Anthranilate Synthetase

PARTIAL PURIFICATION AND SOME KINETIC STUDIES ON THE ENZYME FROM ESCHERICHIA COLI

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SUMMARY

Anthranilate synthetase was purified by ammonium sulfate precipitation and gel filtration from extracts of an episome-bearing Escherichia coli mutant grown under conditions of tryptophan pathway derepression. This purification represented an 18-fold increase in specific activity over the activity of the derepressed mutant extract. Starch gel electrophoresis and ultracentrifugation revealed only slight contamination of the anthranilate synthetase protein. An $n_{o, w}$ of 10.7 S was obtained for the native enzyme.

Kinetic studies with anthranilate synthetase have been initiated. The reaction catalyzed by this enzyme requires two substrates, chorismate and L-glutamine. The reaction mechanism was found to be sequential, and the presence of one substrate on the active site of the enzyme does not affect the binding of the second substrate. In the presence of the feedback inhibitor, L-tryptophan, L-glutamine utilization is noncompetitively inhibited. In addition, L-tryptophan is a partially competitive inhibitor of chorismate utilization.

EXPERIMENTAL PROCEDURE

Organisms—E. coli A2/F'A2 was derived from a λ-sensitive, episome-bearing strain received from Dr. R. Somerville. In

Despite the complexity of the reaction, this is the name currently used to describe the enzymic function which converts chorismate to anthranilate (6, 7).
addition to two colicinogenic factors, this episome carries the try operon and certain other closely linked markers (11). The try A2 mutation, causing an inability to produce the A subunit of tryptophan synthetase (12), was introduced by transduction into a λ-sensitive E. coli K-12 strain. This strain was then mated with the episome donor to yield a try A2/F+ exconjugant. After ultraviolet irradiation of the try A2/F+ strain, try A2/F’try A2 (A2/F’A2) recombinants appeared at a low frequency. These were identified by their auxotrophy and by their ability to transfer cysB+ and several try markers (including try A2) by conjugation. A. aerogenes 62-1 was received from Dr. F. Gibson.

Growth of Cells and Preparation of Extract—E. coli A2/F’A2 was grown in the minimal medium of Vogel and Bonner (13) supplemented with 30 μM indole and 17.2 mM glucose. Sixty-liter cultures were grown at 37° under maximum aeration in a Biogen until growth ceased because of indole disappearance. At this point, the organisms became derepressed for enzymes of the tryptophan pathway (14).

The cells were harvested by centrifugation, washed in 0.1 M Tris buffer, pH 7.5, and resuspended in a volume of the same buffer equal to 4 times their wet weight. Succeeding operations were performed at 0-5°. The suspension of cells was disrupted with a Branson sonic probe. Cell debris was removed by centrifugation for 20 min at 12,000 X g.

Chorismate Preparation—Chorismic acid was obtained from the growth filtrate of A. aerogenes 62-1 as described by Gibson (4). Instead of preparing a barium salt, free chorismic acid was crystallized from an ether-petroleum ether mixture. The crystals were dissolved in water just before use.

Enzyme Assay—Anthranilate formation was measured by noting an increase in fluorescence at 390 μμ in a recording spectrofluorophotometer. Fluorescence was activated by light at 313 μμ. The spectrofluorophotometer used (15) was sensitive to the appearance of 0.1 to 200 μμoles of anthranilate in the reaction mixture. In this instrument, the ratio of fluorescent light to incident light is recorded continuously. Fluorescent light is collected from the illuminated face of the cuvette, not at 90° from within the cuvette. These features permit a sensitive, continuous monitoring of anthranilate appearance that is independent of fluctuations in intensity of the light source and insensitive to changes in absorbance of the reaction mixture.

The reaction mixture contained Tris buffer (pH 7.5), 16.5 μμoles; EDTA, 0.66 μμole; magnesium chloride, 4.0 μμoles; chorismate, 0.2 μμole; glutamine, 10.0 μμoles; and enzyme in a total volume of 2.0 ml. The reaction was initiated by the addition of enzyme and the reaction mixture was maintained at 37°. Standard curves relating fluorescence intensity to anthranilate concentration were prepared. Reaction rates were calculated from the increase in anthranilate during the first 1 to 2 min of the reaction. One unit of activity represents the formation of 1.0 μμole of anthranilate in 20 min. Specific activity is units of activity per mg of protein.

Enzyme activity in starch gels was located by the fluorescence of the product under near ultraviolet illumination when a small amount of the substrate mixture had been allowed to diffuse into the horizontally sliced gel.

Protein Determination—Protein was determined colorimetrically (16) with bovine serum albumin as the standard.

Starch Gel Electrophoresis—This procedure was performed according to Smithies’ method (17) with the use of horizontal trays. The buffer used for gel preparation was 0.03 M sodium borate, pH 8.43, and the reservoir buffer was 0.3 M sodium borate, pH 8.5. The temperature of the gel remained below 20° during an electrophoresis time of 4 hours at 11 volts per cm. Protein bands were visualized by staining the horizontally sliced gel with Buffalo black (17).

Gel Filtration—Dry Sephadex G-200 was permitted to swell for several days in the elution buffer. After a glass column with a sintered glass disc was filled to one-half its volume with the elution buffer, a small amount of Sephadex G-25 was layered on the glass disc. The column was then packed with Sephadex G-200 by allowing a slurry to flow slowly down the inner wall until the desired bed size (diameter, 6.5 cm; height, 46 cm) was attained. A small amount of Sephadex G-25 was then layered on top of the G-200 bed and the column was equilibrated with elution buffer for a minimum of 16 hours. The flow rate of the column was 0.45 ml per cm² per hour. The elution buffer was composed of 20 mM Tris, pH 7.5, containing 0.1 mM EDTA and 1.0 mM 2-mercaptoethanol.

RESULTS

Partial Purification of Enzyme—Five milliliters of 1 mM manganese chloride were added with stirring to each 100 ml of cell-free extract. The suspension was centrifuged immediately for 20 min at 12,000 X g. To each 100 ml of supernatant fluid 14 g of solid ammonium sulfate were added slowly with stirring. The precipitate was removed by centrifugation for 20 min at 12,000 X g. The supernatant fluid was then treated similarly with 7 g of ammonium sulfate and centrifuged. This second precipitate was resuspended in a minimal amount of 20 mM Tris buffer, pH 7.5, containing 0.1 mM EDTA and 1.0 mM 2-mercaptoethanol.

Further purification was accomplished by gel filtration on Sephadex G-200. Ten milliliters of the enzyme suspension were layered under buffer onto a Sephadex G-200 column prepared as described above. The elution pattern showed three major protein peaks; the middle peak contained anthranilate synthetase activity. Those fractions with a constant specific activity were combined and concentrated by precipitation with 28 g of solid ammonium sulfate per 100 ml. Enough elution buffer was added to the precipitate to make a slurry, and this sample (4 to 5 ml) was added to the top of the same Sephadex G-200 column. The elution pattern of the second gel filtration (Fig. 1) showed a

Fig. 1. Elution pattern of anthranilate synthetase from the second Sephadex G-200 column. Fractions 105 through 115 were collected and concentrated as described in the text. □, enzyme activity; ●, protein.
single major protein peak containing anthranilate synthetase activity with small inactive satellite peaks on either side. Again fractions with constant specific activity were collected and concentrated by ammonium sulfate precipitation. The precipitate was dissolved in a minimal amount of elution buffer and centrifuged at 12,000 × g for 20 min. Solid ammonium sulfate was added to the supernatant fluid until a precipitate was formed. The activity in such a suspension was stable for several weeks at 3°. Units of activity and specific activity were measured after each purification step and are recorded in Table I.

Chromatography on cellulose ion exchange columns has so far been unsuccessful, resulting in a 40 to 50% decrease in specific activity and a recovery of 10 to 30% of the enzyme activity added to the column. When fractions from the two edges of the activity peak were mixed and assayed for enzyme activity, additive results were obtained, indicating that the loss of activity was not due merely to the separation of active components.

Effect of Hydrogen Ion Concentration—A series of eight reaction mixtures at different hydrogen ion concentrations was used to study the pH dependence of the anthranilate synthetase-catalyzed reaction. In these, 0.05 ml of 1.0 M Tris-chloride at various pH values from 6.0 to 9.5 was substituted for the usual buffer in the reaction mixture. The actual pH of each reaction mixture was measured before adding enzyme. Anthranilate standard curves were obtained at each pH to correct for any change in fluorescence yield over the pH range. A typical pH dependence curve was obtained with an optimum at 7.5.

Effect of Sulfhydryl Inhibitors—A reaction mixture containing 52 units of enzyme activity but lacking either chorismate, glutamine, or both was incubated for 15 min at room temperature in the absence and in the presence of varying concentrations of iodoacetic acid and p-chloromercuribenzoate. The proper substrate or substrates were then added and activity was measured. A 50% decrease in enzymatic activity was obtained with 10 mM iodoacetic acid or 60 μM p-chloromercuribenzoate; no activity remained after incubation in the presence of 90 μM p-chloromercuribenzoate. The presence of saturating quantities of either substrate during the incubation period had little effect on the inhibition.

Starch Gel Electrophoresis—The anthranilate synthetase preparation obtained after each gel filtration step in the purification procedure was subjected to electrophoresis on starch gel as described by Smithies (17). The pattern obtained (Fig. 2) reveals only a small quantity of contaminating protein in the more purified anthranilate synthetase preparation (Channel B). Enzyme activity was detected as a sharp band coincident with the protein band in Channel B. In Channel A, excessive trailing of activity was noted and no distinct activity band could be distinguished. If any large amount of contaminating protein is present in Channel B, it has the same electrophoretic mobility in this system as anthranilate synthetase.

### Table I

<table>
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<th>Purification of anthranilate synthetase</th>
<th>Enzyme fraction</th>
<th>Total volume</th>
<th>Total enzyme*</th>
<th>Total protein</th>
<th>Specific activity*</th>
<th>Yield %</th>
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<tr>
<td>Cell-free extract</td>
<td>500 ml</td>
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<td>15.00</td>
<td>2.9</td>
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<td>Ammonium sulfate precipitate</td>
<td>12 g</td>
<td>15,250</td>
<td>1.44</td>
<td>10.6</td>
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<td>9,600</td>
<td>0.24</td>
<td>40.0</td>
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</tr>
<tr>
<td>Second Sephadex fractionation</td>
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<td>3,320</td>
<td>0.06</td>
<td>52.0</td>
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</table>

* Units of activity and specific activity are defined in the text.
Ultracentrifugation—Ultracentrifugation measurements of the most purified enzyme preparation were made in a Spinco model E ultracentrifuge. The profile presented in Fig. 3 reveals a single principal protein boundary preceded by several minor boundaries judged to be contaminant protein. No quantitative measurement of the minor components has been made.

Sedimentation velocity was measured at protein concentrations of 4.0, 8.0, and 11.0 mg per ml. A linear relationship was obtained when $s_{20,w}$ was plotted against protein concentration and the corrected sedimentation coefficient ($s_{20,w}$) was 10.7 S.

Effect of Sodium Dodecyl Sulfate on Sedimentation Velocity—Several sedimentation velocity studies were performed in the presence of 50 mM sodium dodecyl sulfate. The enzyme formed a single symmetrical boundary in the ultracentrifuge as shown in Fig. 4. Sodium dodecyl sulfate has been used to dissociate proteins into subunits (18, 19), presumably without cleaving covalent bonds, and possesses the advantage over other agents of being effective at relatively low concentrations. Thus studies of sedimentation can be made without markedly affecting the buoyancy term as would be the case with agents such as urea (18). Some or all of the detergent may attach to the protein, however, causing a slight increase in the sedimentation coefficient. When sedimentation measurements were plotted against protein concentrations at 3, 6, and 10 mg of protein per ml, an $s_{20,w}$ of 2.8 S was obtained. This value should be considered a maximum estimate.
Two Substrate Kinetics—The following kinetic studies were made with the use of the enzyme fraction from the first Sephadex column (Channel A, Fig. 2). Initial rate studies were performed and plotted as described by Florini and Vestling (20) for bireac- tant sequential mechanisms. Fig 5 is a double reciprocal plot showing the initial rates of anthranilate formation as a function of chorismate concentration at several fixed concentrations of glutamine. The double reciprocal plots are linear and all lines converge at a single point to the left of the ordinate. Fig. 6 shows replots of the slopes and intercepts obtained from Fig. 5 against the reciprocals of the several glutamine concentrations. From the intercept values (Fig. 6), the $K_m$ of glutamine is calculated to be 0.36 mM. Fig. 7 shows double reciprocal plots of initial rates with glutamine as the variable substrate and chorismate the changing fixed substrate. All lines converge at a single point to the left of the ordinate. Replots of slopes and intercepts are presented in Fig. 8. Again the double reciprocal plots as well as replots of slopes and intercepts are linear (21). The $K_m$ for chorismate is calculated from Fig. 8 to be 1.2 $\mu$m.

Feedback Inhibition Kinetics—Other investigators have examined anthranilate synthetase from Aerobacter aerogenes (5, 6) and Neurospora crassa (7), and in each case the activity has been sensitive to feedback inhibition by tryptophan. To study the effect of this inhibitor on the apparent $K_m$ and $V_{max}$, initial reaction velocities were measured over a series of substrate concentrations in the presence and in the absence of tryptophan. Fig. 9 is a plot of initial velocities against glutamine concentrations with the chorismate concentration fixed at 0.1 mM. These data show that tryptophan is a noncompetitive inhibitor of glutamine in this reaction.

When the initial velocity of anthranilate formation is plotted against varying concentrations of chorismate at a constant glutamine concentration (5.0 mM) in the presence and absence of tryptophan, a more complicated relationship is obtained (Fig. 10). The reciprocal plot (Fig. 10D) in the absence of inhibitor is linear. In the presence of tryptophan, the reciprocal plot would appear to yield a curved line for substrate concentrations less than 10 $\mu$m. However, the substrate saturation curve (Fig. 10A) in the presence of tryptophan approximates a straight line rather than a sigmoid curve at low substrate concentrations. A more de-
tailed discussion of these plots is presented in a later section. The data in Fig. 10 show that tryptophan is competitive with chorismate, for lines in the reciprocal plot extrapolate to the same $V_{\text{max}}$ value.

In simple competitive inhibition, the inhibitor occupies the active site; therefore, the enzyme-inhibitor complex has no affinity for the substrate and cannot act as a catalyst. At very high concentrations such an inhibitor would stop the reaction completely. Fig. 11 shows that tryptophan cannot completely inhibit anthranilate synthetase activity. With increasing tryptophan concentrations, a limit is reached beyond which additional tryptophan has no effect on reaction velocity. Thus competition is only partial in this case, and the enzyme-inhibitor complex shows catalytic activity even though substrate affinity has been markedly decreased.

Using the basic Michaelis assumptions, Atkinson, Hathaway, and Smith (22) derived Equation 1

$$\log \left[ \frac{v}{V_{\text{max}} - v} \right] = n \log S - \log K$$

(1)

**Fig. 9.** A, effect of glutamine concentration on anthranilate synthetase activity in the presence and absence of tryptophan. Chorismate concentration was fixed at 0.1 mM. Tryptophan was present in concentrations indicated. $S$ represents the millimolar concentration of glutamine; $v$ is expressed in millimicromoles of anthranilate produced per min. $B$, double reciprocal plots of the same data.

**Fig. 10.** A, effect of chorismate concentration on anthranilate synthetase activity in the presence and absence of tryptophan. The glutamine concentration was fixed at 5.0 mM. Chorismate was present in concentrations indicated. $S$ represents the micromolar concentration of chorismate. $v$ is expressed in millimicromoles of anthranilate produced per min. $B$, double reciprocal plots of the same data.

**Fig. 11.** Tryptophan inhibition at various fixed concentrations of chorismate. The glutamine concentration was fixed at 5.0 mM. Chorismate concentrations are presented above each curve. $I$ represents the micromolar concentration of tryptophan.

**Fig. 12.** Anthranilate synthetase activity as a function of chorismate concentration, plotted according to the "empirical Hill equation" (see text). The glutamine concentration was fixed at 5.0 mM. $S$ represents chorismate concentrations varied from 1.0 to 50 $\mu$M. $v$ is expressed in millimicromoles of anthranilate produced per min. Tryptophan concentrations were: $\bullet$, none; $\square$, 2.5 $\mu$M; $\triangle$, 25 $\mu$M.

which they suggest indicates the number of interacting substrate-binding sites for yeast DPN-isocitrate dehydrogenase. This equation, the empirical Hill equation (23), has been used in analyzing the kinetics of several feedback-sensitive enzymes that show substrate-cooperative effects (22, 24, 25). A plot of $\log \left[ v / V_{\text{max}} - v \right]$ against $\log S$ should give a straight line of slope $n$, where $n$ is the measure of an interaction coefficient between substrate-binding sites. Such a plot is presented in Fig. 12 in an attempt to clarify the relationship shown in Fig. 10B. Slopes equal to 1 are obtained in the absence of inhibitor and in the presence of 2.5 $\mu$M L-tryptophan; data for the latter experiment were not included in Fig. 10. In the presence of 25 $\mu$M tryptophan, however, two slopes are indicated. For substrate concentrations under 10 $\mu$M, $n = 1.35$, but for substrate concentrations greater than 10 $\mu$M, $n = 1$. 


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grown under conditions of tryptophan pathway derepression.

tryptophan was varied from 0.025 mM to 0.375 mM. The slope

fixed at 5.0 mu. I represents tryptophan concentrations varied

from 0.025 to 0.375 mm. r is expressed in millimicromoles of anthranilate produced per min. $V_o$ is enzyme activity in the absence of inhibitor. Chorismate concentrations were: □, 0.2 mm; ○, 0.08 mm.

A similar plot has been used to determine interacting inhibitor-binding sites (24, 26). Fig. 13 is a plot of log $[V/V_o - v]$ as a function of log $I$ at two fixed concentrations (0.08 and 0.2 mm) of chorismic acid. The glutamine concentration was fixed and tryptophan was varied from 0.025 mm to 0.375 mm. The slope equals $-1$ in each case, indicating an absence of interaction.

**DISCUSSION**

**Enzyme Purification**—An 18-fold purification of anthranilate synthetase was obtained from crude extracts of E. coli A2/F4A2 grown under conditions of tryptophan pathway derepression. It should be noted that extracts of wild type E. coli growing in minimal medium contain less than 1.5% of the activity shown by our starting material (27).

The results of starch gel electrophoresis and ultracentrifugation taken together suggest that the enzyme at this purity represents the predominant protein present in the preparation. But it is still not possible to decide whether one enzyme or an enzyme complex is responsible for catalyzing the conversion of chorismate to anthranilate.

**Sedimentation Studies**—Sedimentation measurements on the native enzyme provided an $s_{20,w}$ of 10.7. In the presence of sodium dodecyl sulfate the $s_{20,w}$ was 2.8. The dependence of the sedimentation coefficient on concentration was measured in each case using the equation

$$s = \frac{s_0}{1 + Kc}$$

where $s$ is the sedimentation coefficient at concentration $c$, $s_0$ is the sedimentation coefficient at infinite dilution, and $K$ is a constant. This equation and the factors influencing it have been discussed in detail by Schachman (28). The values of $K$ for the native enzyme and treated enzyme are 0.39 and 0.56, respectively. The large decrease in $s_{20,w}$ accompanied by a small increase in $K$ in the presence of sodium dodecyl sulfate might suggest a decreased molecular weight for the treated enzyme, but such extrapolations are too uncertain to allow any definite statements about subunit structure.

**Enzyme Kinetics**—Cleland (29) has shown that results of the type described in Figs. 5 to 8, where the ratio of the apparent Michaelis constant to apparent maximum velocity (Figs. 6 and 8) varies as the concentration of fixed substrate is changed, are consistent with sequential reaction mechanisms. The anthranilate synthetase reaction mechanism appears to be sequential "bi-ter," i.e. a sequential mechanism having two substrates and three products. This kinetics is in agreement with the mechanisms postulated by Levin and Sprinson (1) and by Srivivasan (9).

Florini and Vestling (20) point out that the ordinates of the points of intersection for plots such as those represented by Fig. 5 and Fig. 7 are an indication of the effect of one substrate on the binding of the other substrate. All lines in Figs. 5 and 7 cross at a single point on the abscissa. Thus the $K_m$ is the same for one substrate at several fixed concentrations of the second substrate. These data are consistent with Alberts's special case of Mechanism 1 (30), where the presence of one substrate has no effect on the complex formation of the second substrate.

The kinetic data presented in Figs. 9 to 11 show that both substrates of anthranilate synthetase are affected by feedback inhibition. This inhibition is noncompetitive against glutamine and partially competitive against chorismate. These kinetic results suggest that there are distinct binding sites for substrates and inhibitor. More direct evidence for separate binding sites has not been obtained in this system, however.

It has been noted (31, 32) that homotropic effects of the substrate have been observed with regulatory enzymes in which the allosteric ligand affects preferentially the affinity for substrate (the $K_s$ systems). With anthranilate synthetase, the noncompetitively inhibited substrate, glutamine, does not exhibit a homotropic effect under the experimental conditions tested. But neither does the competitively inhibited substrate, chorismate, exhibit a homotropic effect in the absence of inhibitor or in the presence of 2.5 $\mu$M inhibitor (Figs. 10 and 12). In the presence of 25 $\mu$M inhibitor, the substrate saturation curve (Fig. 10A) still does not show the sigmoid character recognized as a common characteristic of regulatory enzymes belonging to the $K_s$ system, but the same data when presented in a double reciprocal plot (Fig. 10B) give a curve with increasing slope at low substrate concentrations. This curve was seen consistently in several independent experiments. At this concentration of inhibitor, the Hill plot (Fig. 12) is not linear. The data are represented in Fig. 12 as two straight lines with a break at 10 $\mu$M chorismate. The slope, $n$, equals 1.0 for values above 10 $\mu$M chorismate and 1.35 for values below 10 $\mu$M chorismate. These data may indicate slight substrate cooperation, but this interpretation depends on the accuracy of the values at very low substrate concentrations. Alternatively, in this case, the nonlinearity of the Hill plot may reflect the inability of the Hill equation to provide an accurate measure of interacting substrate-binding sites. Perhaps a study of feedback inhibition kinetics under different experimental conditions, for example, by varying the pH and the $Mg$ concentration, will yield more conclusive evidence on the question of substrate cooperation in anthranilate synthetase.

Fig. 13 indicates that within the range of inhibitor and substrate concentrations used in these experiments there are no interacting inhibitor-binding sites on this enzyme.

It should be noted that $Mg^{2+}$ stimulates anthranilate synthetase activity and has been added consistently to the reaction mixture in saturating concentrations. It is quite possible that $Mg^{2+}$ will be found to have an effect on the kinetics of the reaction in the presence or absence of the feedback inhibitor.
Acknowledgment—We wish to express our appreciation to Dr. G. Weber who designed the recording spectrofluorophotometer used in this work and gave advice and assistance in its construction.

REFERENCES
9. Srinivasan, P. R., Biochemistry, 4, 2860 (1965).
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