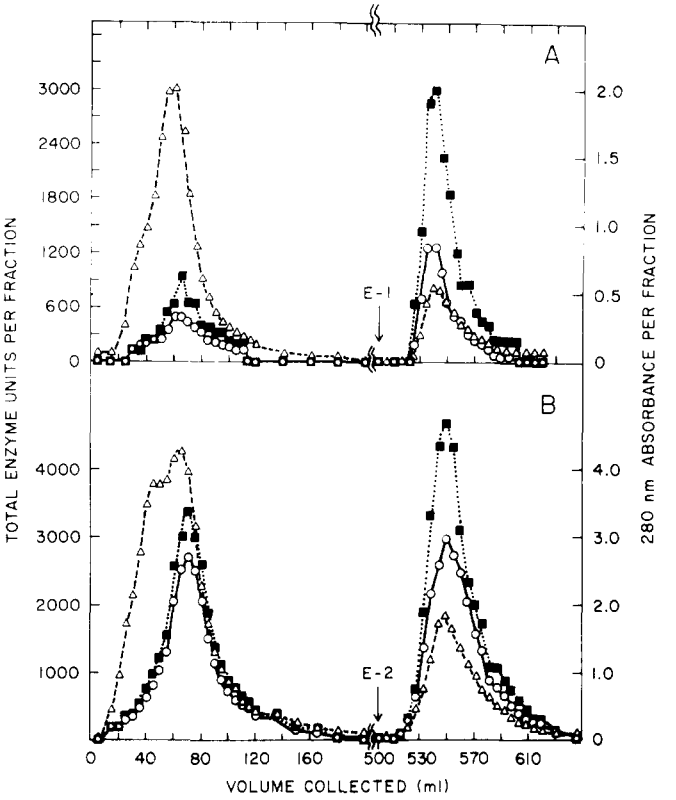


Fig. 1. Binding of the enzyme complex to the two anthranilate affinity matrices. Enzyme assays, matrix synthesis, and column methodology are described under Materials and Methods. (A) Binding to matrix A. (B) Binding to matrix B. E-1, elution by triethanolamine, pH 9.5, buffer; E-2, elution by carbonate/bicarbonate, pH 9.5, buffer; ○, glutamine-dependent anthranilate synthetase activity; ■, phosphoribosyltransferase activity; △, 280 nm absorbance. Matrix A had 3 μmol of anthranilate and matrix B had 9 μmol of anthranilate bound per ml of packed gel volume. Matrix B was saturated in order to determine its enzyme complex capacity.

strength in the form of 1 mM potassium phosphate, pH 7.4. Buffer 1 containing 1 M NaCl eluted no active enzyme complex and only a trace of protein. Active enzyme complex of expected yield could be eluted by high pH after a 1 M NaCl wash. Thus, the anthranilate matrix did not act as an ion-exchange resin. Buffer 1 containing 0.1 M L-glutamine also did not elute any active enzyme complex.

Neither agarose nor succinyl-HMD-agarose columns bound the enzyme complex. HMD-agarose bound the enzyme complex quite well but the active complex was eluted by 0.5 M NaCl in Buffer 1. Rerunning the eluted protein on an anthralinate matrix did not appreciably improve the phosphoribosyltransferase activity. In one experiment, enzyme complex, eluted from matrix A, with a phosphoribosyltransferase specific activity of 770, was rerun on the same matrix. The specific activity increased to only 850 after the second column with an 85% recovery of phosphoribosyltransferase units. Only a trace of protein was detected in the wash fractions.

*Alteration of enzyme complex*

A typical column profile of an anthranilate matrix is depicted in Fig. 1A. The eluted phosphoribosyltransferase units represent a 62% yield of units applied while only 34% of the applied glutamine-dependent anthranilate synthetase units could be recovered. The yield of glutamine-dependent anthranilate synthetase units eluted from either matrix A or B in 10 experiments averaged 24% less than the recovered phosphoribosyltransferase units. The percent decrease depended on the time it took to assay the enzyme complex after elution from an anthranilate matrix. For convenience, enzyme complex that has been eluted from an anthranilate matrix will be referred to hereafter as the altered enzyme complex.

A comparison between the catalytic stability of the normal and altered enzyme complexes is shown in Fig. 2. A quantitative relationship among the enzyme complex activities is described by following the ratio of activities. The glutamine-dependent anthranilate synthetase activity of the altered enzyme complex is much less stable when compared to the same activity of the normal enzyme complex. The altered enzyme complex lost 90% of its glutamine-dependent anthranilate synthetase activity after 2 days. $NH_3$-dependent anthranilate synthetase and phosphoribosyltransferase activities of the altered enzyme complex differ only slightly from those activities of the normal enzyme complex.

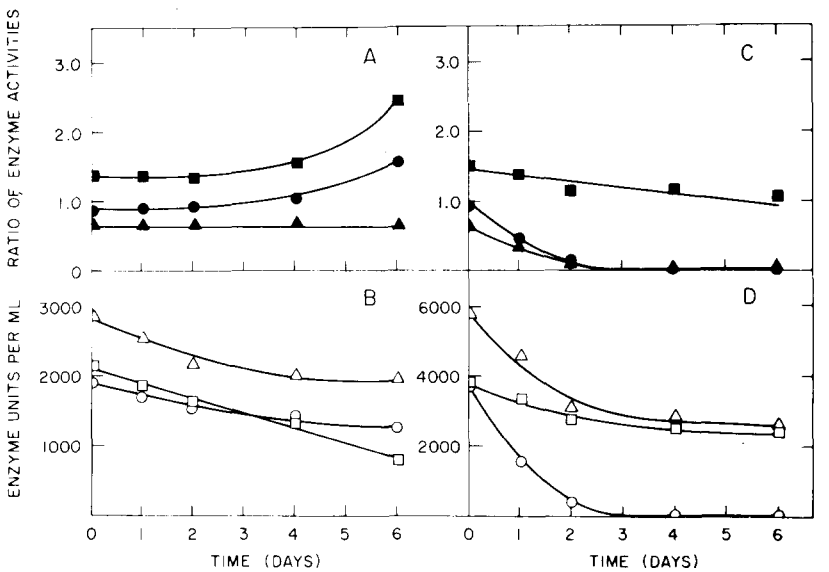


Fig. 2. Stability of catalytic activity of the normal and altered enzyme complexes. Zero time refers to the period immediately after ultracentrifugation for the normal enzyme complex or immediately after elution from an anthranilate matrix for the altered enzyme complex. Both enzyme complexes were stored at 0—4° C in Buffer 2. Methods of assay are described under Materials and Methods. (A) Ratio of enzyme activities for normal enzyme complex. •, ratio of glutamine-dependent anthranilate synthetase to phosphoribosyltransferase activities; ■, ratio of $NH_3$-dependent anthranilate synthetase to phosphoribosyltransferase activities; ▲, ratio of glutamine-dependent anthranilate synthetase to $NH_3$-dependent anthranilate synthetase activities . (B) Enzyme units per ml for normal enzyme complex. ○, glutamine-dependent anthranilate synthetase activity; △, $NH_3$-dependent anthranilate synthetase activity; □, phosphoribosyltransferase activity. (C) Ratio of enzyme activities for altered enzyme complex. Symbols are as in A. (D) Enzyme units per ml for altered enzyme complex. Symbols are as in B.

Incubation of the normal enzyme complex with elution buffers for 25 h or with agarose, HMD-agarose or succinyl-HMD-agarose for 48 h did not generate any altered-type enzyme complex. In order to produce an unstable glutamine-dependent anthranilate synthetase activity, there is an apparent need for the enzyme complex to bind to and be eluted from an anthranilate matrix. Enzyme complex maintained on a large column of anthranilate matrix for 48 h and then eluted and quickly assayed, possessed a normal ratio of glutamine-dependent anthranilate synthetase to phosphoribosyltransferase activities of 0.76. Inactivation of the enzyme complex does not occur until after the enzyme complex is eluted from an anthranilate matrix.

Anthranilate is sparingly soluble in water, reaching only 10-15 mM concentrations in a saturated solution at 0—4° C. Normal enzyme complex incubated for 48 h in Buffer 1 containing 8 mM anthranilate and then incubated 24 h in the same solution diluted 1 : 1 with carbonate/bicarbonate elution buffer (final pH 9.1) showed no alteration in activities. However, the anthranilate matrix milieu cannot be duplicated in solution. The minimum concentration of anthranilate per $cm^3$ of solid agarose, estimated by drying an aliquot of matrix B and measuring the volume of the residue, is equivalent to 210 mM. This is, of course, because the agarose-bound anthranilate is not in solution but is localized on the surface of the agarose where the matrix enzyme complex interaction is taking place.

*Characterization of altered enzyme complex*

There is a direct parallel between the loss of phosphoribosyltransferase region I glutaminase activity and the loss of glutamine-dependent anthranilate synthetase activity (Table I). The fact that there is only a slight decrease in

TABLE I

COMPARISON BETWEEN THE CATALYTIC ACTIVITY AND REGULATION OF THE PARTIALLY PURIFIED NORMAL AND ALTERED ENZYME COMPLEXES

Enzyme assays are described under Materials and Methods. The normal enzyme complex was in Buffer 2 and the altered enzyme complex in Buffer 2 containing 10 mM L-glutamine. The altered enzyme complex was assayed 8 days after elution from an anthranilate matrix.

| | Normal enzyme complex | Altered enzyme complex | Decrease (%) |
|---|---|---|---|
| Ratio of AS-gln to AS-$NH_3$* | 0.61 | 0.06 | 90 |
| Ratio of glutaminase to AS-$NH_3$** | 0.23 | 0.02 | 91 |
| Ratio of AS-Gln to phosphoribosyltransferase*** | 0.81 | 0.06 | 93 |
| Ratio of glutaminase to phosphoribosyltransferase† | 0.30 | 0.02 | 93 |
| Ratio of AS-$NH_3$ to phosphoribosyltransferase†† | 1.33 | 1.10 | 17 |
| Percent tryptophan inhibition of phosphoribosyltransferase††† | 59 | 54 | 8 |

* Ratio of glutamine-dependent anthranilate synthetase to $NH_3$-dependent anthranilate synthetase activities.

** Ratio of phosphoribosyltransferase region I glutaminase to $NH_3$-dependent anthranilate synthetase activities.

*** Ratio of glutamine-dependent anthranilate synthetase to phosphoribosyltransferase activities.

† Ratio of phosphoribosyltransferase region I glutaminase to phosphoribosyltransferase activities.

†† Ratio of $NH_3$-dependent anthranilate synthetase to phosphoribosyltransferase activities.

††† Percent inhibition by 20 μM tryptophan of phosphoribosyltransferase activity.

tryptophan inhibition of the altered enzyme complex indicates that the complex has not dissociated and regulation within the complex has not been disrupted.

The apparent $K_m$ for L-glutamine was determined for the normal and altered enzyme complexes from the intercepts of double-reciprocal plots of initial velocity and glutamine concentration in the presence of excess $Mg^{2+}$ and chorismate. Altered, (4 days after elution) and normal enzyme complexes both were found to have an apparent $K_m$ of 1.2 mM.

It has been shown with the anthranilate synthetase phosphoribosyltransferase enzyme complex of *Escherichia coli* that glutamine reverses tryptophan inhibition of phosphoribosyltransferase activity in the absence of chorismate [22]. Glutamine was found to reverse tryptophan inhibition of the normal enzyme complex (Fig. 3) while not inhibiting phosphoribosyltransferase activity. The concentration of glutamine required for one-half maximal reversal of tryptophan inhibition of phosphorybosyltransferase was 2 mM. Glutamine did not reverse tryptophan inhibition of the altered enzyme complex and apparently had a small inhibitory effect on the phosphoribosyltransferase (Fig. 3).

*Reversal and protection*

Dialyzing the altered enzyme complex against 1000 volumes of Buffer 1 for 20 h or against 450 volumes of Buffer 1 containing 30% glycerol for 13 h, or gel filtration on Biogel A5m did not reactivate lost glutamine-dependent

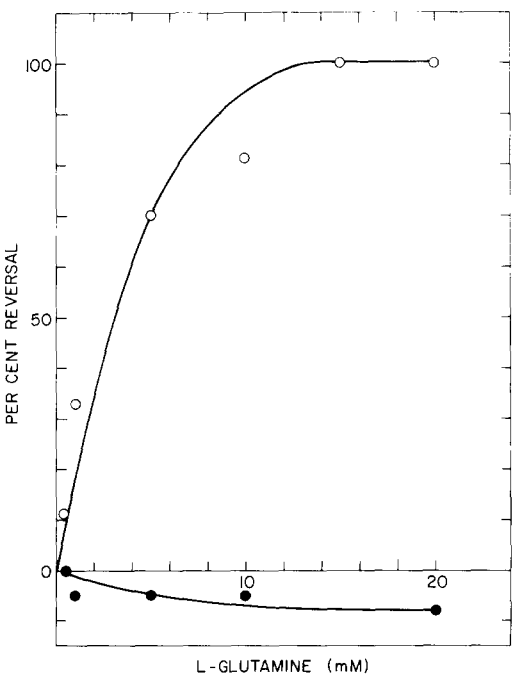


Fig. 3. Effect of L-glutamine on tryptophan inhibition of the phosphoribosyltransferase activity of normal and altered enzyme complexes. Inhibition was by 4 $\mu$M tryptophan. The phosphoribosyltransferase assay is described under Materials and Methods. ●, altered enzyme complex 4 days after elution; ○, normal enzyme complex.

anthranilate synthetase activity. Incubation of the altered enzyme complex in nine volumes of anthranilate matrix wash fraction for 48 h or in 0.1 M potassium phosphate, pH 7.4, containing all 0.1 M combinations of EDTA, $MgCl_2$ (not with EDTA), L-glutamine and 2-mercaptoethanol for 3 and 23 h also failed to restore lost activity.

Inactivation of the glutamine-dependent anthranilate synthetase activity could be slowed down by storing the altered enzyme complex in Buffer 2 containing either 0.1 M L-glutamine or 30% glycerol or as an $(NH_4)_2SO_4$ precipitate. Complete protection was achieved by storing the altered enzyme complex in Buffer 2 containing 50% glycerol at −18° C.

*Properties of altered and normal enzyme complexes*

The glutamine-dependent anthranilate synthetase and phosphoribosyltransferase activities of the altered enzyme complex migrated on a Biogel A5m gel filtration column as a single concerted peak with a molecular weight of 280 000 (Fig. 4). The small enzyme complex peak at the void volume is probably either the remainder of a membrane-bound form [11,12] or an aggregate. Standard polyacrylamide gel electrophoresis of the purified altered enzyme complex, stained for protein, showed one major band containing 60% of the protein and 11 minor bands. The subunit molecular weight of purified altered enzyme complex was estimated by sodium dodecyl sulfate electrophoresis. One major band, representing 80% of the protein and corresponding to a molecular weight of 62 000 (S.D. ±2900), and five minor bands were visible.

Normal enzyme complex migrated on a Biogel A5m gel filtration column as a major peak with a molecular weight of 280 000 and a minor peak at the void volume. Standard polyacrylamide gel electrophoresis of the purified nor-

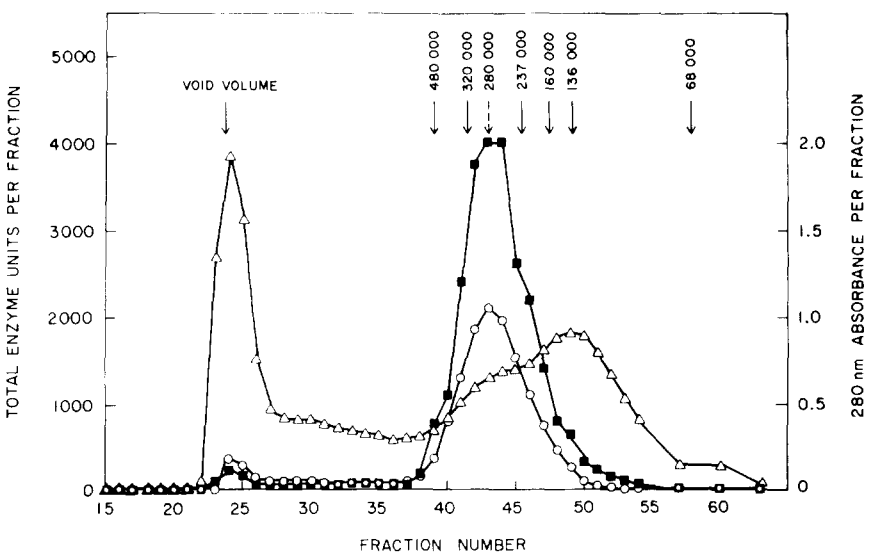


**Fig. 4. Biogel A5m fractionation of altered enzyme complex 1 day after elution. Enzyme assays, column methodology and column calibration for estimation of molecular weight are described under Materials and Methods. ○, glutamine-dependent anthranilate synthetase activity; ■, phosphoribosyltransferase activity; △, 280 nm absorbance.**

TABLE II

COMPARISON OF PURIFIED NORMAL AND ALTERED ENZYME COMPLEXES

Description of ratios is in footnote to Table I. Both normal and altered enzyme complexes were assayed immediately following the last purification step.

| | Normal enzyme complex | Altered enzyme complex |
|---|---|---|
| Ratio of AS-Gln to AS-$NH_3$ | 0.60 | 0.36 |
| Ratio of AS-Gln to phosphoribosyltransferase | 0.75 | 0.34 |
| Ratio of AS-$NH_3$ to phosphoribosyltransferase | 1.24 | 0.95 |

mal enzyme complex, stained for protein, showed one major band containing 93% of the protein and six minor bands. Table II compares the purified altered with the purified normal enzyme complex. The purified normal enzyme complex resembles the ultracentrifuge supernatant enzyme complex (Table I) whereas the altered enzyme complex differs radically from both.

## Discussion

Anthranilate matrices A and B bind enzyme complex apparently to the same extent. Even though the enzyme complex binds tightly to both matrices, the matrix capacity is quite low. Assuming a stoichiometry of one to one between the agarose-bound anthranilate and the adhering enzyme complex of molecular weight 280 000, less than 0.03% of the bound anthranilate molecules are utilized as sites for binding enzyme complex. The sites appear to have a wide range of binding affinities as evidenced by the skewed elution peak (Fig. 1).

The observation that the anthranilate matrix leads to the inactivation of phosphoribosyltransferase region I and not region II, the location of the anthranilate binding site, suggests that the binding of the enzyme complex is not via the active site. In support of this suggestion are the findings that: (a) a large number of different proteins bind to the anthranilate matrix; and (b) the anthranilate matrix does not require any special treatment (urea or sodium dodecylsulfate wash) between use suggesting that all of the proteins bind in the same manner and all are eluted by high pH or low ionic strength. An alternative explanation for the mode of binding of enzyme complex is that the anthranilate matrix acts as a hydrophobic affinity column [23]. Proteins on a hydrophobic matrix are characteristically eluted by high pH or low ionic strength and not by high ionic strength [24]. Since many proteins have hydrophobic regions in their structure, many proteins should be able to bind to and be eluted from an anthranilate matrix.

Enzyme complex eluted from an anthranilate matrix acquires an unstable glutamine-dependent anthranilate synthetase due to an alteration of phosphoribosyltransferase region I. The altered enzyme complex ultimately loses all of its glutamine-dependent anthranilate synthetase activity while retaining the phosphoribosyltransferase and $NH_3$-dependent anthranilate synthetase activities and the ability of phosphoribosyltransferase to be inhibited by tryptophan. The extent of alteration of phosphoribosyltransferase region I is unknown. No

large structure of the enzyme complex has been removed. The molecular weight of 280 000 determined by gel filtration for the altered enzyme complex agrees well with the molecular weight of 280 000 determined here for the normal enzyme complex and elsewhere for classically purified enzyme complex by sedimentation equilibrium [2]. The subunit molecular weight of 62 000 for altered enzyme complex is identical to that reported for classically purified enzyme complex [2]. Thus the alteration in phosphoribosyltransferase region I cannot be the result of extensive proteolysis. Attempts to reactivate the altered enzyme complex showed no evidence for the loss of a small molecule(s).

It is not known whether the acidity, hydrophobicity or some other property of the anthranilate matrix is responsible for altering the enzyme complex. Preliminary attempts to acid shock the enzyme complex using HCl have resulted in drastic alterations of all enzyme complex activities. Inclusion of 20% glycerol in the triethanolamine · HCl elution buffer, thus lowering the dielectric constant of the environment and weakening hydrophobic interactions, failed to prevent formation of an altered enzyme complex. However, the anthranilate matrix-enzyme complex interaction may be too strong to be overcome by 20% glycerol. Partial protection by 30% glycerol and complete protection by 50% glycerol suggests that the altered phosphoribosyltransferase region I is stabilized by reducing the polarity of the environment.

It is hypothesized that the alteration of the enzyme complex occurs in two steps. First, phosphoribosyltransferase region I, which may be normally buried in hydrophobic mortise of the enzyme complex, is "exposed" by the anthranilate matrix. The "exposed" region I is then slowly denatured by an antagonistic environment. In support of this hypothesis is the evidence that enzyme complex remained stable when maintained on an anthranilate matrix for 48 h and only after elution underwent slow inactivation. Whether the "exposure" is a result of a conformational change has not been determined.

A solid state effector, such as the anthranilate matrix, should be viewed as both a useful tool and a potential problem. The anthranilate matrix is an effective agent for selectively altering one region of the enzyme complex without covalent modification. The modification and purification of the enzyme complex takes place in one step. It is also important to recognize the possible reactivity of affinity matrices. Chemicals bound to an insoluble support may be present at very high local concentrations and may subject even the most stable protein to an adverse environment. These bound chemicals are not necessarily inert but may possess properties capable of modifying proteins and other molecules. Careful attention should be given to an affinity matrix before it is considered innocuous.

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