Anthranilate Synthetase from *Serratia marcescens*

ON THE PROPERTIES AND RELATIONSHIP TO THE ENZYME FROM *SALMONELLA TYPHIMURIUM*

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SUMMARY

Anthranilate synthetase was purified from *Serratia marcescens*. The enzyme was homogeneous by the criterion of disc gel electrophoresis. Glutamine- and NH$_3$-dependent anthranilate synthetase activities were subject to end product inhibition by tryptophan. Inhibition by tryptophan exhibited positive cooperativity. In the absence of tryptophan, Michaelis-Menten kinetics were obtained for saturation by the two substrates, chorismate and glutamine. Positive cooperativity for chorismate was detected in the presence of tryptophan. These kinetic properties of anthranilate synthetase from *S. marcescens* were similar to those of the multifunctional anthranilate synthetase-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (PR transferase) aggregate from *Salmonella typhimurium*.

A molecular weight of approximately 141,000 was estimated for the oligomeric anthranilate synthetase from *S. marcescens*. Nonidentical subunits of molecular weights approximately 60,000 and 21,000 were detected by disc gel electrophoresis in sodium dodecyl sulfate.

The glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON), and sulfhydryl reagents selectively inactivated glutamine-dependent anthranilate synthetase activity; NH$_3$-dependent activity was largely retained. 14C-Labeled DON and iodotetrazoliumClacetamide were incorporated into the small subunit thus establishing the relationship of this subunit to anthranilate synthetase Component II from anthranilate synthetase-PR transferase of *S. typhimurium*. Approximately 2 moles of DON or iodoacetamide were incorporated per mole of anthranilate synthetase, suggesting that the oligomeric enzyme contains two glutamine-binding subunits (anthranilate synthetase Component II). From a consideration of the approximate size of the native enzyme and its subunits a composition of two chains each of anthranilate synthetase Components I and II is suggested.

A glutaminase activity was detected. This activity may serve to transfer the amide of glutamine from anthranilate synthetase Component II to the catalytic site on Component I. Catalytic properties and structural features of anthranilate synthetase from *S. marcescens* were compared to those of trypsin-treated anthranilate synthetase-PR transferase from *S. typhimurium* (HWANG, L. H., AND ZALKIN, H., J. Biol. Chem., 246, 2338 (1971)). On the basis of similarities between the two enzymes it is suggested that gene fusion could account for the two types of anthranilate synthetase.

Anthranilate synthetase catalyzes the first reaction of tryptophan synthesis in microorganisms. As shown by Equations 1 and 2 either glutamine or NH$_3$ may serve as amino donor.

\[
\text{Chorismate + glutamine } \overset{\text{Mg}^2+}{\longrightarrow} \text{anthranilate + pyruvate + glutamate} \tag{1}
\]

\[
\text{Chorismate + NH}_3 \overset{\text{Mg}^2+}{\longrightarrow} \text{anthranilate + pyruvate} \tag{2}
\]

Anthranilate synthetase from *Salmonella typhimurium* (1), *Escherichia coli* (2), and *Aerobacter aerogenes* (3) is aggregated to the second enzyme in the pathway, anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase. Utilization of glutamine is dependent upon aggregation of anthranilate synthetase subunits (called anthranilate synthetase Component I) with PR transferase subunits (called anthranilate synthetase Component II). Anthranilate synthetase Component I from these organisms catalyzes Reaction 2 but cannot utilize glutamine because the glutamine site is apparently on the anthranilate synthetase Component II subunit (4).

In *Serratia marcescens* (5) as well as in *Bacillus subtilis* (6) and in species of *Pseudomonas* (7) anthranilate synthetase is not aggregated with other enzymes of the tryptophan pathway. It was therefore of interest to compare the enzyme from *S. marcescens* with the anthranilate synthetase-PR transferase from *S. typhimurium*.

It is the purpose of this paper to report the purification to homogeneity of anthranilate synthetase from *S. marcescens*. The enzyme is an oligomer of molecular weight approximately 141,000 and contains nonidentical subunits of molecular weights approximately 60,000 and 21,000. The smaller subunit functions as a glutamine-binding protein. A possibly close relationship be-

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1 The abbreviations used are: PR transferase, anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase; DON, 6-diazo-5-oxo-L-norleucine; SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
between this enzyme and the anthranilate synthetase-PR transferase from *S. typhimurium* is discussed.

**Experimental Procedure**

**Enzyme Purification**—Anthranilate synthetase was purified from *S. marcescens* strain HY 150 (ATCC 27143), a tryptophan auxotroph lacking indoleglycerol-3-P synthetase (5). Cells were grown in 1 liter of media in 2-liter flasks with vigorous shaking for about 17 hours at 30° or 37°. Each flask contained salts (as described in Reference 8, but lacking citrate), 10 g of acid hydrolyzed casein, 4 g of glucose, and 5 mg of tryptophan. Growth was started with 1 ml of an overnight culture grown in the same media. Cells were harvested by centrifugation, washed with 0.05 M potassium phosphate, pH 7.4, and stored at -10° until used.

For a typical enzyme preparation 120 to 170 g of frozen cells were thawed in 4 ml per g of 0.1 M potassium phosphate, pH 7.4, 0.2 mM dithiothreitol, and 0.1 mM EDTA and were then subjected to sonic disruption with a Branson S-125 sonifier. Power output and time were adjusted to yield an extract containing between 18 and 20 mg of protein per ml. The temperature was maintained below 10° during sonic disruption. The crude extract was obtained following centrifugation at 37,000 x g for 45 min. All succeeding steps were performed at 2°-5°.

The extract was adjusted to a protein concentration of 15 mg per ml. A freshly prepared 2% solution of protamine sulfate in 0.1 M potassium phosphate, pH 7.4, was added dropwise with stirring to yield a final concentration of 0.16% of protamine sulfate per mg of protein. Stirring was continued for 15 min after the last addition. The solution was centrifuged at 37,000 x g for 10 min and the sediment was discarded. To the supernatant solution was added 230 mg of ammonium sulfate per ml. Stirring was continued for 15 min after the last addition and the solution was then centrifuged at 37,000 x g for 10 min. The precipitate was dissolved in 0.05 M Buffer A (similar to 0.1 M Buffer A except for the concentration of potassium phosphate) and was dialyzed overnight against approximately 50 volumes of the same buffer solution. After dialysis the protein solution was diluted with 0.05 M Buffer A to a protein concentration of 10 to 12 mg per ml and was applied to a column (3 x 50 cm) of DEAE-cellulose, previously equilibrated with the same buffer solution. The enzyme was eluted with about 50 to 75 ml of 0.05 M potassium phosphate, pH 7.4, and contained 0.2 mg dithiothreitol. The enzyme was eluted from the column with this buffer solution, and fractions of constant specific activity were pooled. The pooled enzyme could be stored at -10°. An initial drop in specific activity was always observed but, thereafter, the activity was stable for several months. A typical purification starting with 166 g of frozen cell paste is summarized in Table I.

**Materials**—*S. marcescens* strain HY 150 was obtained from Dr. W. L. Belser. Unlabeled and 14C-labeled DON were preparations that were synthesized and used previously (4). Chorismic acid was made according to the procedure of Gibson (9). Iodo-[1-14C]acetamide (523 mCi per mmole) was obtained from Amersham-Searle. Radioisotopic purity (> 95%) was verified by thin layer chromatography on silica gel with the use of benzene-dioxane-acetic acid (90:25:4) and methanol-acetone (50:50) solvent systems. Other chemicals were obtained from commercial suppliers and were used without further purification.

**Disaccharide Electrophoresis**—Electrophoresis of the native enzyme was performed by the method of Davis (10). Electrophoresis in SDS was according to the method of Shapiro, Viñuela, and Maizel (11). For electrophoresis in urea, the method of Davis (10) was modified by inclusion of 8 M urea in the buffers and gels. Sample and spacer gels were not used. Sucrose, final concentration 20%, or a drop of glycerol was added to increase the density of enzyme solutions which were applied directly to the separating gel. Coomassie blue and acetic acid-methanol-water (12) were used for staining and destaining, respectively, in all experiments.

**Measurement of Incorporation of 14C DON and 14C Amino Acids into Protein Subunits**—Anthranilate synthetase was labeled with radioactive DON or iodoacetamide as described in the appropriate figure legends. The enzymes were then denatured with 1% SDS and 1% mercaptoethanol or with 8 M urea and the subunits separated by SDS or urea gel electrophoresis. The gels were stained, destained, and then exhaustively washed in acetic acid-methanol-water as reported previously (4). The gels were sliced, solubilized by treatment with H2O2, and counted for radioactivity (4).

**Sucrose Gradient Centrifugation**—The techniques and calculations described by Martin and Ames (13) were followed. Yeast alcohol dehydrogenase, *ε* and molecular weight 141,000 (14), was used as a reference protein. In most experiments the sedimentation profile for anthranilate synthetase was superimposable with that of yeast alcohol dehydrogenase.

**Enzyme Assays**—Glutaminase activity of anthranilate synthetase was assayed by the hydroxamate method used previously (4). Methods for assaying glutamine-dependent anthranilate synthetase (15) and NADH-dependent anthranilate synthetase (16) have been described. A unit of activity corresponds to the formation of 1 nmole of anthranilate per min under the conditions of assay. In all figures, v, is expressed as enzyme units. Specific activity is expressed as units of activity per mg of protein. Protein was determined by the method of Lowry as described by Layne (17) and related to dry weight. All experiments relating to the subunit composition were done with homogeneous enzyme. Kinetic experiments were performed with DEAE-cellulose or hydroxylapatite edge fractions.

**Determination of Dry Weight of Enzyme**—A sample of enzyme containing about 20 mg of protein was dialyzed against 500 volumes of 5 M potassium phosphate, pH 7.4. The buffer solution was replaced every 24 hours and dialysis continued for 4 days. Protein was determined by the method of Lowry with the use of
**TABLE I**

Summary for purification of anthranilate synthetase
Details are provided under “Experimental Procedure.”

| Fraction                  | Volume | Protein | Activity  | Specific activity |
|---------------------------|--------|---------|-----------|-------------------|
|                            | ml     | mg      | units     | units/mg          |
| Extract                   | 690    | 9,750   | 1,100,000 | 113              |
| Protamine sulfate         | 740    | 9,800   | 900,000   | 101              |
| Ammonium sulfate          | 96     | 2,160   | 770,000   | 356              |
| DEAE-cellulose            | 18     | 440     | 990,000   | 2,250            |
| ECTEOLA-cellulose         | 18     | 121     | 577,000   | 4,700            |
| Hydrolylpaste             |        | 67      | 496,000   | 7,400           |

* Specific activity declined to 3,400 to 4,000 after 1 day and thereafter remained constant.

**RESULTS**

*Enzyme Purification*—The results of a typical purification are summarized in Table I. After storage for 1 day at 2° or at -10° the specific activity declined to 3400 to 4000 units per mg and remained constant for several months. Following the hydroxylapatite purification step the enzyme was homogeneous by the criterion of disc gel electrophoresis.

**Kinetics for Substrate Saturation and Inhibition by Tryptophan**—Similar to other glutamine amidotransferases (18) and anthranilate synthetase enzymes from other species (1–3, 6, 7), the S. marcescens anthranilate synthetase utilized NH₃ in addition to glutamine. Under optimal assay conditions the ratio of the glutamine- to the NH₃-dependent activity was approximately 1.0.

Substrate saturation data in the form of Lineweaver-Burk plots (19) are shown in Figs. 1 and 2. The data in Fig. 1A show saturation of glutamine-dependent anthranilate synthetase activity by chorismate. The chorismate levels are ••••••, 50 μM; ○---○, 20 μM; ■---■, 5 μM; and □---□, 2 μM. Standard assay conditions were employed except for the use of 0.05 m triethanolamine-chloride, pH 8.5, and variation of the substrate concentrations. The glutamine and ammonium sulfate concentrations are in millimolar units. *v* is in enzyme units.

**Substrate Saturation**

Fig. 1. Lineweaver-Burk plots showing saturation of glutamine- and NH₃-dependent anthranilate synthetase activities by chorismate. *A,* dependence of glutamine-dependent anthranilate synthetase activity upon chorismate concentration (micromolar) at fixed glutamine concentrations of •---•, 5 mM; ○---○, 0.5 mM; and □---□, 0.1 mM. *B,* dependence of NH₃-dependent anthranilate synthetase activity upon chorismate concentration (micromolar) at fixed ammonium sulfate concentrations of •---•, 50 mM; ○---○, 5 mM; and □---□, 2 mM. Standard conditions were employed for assay of both activities except for the use of 0.05 m triethanolamine-chloride, pH 8.5, and variation of the substrate concentrations. *v* is in enzyme units.

Fig. 2. Lineweaver-Burk plots for saturation of anthranilate synthetase by *A,* glutamine and *B,* ammonium sulfate at several fixed levels of chorismate. The chorismate levels are ••••••, 50 μM; ○---○, 20 μM; ■---■, 5 μM; and □---□, 2 μM. Standard assay conditions were employed except for the use of 0.05 m triethanolamine-chloride, pH 8.5, and variation of the substrate concentrations. The glutamine and ammonium sulfate concentrations are in millimolar units. *v* is in enzyme units.

**Inhibition of Substrate**

Fig. 3. Inhibition of glutamine- (•---•) and NH₃-dependent (○---○) anthranilate synthetase activities by tryptophan. Standard assay conditions were modified by using 0.05 m triethanolamine-chloride, pH 8.5, and including tryptophan. The inset shows the data of the main figure plotted according to the modified Hill equation, log *v*ᵣ/*v*ₙ = log *K* - *n*'* log *I*. The symbols are *v*ᵣ velocity in the presence of tryptophan; *v*ₙ velocity in the absence of tryptophan; *K*, a constant; *n*’, the interaction coefficient; *I*, the inhibitor concentration (tryptophan in this case).

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Substrate saturation data in the form of Lineweaver-Burk plots (19) are shown in Figs. 1 and 2. The data in Fig. 1A show saturation of glutamine-dependent anthranilate synthetase by chorismate at three fixed levels of glutamine. Saturation by chorismate at three fixed levels of ammonium sulfate is shown in...
The phenomenon will be treated below. In contrast to the data in Fig. 1A obtained from assays which were performed in triethanolamine-chloride, pH 8.5, including tryptophan and varying the substrate indicated. Standard assay conditions for glutamine-dependent anthranilate synthetase were modified by using 0.05 M triethanolamine-chloride, pH 8.5, including tryptophan added. Standard assay conditions for glutamine-dependent anthranilate synthetase were modified by using 0.05 M triethanolamine-chloride, pH 8.5, including tryptophan and varying the substrate indicated.

Saturation by glutamine and ammonium sulfate at fixed levels of chorismate is summarized in Fig. 2. Absence of curvature precludes cooperative interactions for these substrates. Values of \( K_m \) for chorismate of 18 mM and 15 mM were calculated from secondary plots for the reactions with glutamine and ammonium sulfate, respectively. In the above experiments both activities were assayed in triethanolamine-chloride, pH 8.5. The \( K_m \) for chorismate for glutamine-dependent anthranilate synthetase was 5-fold lower when determined with potassium phosphate, pH 7.4.

Both the glutamine- and NHB-dependent activities were completely inhibited by low concentrations of tryptophan. Concave upward curvature in plots of \( 1/V \) against tryptophan concentration (Fig. 3) and slopes, \( n' \), greater than 1 in Hill plots (20) indicate positive cooperativity for tryptophan sites. For the experiment shown in Fig. 3, 50% inhibition of glutamine-dependent anthranilate synthetase activity required 7 \( \mu \)M tryptophan. The effect of tryptophan on substrate saturation of glutamine-dependent anthranilate synthetase activity is shown in Fig. 4. Concave upward curvature (Fig. 4A) is consistent with positive cooperativity for chorismate in the presence of tryptophan. End product inhibition increased the chorismate concentration required for half-maximal velocity (\( S_{1/2} \)) and also decreased the \( V_{max} \) (Fig. 4A). Inhibition by tryptophan therefore may not be the result of simple competition for the chorismate site. Tryptophan inhibition was clearly noncompetitive with respect to glutamine (Fig. 4B). Similar data were obtained for tryptophan inhibition of NHB-dependent anthranilate synthetase activity (not shown).

The data in Fig. 4A suggest that inhibition by tryptophan was not a result of simple competition for the chorismate site. Evidence supporting a multisite model with distinct substrate and end product regulatory sites was provided by the data in Fig. 5. Lineweaver-Burk plots for chorismate saturation of glutamine-dependent anthranilate synthetase performed in potassium phosphate buffer, pH 7.4, show deviations from linearity indicative of substrate inhibition. One interpretation for substrate inhibition is that at high concentrations, chorismate interacts with the tryptophan regulatory site. The data in Fig. 5 show that a low concentration of tryptophan relieves substrate inhibition by chorismate. Tryptophan may displace chorismate from a regulatory site.

Effect of pH on Enzyme Activity and Inhibition by Tryptophan—
The pH optima for enzyme activity and inhibition by tryptophan are shown in Fig. 6. Optimal activity for glutamine-dependent anthranilate synthetase was at about pH 7.6 while that for the...
Selective inactivation of glutamine-dependent anthranilate synthetase activity by DON was performed in 0.2-ml reaction mixtures containing 0.05 M potassium phosphate, pH 7.4, 3.2 μM DON, 1.0 mM EDTA, 57 μg of enzyme, and other additions as noted. Incubation was at 25°. Aliquots of 0.01 ml were removed at different times and diluted 100-fold into assay mixtures for glutamine- and NH₃-dependent anthranilate synthetase activities. Pseudo first order rate constants for inactivation were calculated from data similar to that shown in Fig. 9.

### Table II

| Additions | k (min⁻¹) |
|-----------|-----------|
| None      | 0.03      |
| Chorismic, 0.1 mM | 0.09     |
| Chorismic, 0.1 mM, plus glutamine, 5 mM. | 0.09     |
| Tryptophan, 50 μM. | 0.02     |

**Fig. 7.** Selective inactivation of glutamine-dependent anthranilate synthetase activity by DON (O—O) and iodoacetamide (□—□). NH₃-dependent anthranilate synthetase activity was relatively unaffected by treatment with DON (●—●) or iodoacetamide (■—■). Treatment with DON was performed at room temperature in a reaction mixture (0.2 ml) containing 3.3 μM DON, 0.05 M potassium phosphate, pH 7.4, 50 μM chorismic, and 49 μg of enzyme. For inactivation with 2.5 mM iodoacetamide the buffer was 0.05 M potassium phosphate, pH 8.0. Other conditions were similar. Aliquots were removed and diluted 100-fold into standard assay mixtures for glutamine- and NH₃-dependent anthranilate synthetase.

**NH₃-dependent reaction was at pH 8.5 or higher. Inhibition of both activities by 10 μM tryptophan was maximal at high pH.**

**Molecular Weight and Subunit Composition.—**The molecular weight of anthranilate synthetase estimated by sucrose gradient centrifugation was 141,000 relative to yeast alcohol dehydrogenase. In seven experiments the range was 132,000 to 145,000. Nonidentical polypeptide chains were detected by disc gel electrophoresis in urea or in SDS. The molecular weights of the polypeptide chains were estimated to be approximately 60,000 and 21,000 by SDS gel electrophoresis.

Evidence supporting a subunit composition of two polypeptide chains of molecular weight approximately 60,000 plus two chains of molecular weight approximately 21,000 was obtained from ligand-binding studies (see below) and from intramolecular cross-linking. Cross-linking with dimethyl suberimidate (21) was used as one approach to distinguish between anthranilate synthetase oligomers containing one or two small polypeptide chains. Most of the eight species that should result from random intramolecular cross-linking of a tetramer containing subunits of two sizes were detected by SDS gel electrophoresis. Significantly, a protein chain of molecular weight approximately 40,000 was found following treatment with dimethyl suberimidate. Formation of this species was independent of protein concentration and therefore suggested that oligomeric anthranilate synthetase might contain two protein chains of molecular weight approximately 21,000. Further evidence for a tetramer containing two large and two small subunits is presented below.

**Selective Inactivation of Glutamine-dependent Anthranilate Synthetase Activity.—**Evidence for a glutamine site distinct from the site for NH₃ was obtained by selective inactivation of glutamine-dependent anthranilate synthetase activity. Such inactivation was obtained by treatment with the glutamine analogue DON or with sulfhydryl reagents. The data in Fig. 7 show apparent first order kinetics for inactivation of glutamine-dependent anthranilate synthetase activity by 3.3 μM DON and 2.5 mM iodoacetamide. Little or no inactivation of NH₃-dependent anthranilate synthetase was obtained. Similar but less reproducible results were obtained with p-mercuribenzoate and DTNB. For these experiments the half-times for inactivation by 3.3 μM DON and 2.5 mM iodoacetamide were 1.6 and 10 min, respectively.

**Inactivation of glutamine-dependent anthranilate synthetase activity by DON required chorismate and was retarded by glutamine and by tryptophan (Table II).** Inclusion of 1 mM EDTA in reaction mixtures completely prevented anthranilate synthesis. Similar effects were noted for inactivation of glutamine-depend-
The requirement for chorismate was ascribed to ordered binding of first chorismate and then glutamine. Tryptophan caused reversal of enzyme activity (feedback inhibition) and DON inactivation either by preventing chorismate from binding or from exerting the conformational change required to allow glutamine (or DON) to bind. Recent experiments have verified directly that tryptophan prevents binding of chorismate to anthranilate synthetase-PR transferase from S. typhimurium (22).

Qualitatively similar effects of chorismate and tryptophan but of lesser magnitude were obtained with the use of iodoacetamide to inactivate glutamine dependent anthranilate synthetase activity. Stimulation of the pseudo first order rate constant for inactivation by chorismate and inhibition by tryptophan was approximately 50% in each case.

Characterization of Glutamine-binding Protein—By analogy with trypsin-treated anthranilate synthetase-PR transferase from S. typhimurium (23) it was anticipated that the site alkylated by DON and iodoacetamide was on the small polypeptide chain of molecular weight approximately 21,000. Anthranilate synthetase was incubated with [14C]DON until greater than 90% inactivation of glutamine-dependent anthranilate synthetase activity was obtained. Following SDS gel electrophoresis [14C]DON was detected exclusively in the small subunit (Fig. 8). No radioactivity over the background level was detected in the region of the gel containing the large polypeptide chain. Similar results were obtained with iodo[14C]acetamide except that in some experiments small amounts of radioactivity were incorporated into the large polypeptide chain.

The kinetics for incorporation of iodo[14C]acetamide into the polypeptide chain of molecular weight approximately 21,000 are shown in Fig. 9. It is apparent that incorporation of radioactivity into the small polypeptide chain closely paralleled inactivation of glutamine-dependent enzyme activity. It is therefore concluded that alkylation of a group on the 21,000 molecular weight protein chain caused inactivation of glutamine-dependent anthranilate synthetase activity. In this experiment there was negligible inactivation of NH$_3$-dependent enzyme activity al-
From these experiments it is apparent that a site on the small subunit that is susceptible to alkylation by DON and iodoacetamide is required for glutamine-dependent anthranilate synthetase activity. NH₃-dependent anthranilate synthetase activity is completely independent of this site. By analogy with trypsin, treated anthranilate synthetase from S. typhimurium and with the enzyme from Pseudomonas (7) and in keeping with current nomenclature (2) the large and small subunits can be designated anthranilate synthetase Components I and II, respectively.

The foregoing experiments while clearly establishing anthranilate synthetase Component II as the glutamine-binding protein were not satisfactory for determining the number of sites alkylated by DON or iodoacetamide. Quantitative recovery of radioactive material and enzyme was difficult following SDS gel electrophoresis. The number of DON sites for anthranilate synthetase was estimated by the kinetic method of Naganur, Zalkin, and Henderson (4). A typical experiment is shown in Fig. 11. In that method glutamine-dependent anthranilate synthetase activity was determined in reaction mixtures containing different molar ratios of enzyme to DON. The amount of DON required to achieve complete inactivation was obtained by extrapolation and as shown in Fig. 11 was 2.2 moles of DON per mole of enzyme.

The number of sites on anthranilate synthetase Component II that were specifically required for utilization of glutamine and were alkylated by iodoacetamide was determined. Anthranilate synthetase was treated with iodo[¹⁴C]acetamide until 90% inactivation of glutamine-dependent activity. Under such conditions 100% of the NH₃-dependent anthranilate synthetase activity was retained. The reaction mixture was applied to a column of Sephadex G-25 in order to separate alkylated enzyme from unreacted iodo[¹⁴C]acetamide. The recovered enzyme fraction was assayed for protein and counted for radioactivity. The number of sites calculated according to this procedure was 1.8 per enzyme.

Inactivation of glutamine-dependent anthranilate synthetase activity by sulfhydryl reagents and incorporation of iodoacetamide into the enzyme indicates sulfhydryl groups are essential for utilization of glutamine. It has also been shown that DON alkylates cysteine residues of other glutamine amidotransferases (4, 24, 25). Titration with DTNB was used to confirm that specific inactivation of S. marcescens glutamine-dependent anthranilate synthetase by DON and iodoacetamide resulted from alkylation of cysteine groups. The data in Table III show the number of titratable sulfhydryl groups in the native and alkylated enzyme samples. Following inactivation of glutamine-dependent anthranilate synthetase by DON approximately 1.5 cysteine groups per enzyme were blocked and were unavailable for titration with DTNB. Interestingly, these residues were largely inaccessible to

### Table III

| Treatment                | Molar SDS | Plus SDS |
|--------------------------|-----------|----------|
| None                     | 1.64      | 14.9     |
| DON treated              | 1.40      | 13.4     |
| Iodoacetamide treated    | 0.93      | 12.1     |
Glutaminase activity of anthranilate synthetase

| Reaction conditions                          | Activity (µmol/30 min) |
|---------------------------------------------|------------------------|
| Complete                                    | 1.06                   |
| Minus chorismate                            | 0.24                   |
| Plus tryptophan                             | 0.45                   |
| Minus native enzyme plus iodoacetate-treated enzyme plus chorismate | 0.32                   |

DTNB in the active enzyme and were only detected after denaturation with SDS. Similar results were previously obtained for anthranilate synthetase-PR transferase from S. typhimurium (4). In the iodoacetamide-treated sample 2.8 cysteine residues per enzyme were blocked, and only one-fourth of this number were accessible prior to denaturation with SDS. It is possible that further nonspecific alkylation by iodoacetamide occurred during the initial period of dialysis (see legend to Table III) and could thus account for the higher than expected number of blocked cysteine groups.

Glutaminase Activity—It has been suggested that a glutaminase activity functions to transfer the amide of glutamine from anthranilate synthetase Component I to the NH₃ site on anthranilate synthetase Component I in the S. typhimurium anthranilate synthetase-PR transferase (4). The data in Table IV show that Serratia anthranilate synthetase has glutaminase activity. This activity was dependent upon chorismate and was inhibited by tryptophan. Upon inactivation of glutamine-dependent anthranilate synthetase activity with iodoacetamide, the glutaminase was largely inhibited.

**DISCUSSION**

Anthranilate synthetase from different bacterial species has been characterized to varying degrees. Two main types of this enzyme have thus far been detected in bacteria; type I anthranilate synthetases are oligomeric proteins that are isolated free from other enzymes of the tryptophan biosynthetic pathway. Enzymes of type I have been isolated from B. subtilis (6), various species of *Pseudomonas* (7), and from *S. marcescens* (5). Type II anthranilate synthetases are oligomeric proteins found in association with the second enzyme of the tryptophan biosynthetic pathway, PR transferase. Enzymes of type II have been isolated and at least partially characterized from *E. coli* (2), *A. aerogenes* (3), and *S. typhimurium* (1, 4). While some variation in subunit association among enzymes from different species of *Pseudomonas* has been detected (7), **in vitro** complementation experiments show the basic similarities between enzymes in each of the two main classes (7, 26). It is therefore illuminating to compare the anthranilate synthetase from *S. marcescens*, representative of type I, with the anthranilate synthetase-PR transferase of *S. typhimurium*, representative of type II.

Anthranilate synthetase-PR transferase from *S. typhimurium* is a multifunctional enzyme aggregate of molecular weight 280,000 (15, 22). Anthranilate synthetase Component I of this aggregate is a polypeptide chain of molecular weight 64,000 which catalyzes anthranilate formation from chorismate and NH₃ (16, 27). This activity is subject to inhibition by tryptophan. Anthranilate synthetase Component II is bifunctional. This protein of molecular weight approximately 60,000 to 64,000 contains the glutamine site for glutamine-dependent anthranilate synthetase activity and the site for PR transferase activity (1, 28). The enzyme aggregate contains two subunits each of Components I and II.

Treatment of the multifunctional anthranilate synthetase-PR transferase (type II enzyme) with trypsin (23) results in formation of a type I anthranilate synthetase. As a result of digestion with trypsin, a major portion of anthranilate synthetase Component II containing the PR transferase activity is removed, leaving an enzyme of molecular weight approximately 141,000 and containing two subunits of unaltered anthranilate synthetase Component I and two subunits of digested anthranilate synthetase Component II. The digested anthranilate synthetase Component II has a molecular weight of approximately 15,000 to 19,000, and all properties of the glutamine-dependent reaction appear identical with those of the native multifunctional enzyme.

The properties of trypsin-treated anthranilate synthetase from *S. typhimurium* are remarkably similar to those of the *S. marcescens* enzyme reported in this investigation. The similarities include (a) similar approximate molecular weights (141,000) for the oligomeric enzymes, and (b) similar molecular weights for the two types of subunits. Also, (c) both enzymes appear to contain two subunits of each type and (d) the small subunits in each case are alkylated by the glutamine analogue DON. Alkylation of the small subunit causes inactivation of glutamine-dependent anthranilate synthetase activity but NH₃-dependent activity is retained. (e) Both enzymes have glutaminase activity which could function to transfer the amide of glutamine from the glutamine-binding protein (anthranilate synthetase Component II) to the NH₃ site on anthranilate synthetase Component I. (f) Both enzymes exhibit similar regulatory features, positive cooperativity for tryptophan inhibition and positive cooperativity for chorismate in the presence of tryptophan. (g) Both enzymes have similar *Kₘ* values for chorismate and glutamate. The *Kₘ* values for chorismate and glutamate determined in potassium phosphate buffer were 2.3 µM and 0.50 mM, respectively, for the enzyme from *S. marcescens*, and 3.7 µM and 0.67 mM, respectively, for the enzyme from *S. typhimurium*. (h) Inhibition by tryptophan is maximal at pH greater than 8.0.

The recent report by Youngo, Kohno, and Roth (29) on gene fusion in the *his* operon of *S. typhimurium* suggests a mechanism for evolution of Type II anthranilate synthetase from Type I enzyme. It has been speculated that upon evolution of a glutamine-binding protein, aggregation of this protein occurred with a primitive NH₃-dependent enzyme (4). It appears possible that in some cases the gene for the glutamine-binding protein was inserted into the trp operon and became fused to the gene for PR transferase. Such a fusion would produce a bifunctional anthranilate synthetase Component II. According to the idea of Youngo et al. (29) such a bifunctional enzyme may contain a glutamine-binding peptide connected to a PR transferase protein by a flexible arm. Digestion by trypsin could occur in the region of the flexible arm and thus remove the PR transferase protein seg-
ment from the anthranilate synthetase Component I-ant,hranilate synthetase Component II oligomer.

Anthranilate synthetase from S. mucrescens has also been purified and characterized by Robb, Hutchinson, and Belser (30). Their results and conclusions are in general agreement with those reported here.

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