Anthranilate Synthetase. Purification and Properties of Component I from *Salmonella typhimurium**

H. Zalkin and D. Kling

ABSTRACT: Anthranilate synthetase component I has been purified from *Salmonella typhimurium*. In contrast to the native enzyme which is aggregated with component II, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase, component I is inactive with glutamine as amino donor for anthranilate synthesis. Component I utilizes NH_4^+. Glutamine and glutamate inhibit anthranilate synthesis and are competitive with NH_4^+, suggesting that component I retains a glutamine binding site. Initial velocity and product inhibition patterns are linear and are consistent with a sequential mechanism.

The enzyme retains sensitivity to end-product inhibition by tryptophan. Reciprocal plots for inhibition by tryptophan are linear and show that tryptophan is competitive with chorismate and noncompetitive with NH_4^+. There are no indications for homotropic or heterotropic interactions. The enzyme is inactivated by *p*-mercuribenzoate and *N*-ethylmaleimide. Partial reversal of *p*-mercuribenzoate inhibition by dithiothreitol indicates that one or more sulfhydryl groups are essential for activity. Component I is also rapidly inactivated by a substrate analog, bromopyruvate. Inactivation is prevented by chorismate and by tryptophan under some conditions. It is postulated that a basic group on the enzyme, susceptible to alkylation by bromopyruvate, participates in the removal of a proton from chorismate. Since the third proton of the pyruvate that is produced in the reaction is not derived from the solvent, it appears that the proton removed from chorismate may be transferred to the leaving enolpyruvyl group to form pyruvate. Gel filtration on Sephadex G-100 provides an estimate of 63,000 for the molecular weight of component I. The protein recombines with component II and reactivity with glutamine is restored.

Anthranilate synthetase catalyzes the first reaction specific to tryptophan biosynthesis, the glutamine- or ammonia-dependent conversion of chorismate into anthranilate (Gibson and Gibson, 1964; Gibson *et al.*, 1967). The reaction, with glutamine as amino donor, is seen in eq 1. In *Escherichia coli* (Ito and

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

Yanofsky, 1966), *Aerobacter aerogenes* (Egan and Gibson, 1966), and *Salmonella typhimurium* (Bauerle and Margolin, 1966), anthranilate synthetase is associated with the next enzyme of the pathway, PR transferase.[1] This enzyme complex is considered to be the native form of anthranilate synthetase in these bacteria. Native anthranilate synthetase has been purified from *E. coli* (Baker and Crawford, 1966) and *A. aerogenes* (Egan and Gibson, 1966). Anthranilate synthetase, separated from PR transferase, has been obtained in extracts of *E. coli* (Ito and Yanofsky, 1966) and *S. typhimurium* (Bauerle and Margolin, 1966) and was recently purified from *E. coli* (Ito and Yanofsky, 1967). The two components of the complex, anthranilate synthetase and PR transferase, have been designated component I and component II, respectively (Ito and Yanofsky, 1966). A distinguishing feature of anthranilate synthetase component I is an altered substrate specificity. Whereas the native enzyme will utilize glutamine or NH_4^+ for anthranilate synthesis, component I requires NH_4^+ and is inactive with glutamine.

The purpose of this communication is to report the partial purification of anthranilate synthetase component I and to describe some catalytic and regulatory properties of the enzyme.

Experimental Procedure

Materials and General Methods. Barium chorismate and chorismic acid were prepared as described by Gibson (1964) and by Edwards and Jackman (1965), respectively. Both were converted into the potassium salt and were assayed by enzymatic conversion into anthranilate before use. Enzymes and proteins were of the highest purity available. All other reagents and materials were obtained from commercial suppliers and were used without further purification.

A crude extract of *S. typhimurium trp A148* was used as the source of PR transferase. Cells were grown in salts (Vogel and Bonner, 1956), 0.4% glucose, 2 g/l. of acid-hydrolyzed casein, and 3 mg/l. of tryptophan for 21 hr at 37°. The cells were harvested by centrifugation, washed with 0.05 M potassium phosphate buffer

* From the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907. *Received June 10, 1968.* Supported by Grant GB-5841 from the National Science Foundation. Journal Paper No. 3404 of Purdue Agricultural Experiment Station.

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: PR transferase, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase.

(pH 7.4), and broken by sonic disruption in a solution containing 0.05 M potassium phosphate (pH 7.4), 0.1 mM EDTA, and 0.2 mM dithiothreitol. The crude extract was obtained following centrifugation at 39,000*g* for 45 min at 1°. Reconstitution of the anthranilate synthetase–PR transferase complex was accomplished by mixing 3.6 μg of anthranilate synthetase component I (purified in the absence of glycerol) with 0.39 mg of *trp A148* extract in 0.1 ml at room temperature for 3 min.

Molecular weight estimation by gel filtration was done according to the method of Andrews (1964) using a 1.4 × 36 cm column of Sephadex G-100. The column was equilibrated with 0.05 M Tris-HCl (pH 7.5) plus 1 mM dithiothreitol. Anthranilate synthetase component I (0.12 mg) was applied to the column together with 1.5 mg of horse heart cytochrome *c* (mol wt 12,400), 0.2 mg of adenylate kinase (mol wt 21,000), 2 mg of horseradish peroxidase (mol wt 40,000), 5 mg of bovine serum albumin (mol wt 67,000), and 0.2 mg of yeast hexokinase (mol wt 96,600). Fractions of 12 drops were collected and enzymes were located in fractions by enzymatic assay. Methods for the assay of adenylate kinase (Oliver, 1955), hexokinase (Kornberg, 1950), and peroxidase (Chance and Maehly, 1955) have been described. Bovine serum albumin was located by its absorbance at 280 mμ and cytochrome *c* by absorbance at 412 mμ. The fraction number containing the peak of activity for each protein is plotted against log molecular weight.

Polyacrylamide disc gel electrophoresis was performed as described by Davis (1964) except that persulfate was removed from the gels by electrophoresis (Mitchell, 1967), using buffer containing 1 mM dithiothreitol, prior to application of the sample. Protein was determined by the method of Lowry *et al.* (1951).

Anthranilate Synthetase Assay. For assay of component I activity, reaction mixtures contained 0.3 mM potassium chorismate, 50 mM ammonium sulfate, 10 mM magnesium chloride, 50 mM triethanolamine-HCl (pH 8.9), 2 mM thioglycerol, and a limiting amount of enzyme. The final pH was 8.5–8.6. For assay of the native glutamine-reactive activity, reaction mixtures contained 0.3 mM potassium chorismate, 5 mM glutamine, 10 mM magnesium chloride, 50 mM potassium phosphate (pH 7.4), and a limiting amount of enzyme. In each case the final volume was 1.0 ml. The initial rate of anthranilate formation was determined at 22–23° using an Aminco-Bowman spectrophotofluorimeter. The activation wavelength was 325 mμ and emission wavelength, 400 mμ. For some experiments a standard curve relating fluorescence intensity to anthranilate concentration was used. Any deviations from this assay procedure are specifically noted in the tables and figures.

A unit of activity represents the formation of 1 mμmole of anthranilate/min. Specific activity is defined as units per milligram of protein. Initial velocities are expressed as millimeters of relative fluorescence per minute for the data in all figures.

Inhibition by NH_4^+ Analogs. Inhibition by 0.1 M neutralized *N*-methylhydroxylamine was obtained in reaction mixtures containing 0.5 mM potassium chorismate, 10 mM magnesium chloride, 0.1 M triethanolamine-HCl (pH 8.9), 2 mM thioglycerol, 10 mM ammonium sulfate, and enzyme. To observe "hydroxamate" formation, incubation was for 20 min at 25°. The reaction was terminated with 0.25 ml of a mixture containing equal amounts of 24% trichloroacetic acid, 6 N hydrochloric acid, and 10% ferric chloride in 0.02 N hydrochloric acid (Woolfolk *et al.*, 1966). The color due to "hydroxamate" formation was measured at 500 mμ. Inhibition of anthranilate synthetase activity by methylamine, dimethylamine, trimethylamine, and diethylamine (all at final concentration of 25 mM) was observed in standard assay mixtures with ammonium sulfate reduced to 2 mM.

Inactivation by Bromopyruvate. Anthranilate synthetase component I (115 μg) was incubated with 10 mM bromopyruvate and 0.04 M potassium phosphate buffer in a final volume of 0.1 ml at room temperature. Aliquots (0.02 ml) were removed at timed intervals and added directly to 0.98 ml of the standard assay solution containing 4 mM thioglycerol. Inactivation by *p*-mercuribenzoate and *N*-ethylmaleimide was done in an identical way.

Formation of Pyruvate in $[^3H]H_2O$. One reaction mixture contained 0.70 mM potassium chorismate, 0.1 M triethanolamine-HCl (pH 8.9), 20 mM magnesium chloride, 4 mM thioglycerol, 0.16 M ammonium sulfate, 0.04 ml of $[^3H]H_2O$ (1.41×10^8 cpm), and 46 μg of anthranilate synthetase component I. The volume was 0.5 ml. A control reaction mixture was identical except that chorismate was replaced by 20 mM pyruvate. The pyruvate kinase reaction was used for a positive control. The reaction mixture contained 30 mM potassium phosphoenolpyruvate, 0.13 M Tris-HCl (pH 7.5), 26 mM magnesium chloride, 26 mM potassium chloride, 26 mM ADP, 0.02 ml of $[^3H]H_2O$ (7.22×10^7 cpm), and 100 μg of pyruvate kinase. Final volume was 0.38 ml. All reaction mixtures were incubated for 30 min at 37°. Reactions were terminated by addition of 9.5 ml of cold water and were applied to 0.8 × 8 cm columns of Dowex 1 (Cl^-). Columns were washed with 10 ml of water and the pyruvate was obtained by stepwise elution with ammonium chloride. The amount of pyruvate in each fraction was determined by enzymatic assay. Fractions containing pyruvate were lyophilized, dissolved in 0.5 ml of water, and 0.2-ml aliquots were counted for radioactivity in a dioxane–naphthalene system in a Beckman CPM-100 liquid scintillation counter.

Enzyme Preparation. Anthranilate synthetase component I was purified from *S. typhimurium trp BEDC43.* Cells were grown for 24 hr at 37° in salts (Vogel and Bonner, 1956), 0.4% glucose, and 2 mg/l. of tryptophan. These growth conditions allow derepression of anthranilate synthetase. Cells were harvested by centrifugation and washed with 0.05 M potassium phosphate buffer (pH 7.4). Cells were suspended in buffer containing 0.05 M potassium phosphate (pH 7.4), 0.1 mM EDTA, and 0.2 mM dithiothreitol and were sonicated with a Branson S-125 sonifier. Particulate material was sedimented by centrifugation at 39,000*g* for 45 min at 1° and discarded. The protein concentration was adjusted

TABLE I: Summary of Purification of Anthranilate Synthetase.[a]

Fraction	Vol (ml)	Protein Concn (mg/ml)	Act. (units)	Sp Act. (units/mg)	Yield (%)
I. Extract	400	14.1	87,600	15.5	
II. Protamine sulfate	418	11.5	69,200	14.4	79
III. Ammonium sulfate	50	36.2	62,600	34.6	71
IV. Sephadex G-200	205	1.5	20,100	65.2	23
V. DEAE-cellulose	22	2.3	19,400	384	22

[a] See Experimental Procedure for details.

to about 15 mg/ml. All succeeding steps were performed at 0–5°.

A freshly prepared 2% solution of protamine sulfate in 0.1 M potassium phosphate buffer (pH 7.0) was added slowly with gentle stirring to a final concentration of 0.17 mg of protamine sulfate/mg of protein. Stirring was continued for 15 min after the last addition of protamine sulfate. The solution was centrifuged at 13,000*g* for 20 min and the sediment was discarded. Ammonium sulfate (2.43 g/100 ml of extract) was added slowly with stirring. Stirring was continued for 20 min after the last addition and the solution was then centrifuged at 13,000*g* for 20 min. The sediment was dissolved in buffer solution A, which contained 0.05 M Tris-HCl (pH 7.4), 1 mM thioglycerol, 0.1 mM EDTA, and 0.05 mM tryptophan. The protein concentration was adjusted to 35–40 mg/ml and up to 50 ml was applied to a 5 × 90 cm column of Sephadex G-200. Elution was with buffer solution A. Fractions containing the highest specific activity were pooled and diluted with 0.25 volume of glycerol. The combined fraction containing about 300 mg of protein was applied to a 2.5 × 40 cm DEAE-cellulose column that had been equilibrated with buffer solution A containing 20% glycerol. Elution was with a linear ammonium chloride gradient using 1 l. of buffer solution A plus 20% glycerol in the mixing chamber and 1 l. of the same solution containing 0.5 M ammonium chloride in the reservoir. Fractions with highest activity were pooled and glycerol was added to a final concentration of 35%. The NH_4^+ concentration in the enzyme solution was determined by Nesslerization. The enzyme was stored at −20°. A summary of a typical purification is shown in Table I. Polyacrylamide disc electrophoresis of the purified protein showed one major fast-moving band accounting for about 80% of the protein and a slower moving band accounting for the remaining protein. When persulfate was not removed from the gels, prior to sample application, the two bands contained about equal amounts of protein.

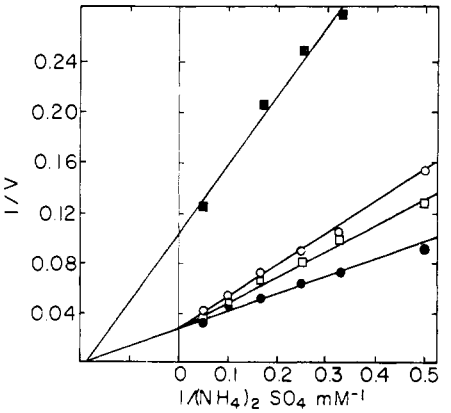

FIGURE 1: Double-reciprocal plot of inhibition by glutamine, glutamate, and α-ketoglutarate with $(NH_4)_2SO_4$ as the variable substrate. The symbols are: (●—●) no inhibitor, (□—□) 25 mM glutamate, (○—○) 25 mM glutamine, and (■—■) 25 mM α-ketoglutarate. The Mg^{2+} concentration of the standard assay was increased to 30 mM. Enzyme concentration, 23 μg/ml.

Results

Comparison of NH_4^+ and Glutamine as Substrate. In contrast to the native anthranilate synthetase–PR transferase complex, anthranilate synthetase component I, in crude extracts and in purified preparations, cannot utilize glutamine as amino donor between pH 6.5 and 8.0 (Table II). The crude or purified enzyme can utilize NH_4^+.

Failure of the enzyme to utilize glutamine for anthranilate synthesis could be due to alteration or loss

TABLE II: Comparison of NH_4^+ and Glutamine as a Substrate for Purified Anthranilate Synthetase.[a]

Substrate	pH	Sp Act. (units/mg)
Ammonium sulfate	8.0	198
Glutamine	6.5	<0.1
Glutamine	6.9	<0.1
Glutamine	7.4	<0.1
Glutamine	8.0	<0.1

[a] Reaction mixtures are as described in Experimental Procedure except that the buffer concentration was 0.1 M and was Tris-maleate at pH 6.5 and triethanolamine-HCl for pH 6.9–8.0.

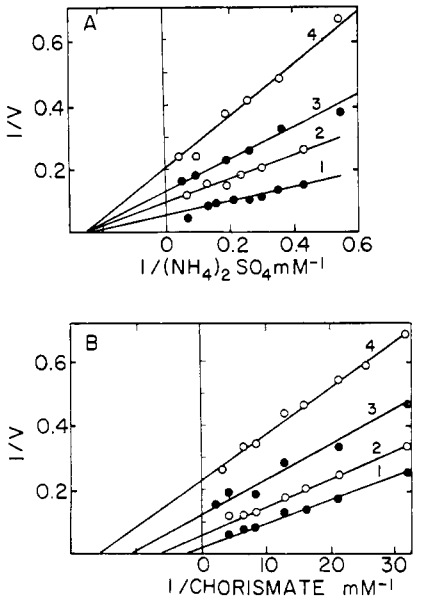

FIGURE 2: Initial velocity patterns. (A) With $(NH_4)_2SO_4$ as the varied substrate. Chorismate concentrations: (1) 0.25, (2) 0.075, (3) 0.05, and (4) 0.025 mM. (B) With potassium chorismate as the varied substrate. $(NH_4)_2SO_4$ concentrations: (1) 50, (2) 5.3, (3) 2.3, and (4) 1.1 mM. Enzyme concentration was 16 μg/ml in both experiments.

of the glutamine binding site. Reciprocal plots of initial velocity at variable NH_4^+ concentration, shown in Figure 1, indicate that glutamine and glutamate inhibit anthranilate synthesis. Inhibition by glutamine and glutamate is competitive with NH_4^+. α-Ketoglutarate also inhibits anthranilate synthesis but this inhibition is noncompetitive with NH_4^+ as well as with chorismate. These data suggest that anthranilate synthetase component I has a glutamine binding site.

Initial Velocity Patterns for Saturation by Substrates and Mg^{2+}. The initial rate of anthranilate formation was measured as a function of variable NH_4^+ concentration at chorismate levels of 0.025, 0.075, and 0.25 mM (Figure 2A). Linear double-reciprocal plots that converge to the left of the ordinate are obtained. Saturation with chorismate at four fixed levels of NH_4^+ also yields linear double-reciprocal plots that converge in the lower left-hand quadrant (Figure 2B). The data in Figure 3 show slope (K/V) and intercept ($1/V$) replots of the primary data from Figure 2. The Michaelis constants obtained from Figure 3A,B are 0.11 mM for chorismate and 24 mM for NH_4^+.

Anthranilate synthetase component I has an absolute requirement for Mg^{2+}. The reciprocal plot shown in Figure 4 is obtained when the Mg^{2+} concentration is varied. Straight lines that change slope at 4.4 mM Mg^{2+} are obtained.

Feedback Inhibition by Tryptophan. Anthranilate synthetase component I activity retains sensitivity to inhibition by tryptophan. A concentration of 0.035 mM tryptophan gives 50% inhibition in the standard assay. Inhibition by tryptophan is competitive with chorismate (Figure 5A) and noncompetitive with NH_4^+ (Figure 5B). There is no indication of deviation from linearity in reciprocal plots. The dependence of anthranilate

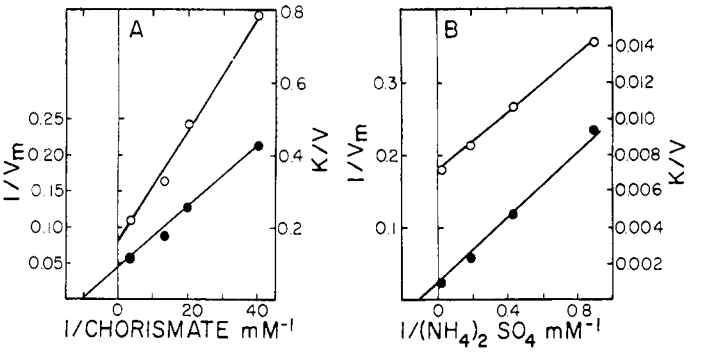

FIGURE 3: Secondary plots of slopes (K/V) and intercepts ($1/V$). (A) With respect to the reciprocal of the chorismate concentration. Values for slopes, intercepts, and chorismate concentrations were taken from Figure 1A. The symbols are: (●—●) $1/V$, (○—○) K/V. (B) With respect to the reciprocal of the $(NH_4)_2SO_4$ concentration. Values for slopes, intercepts and $(NH_4)_2SO_4$ concentrations were taken from Figure 1B. The symbols are: (●—●) $1/V$, (○—○) K/V.

synthetase component I activity upon pH in the absence and in the presence of 0.06 mM tryptophan is shown in Figure 6. The enzyme activity remains 75–80% inhibited by 0.06 mM tryptophan from pH 8.4 to 9.5 even though the catalytic activity changes twofold. Less inhibition is observed as the pH is lowered from 8.4 to 7.6.

Phenylalanine and tyrosine at concentrations as high as 1 mM do not affect anthranilate synthetase component I activity. These amino acids do not modify the tryptophan-mediated inhibition of enzyme activity.

Product Inhibition. Pyruvate is a competitive inhibitor with respect to chorismate (Figure 7A) and noncompetitive with respect to NH_4^+ (Figure 7B). Attempts to study product inhibition by anthranilate have thus far been unsuccessful.

Inhibition by NH_4^+ Analogs. In view of inhibition by hydroxylamine of glutamine-dependent anthranilate synthetase activity in the native anthranilate synthetase–PR transferase complex from *E. coli* (Somerville and Elford, 1967), various NH_4^+ analogs were tested. Hydroxylamine was without effect but *N*-methylhydroxylamine was inhibitory. Inhibition by 0.1 M *N*-methylhydroxyamine was approximately 30% in reaction mixtures containing 20 mM NH_4^+. Addition of ferric chloride and acid resulted in formation of the color at 500 mμ usually associated with hydroxamates. "Hydroxamate" formation was dependent upon chorismate and enzyme; Mg^{2+} and NH_4^+ were not required. "Hydroxamate" was formed with *N*-methylhydroxylamine but not with

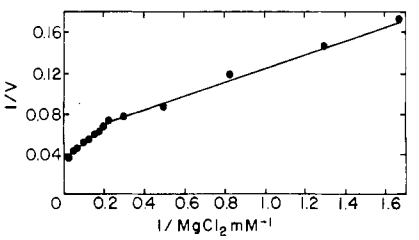

FIGURE 4: Double-reciprocal plot of the effect of varying the $MgCl_2$ concentraion. Enzyme concentration, 16 μg/ml.

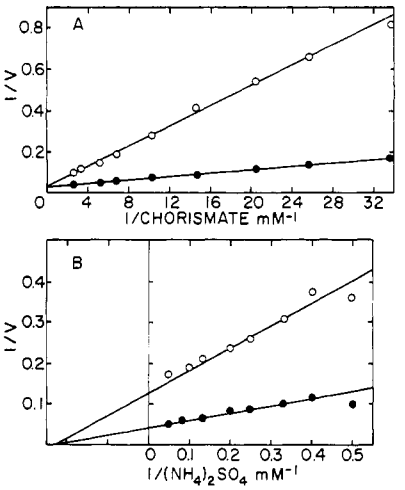

FIGURE 5: Double-reciprocal plots. (A) Showing inhibition by tryptophan with chorismate as variable substrate. The symbols are: (●—●) no tryptophan, (○—○) 0.05 mM tryptophan. (B) Showing inhibition by tryptophan with $(NH_4)_2SO_4$ as variable substrate. The symbols are: (●—●) no tryptophan, (○—○) 0.06 mM tryptophan. Enzyme concentration, 16 μg/ml in both experiments.

hydroxylamine. Tryptophan does not inhibit formation of the "hydroxamate." These results are consistent with formation of a chorismate-*N*-methylhydroxylamine adduct which could absorb at 500 mμ in the presence of Fe^{3+} and acid.

Anthranilate synthetase activity was inhibited by methylamine, dimethylamine, trimethylamine, and diethylamine. Enzyme activity was inhibited from 13 to 20% when the inhibitor concentration was 50 mM and NH_4^+ was 4 mM.

Inactivation by Bromopyruvate. Since pyruvate is a product of the anthranilate synthetase reaction, bromopyruvate is potentially an active site specific alkylating reagent. Anthranilate synthetase was inactivated when incubated with bromopyruvate. The time course of inactivation by 10 mM bromopyruvate at pH 6 is shown in Figure 8. Under these conditions 50% inactivation was achieved in about 6 min and 90% inactivation required 17–20 min. Chorismate protected against inactivation caused by bromopyruvate. There was no Mg^{2+} requirement for protection by chorismate.

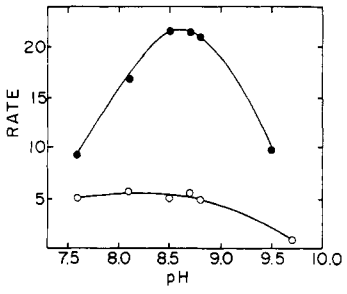

FIGURE 6: Effect of pH on initial rate of anthranilate formation in the presence and absence of tryptophan. The symbols are: (●—●) no tryptophan, (○—○) 0.06 mM tryptophan. Enzyme concentration, 16 μg/ml. Each pH was measured upon completion of the assay. The buffer was 0.1 M triethanolamine-HCl.

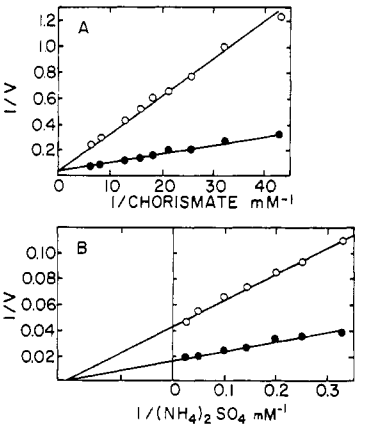

FIGURE 7: Double-reciprocal plots. (A) showing inhibition by pyruvate with chorismate as variable substrate. The symbols are: (●—●) no pyruvate, (○—○) 30 mM pyruvate. (B) Showing inhibition by pyruvate with $(NH_4)_2SO_4$ as variable substrate. The symbols are: (●—●) no pyruvate, (○—○) 30 mM pyruvate. Enzyme concentration was 16 μg/ml in both experiments.

Apparently Mg^{2+} is not required for binding of chorismate. Pyruvate (20 mM) conferred only slight protection which was augmented by 0.4 mM anthranilate. Under identical conditions 10 mM iodoacetate and 10 mM iodoacetamide were without effect.

Tryptophan, which causes end-product inhibition that is competitive with chorismate, also protects against inactivation of anthranilate synthetase component I by bromopyruvate. The data in Table III show that 0.1 mM tryptophan confers significant protection against inactivation by bromopyruvate at pH 6 and also at pH 8.0. There is no effect of Mg^{2+} on the protection afforded by tryptophan. Thus Mg^{2+} does not appear to be required for binding of tryptophan. The alkali lability of chorismate could account for the failure of chorismate to provide significant protection at pH 8.

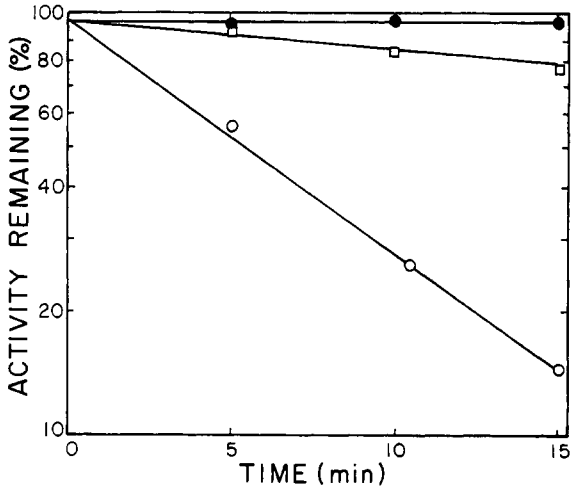

FIGURE 8: Time course of inactivation of anthranilate synthetase by bromopyruvate. The symbols are: (○—○) 10 mM bromopyruvate, (□—□) 10 mM bromopyruvate plus 2 mM chorismate, and (●—●) no bromopyruvate, no chorismate.

TABLE III: Effect of Chorismate and Tryptophan on Inactivation by Bromopyruvate.

Additions (mM)	% Act. Remaining
Experiment 1[a]	
None	20
Chorismate (1)	57
Tryptophan (0.1)	31
Tryptophan (1.0)	40
Experiment 2[b]	
None	52
Chorismate (1.4)	55
Tryptophan (0.1)	68
Tryptophan (1.0)	84

[a] In expt 1, 10 mM bromopyruvate was incubated with 115 μg of anthranilate synthetase component I and 0.04 M potassium phosphate (pH 6.0), and additions were noted in the table for 15 min at room temperature. [b] In expt 2, 1 mM bromopyruvate was incubated with 115 μg of enzyme and 0.04 M potassium phosphate (pH 8.0), and additions were noted for 10 min at room temperature.

Inactivation by Sulfhydryl Reagents. Anthranilate synthetase component I is inactivated by sulfhydryl reagents. Incubation of 46 μg of enzyme for 5 min at room temperature with 0.06 and 0.10 mM *p*-mercuribenzoate gave 41 and 94% inactivation, respectively. In this case enzyme activity was assayed in a mixture devoid of thioglycerol or dithiothreitol. If after incubation with 0.06 and 0.10 mM mercurial, the enzyme was assayed in a reaction mixture containing 2 mM dithiothreitol, inhibition was only 8 and 36%, respectively. Thus, dithiothreitol partially reversed the inhibition. Incubation of 115 μg of enzyme with 1 mM *N*-ethylmaleimide caused 70% inactivation. Chorismate (2 mM) reduced the inactivation to 40% under these conditions. As stated above, 10 mM iodoacetate and 10 mM iodoacetamide were noninhibitory.

Source of the Third Methyl Proton of Pyruvate. Elimination of the enolpyruvyl group of chorismate is accompanied by protonation to form pyruvate. The source of this proton was investigated by performing the anthranilate synthetase reaction in tritiated water. It is seen in Table IV that pyruvate obtained from the pyruvate kinase reaction has the expected incorporation of 1 g-atom of tritium from $[^3H]H_2O$/mole of pyruvate. Pyruvate isolated from the anthranilate synthetase reaction does not acquire a tritium atom from the solvent. This pyruvate sample contained the same low amount of radioactivity as the pyruvate isolated from the control reaction that did not contain chorismate. The third methyl proton of pyruvate must originate from chorismate.

Molecular Weight Estimation. Estimation of the molecular weight of anthranilate synthetase component I by gel filtration according to the method of Andrews (1964) yields the data shown in Figure 9. An extrapolated molecular weight of 63,000 is obtained. Preliminary examination of the enzyme in the ultracentrifuge at a protein concentration of 6.6 mg/ml showed a single symmetrical peak having an $s_{20,w}$ of 4.0 S.

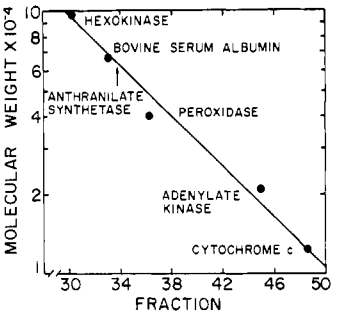

FIGURE 9: Molecular weight estimation of anthranilate synthetase component I by gel filtration. See Experimental Procedure for details.

Reconstitution of the Native Activity. In order to ascertain whether the purified anthranilate synthetase component I was grossly altered, attempts were made to reconstitute the native enzyme by mixing with crude extracts containing PR transferase (component II). Extracts of *trp A148* contain PR transferase and lack anthranilate synthetase component I.

Restoration of glutamine reactivity was the criterion used to show reconstitution of the native enzyme complex. The data summarized in Table V show that component I is inactive with glutamine as substrate. In the presence of component II, PR transferase furnished by the *trp A148* extract, anthranilate synthetase regains activity with glutamine. Addition of the *trp A148* extract also stimulated the rate of anthranilate formation using NH_4^+. More detailed experiments have been deferred until purified PR transferase and native anthranilate synthetase are available.

TABLE IV: Analysis of Pyruvate from Reactions in $[^3H]H_2O$.[a]

Source of Pyruvate	Sp Act. Found (cpm/μmole)	Sp Act. Calcd[b] (cpm/μmole)
Anthranilate synthetase reaction	348	2530
Pyruvate–$[^3H]H_2O$ control[c]	307	2530
Pyruvate kinase reaction	1820	1700

[a] Reaction conditions are described in Experimental Procedure. [b] Calculated for incorporation of 1 g-atom of tritium/mole of pyruvate. [c] This reaction mixture was identical with the one listed above except that chorismate was omitted and was replaced by pyruvate. This reaction would detect a pyruvate–$[^3H]H_2O$ exchange if it had occurred.

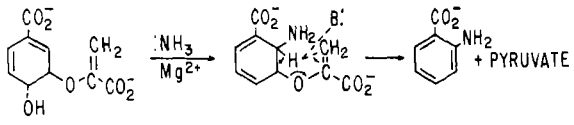

FIGURE 10: Possible mechanism of the anthranilate synthetase reaction. The stereochemistry of the amino group and hydrogen atom on 2,3-dihydro-3-enolpyruvylanthranilate is not specified. B represents a basic group on the enzyme that participates in proton transfer. The dashed lines depict proton transfer facilitated by a basic group on the enzyme.

Discussion

In *E. coli*, *A. aerogenes*, and *S. typhimurium*, native anthranilate synthetase exists as an aggregate with PR transferase, the following enzyme of the tryptophan biosynthetic pathway. The two components of the aggregate have been designated component I and component II, respectively (Ito and Yanofsky, 1966). Two major properties distinguish anthranilate synthetase component I from the native enzyme. Obviously, component I is smaller than the aggregate. A molecular weight estimate of 63,000 is obtained by gel filtration for purified component I whereas the native enzyme from *Salmonella* is composed of two species, both larger than 200,000 (E. J. Henderson and H. Zalkin, unpublished data). Secondly, purified component I has altered substrate specificity. As first described by Ito and Yanofsky (1966), component I no longer can utilize glutamine as amino donor but requires NH_4^+ for activity. The data reported in Figure 1 show that glutamine and glutamate inhibit anthranilate synthesis competitively with NH_4^+. This result suggests that the glutamine binding site is present in purified component I. It further suggests a close relationship between the NH_4^+ and glutamine sites.

Kinetic studies on purified anthranilate synthetase from *S. typhimurium* establish a qualitative similarity to the kinetic properties of the native enzyme from *E. coli* (Baker and Crawford, 1966). Substrate saturation data shown in Figure 2 yield linear reciprocal plots and thus preclude cooperative interactions. Replots of the reciprocals of the maximum velocities and slopes against the reciprocal of the fixed substrate concentration also yield straight lines (Figure 3). Linear variation of the ratio of apparent Michaelis constant to apparent maximal velocity (slope replots in Figure 3) is consistent with a sequential mechanism (Cleland, 1963). Also consistent with such a mechanism is the patterns of product inhibition caused by pyruvate. Pyruvate inhibition is competitive with chorismate and noncompetitive with NH_4^+ (Figure 7).

Inhibition by tryptophan is found to be competitive with chorismate and noncompetitive with NH_4^+ (Figure 5). Linear double-reciprocal plots were always obtained. No kinetic evidence for homotropic or heterotropic interactions was obtained. Attempts to desensitize the enzyme to tryptophan inhibition were unsuccessful. Treatment with *p*-mercuribenzoate or heat inactivation resulted in loss of enzyme activity but no change in sensitivity to tryptophan inhibition. It is therefore presently not possible to specify if the tryptophan binding site is distinct from the catalytic site.

The role of Mg^{2+} is not apparent. Preliminary evidence, based on the ability of chorismate and tryptophan to protect against inactivation by bromopyruvate in the absence of added Mg^{2+}, suggests that Mg^{2+} is not required for binding of either of these compounds to the enzyme. The biphasic double-reciprocal plot (Figure 4) may indicate multiple binding sites for Mg^{2+} or a change in relative rates of intermediate steps in the over-all reaction sequence as Mg^{2+} is varied. The Michaelis constants, obtained from the two linear portions of the double-reciprocal plot, are 1.0 and 5.1 mM.

Inhibition of anthranilate synthetase component I activity by *p*-mercuribenzoate and *N*-ethylmaleimide, and partial reversal of *p*-mercuribenzoate inhibition by dithiothreitol implicates one or more essential sulfhydryl groups. Inhibition of enzyme activity by bromopyruvate but not by iodoacetate or iodoacetamide suggests alkylation of a basic group at the active site. That chorismate protects against inactivation mediated by bromopyruvate is consistent with such a postulate. Alkylation of 2-keto-3-deoxy-6-phosphogluconate aldolase by bromopyruvate has been studied by Meloche (1967).

It is anticipated that a basic group at the catalytic site may participate in proton transfer that leads to aromatization and elimination of enolpyruvate (or pyruvate). Such a mechanism is shown in Figure 10. The first step could involve attack of NH_3 and elimination of the hydroxyl group to form 2,3-dihydro-3-enolpyruvylanthranilate. Completion of the reaction would require abstraction of a proton, and an appropriate electron shift resulting in elimination of enolpyruvate (or pyruvate). The postulated intermediate may be stabilized by using *N*-methylhydroxylamine as an NH_3 analog. Since *N*-methylhydroxylamine inhibits anthranilate synthesis and gives rise to a "hydroxamate" in the presence of ferric chloride at acidic pH, such an intermediate is suggested. Stabilization of the postulated intermediate would occur if proton abstraction were prevented. Since the pyruvate that is produced in the reaction does not acquire a proton from the solvent, it is necessary to conclude that the proton comes from the chorismate. It is therefore possible that abstraction

TABLE V: Reconstitution of Glutamine Reactivity.[a]

trp A148 (mg)	Amino Donor	Anthranilate Synthetase[b] Sp Act.
0	Glutamine	0.40
0.39	Glutamine	94
0	Ammonium sulfate	188
0.39	Ammonium sulfate	307

[a] All reactions contained 3.6 μg of anthranilate synthetase component I. [b] Activities are corrected for small contributions of the *trp A148* extract. Specific activities are based on the amount of component I.

of the proton is facilitated by a basic group on the enzyme and that the proton is transferred to the methylene group of the leaving enolpyruvate. Inhibition of the enzyme by bromopyruvate is interpreted as resulting from alkylation of the basic group that participates in proton transfer. There are precedents for nonenzymatic and enzymatic intramolecular proton transfer reactions that occur without exchange with solvent protons. Racemization of 2-phenylbutyronitrile and 2-*N*,*N*-dimethylamido-9-methyl-7-nitrofluorene catalyzed by the base, tripropylamine, occurs with negligible exchange (Cram and Grosser, 1964). Racemization without exchange has been called isoracemization (Cram and Grosser, 1964). Intramolecular proton transfer in 3-keto steroids, catalyzed by Δ^5-3-keto steroid isomerase from *Pseudomonas testosteroni* proceeds without detectable exchange with solvent protons (Wang *et al.*, 1963; Malhotra and Ringold, 1965). Under certain conditions, proton transfer catalyzed by phosphoglucose isomerase occurs without exchange with solvent protons (Rose and O'Connell, 1961). The main features of the mechanism shown in Figure 10 have been proposed by Levin and Sprinson (1964) and by Srinivasan (1965) for the glutamine-dependent reaction catalyzed by the native anthranilate synthetase–PR transferase complex.

The assumption is made that anthranilate synthetase component I catalyzes an only slightly modified form of the physiological reaction since the purified enzyme can be recombined with crude PR transferase to reconstitute the glutamine-dependent activity that is characteristic of the native enzyme.

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