SUMMARY

The anthranilate aggregate, which catalyzes the first two reactions of tryptophan biosynthesis in *Escherichia coli*, consists of two anthranilate synthetase subunits and two phosphoribosyltransferase subunits. The aggregate remains associated under physiological conditions.

Three lines of evidence indicate that regulation in the aggregate involves conformational changes associated with the binding of the substrates, chorismate and glutamine, and the feedback inhibitor, tryptophan. First, either chorismate or glutamine, the anthranilate synthetase substrates, can pseudocompetitively reactivate tryptophan-inhibited phosphoribosyltransferase. Second, the regulatory ligands alter the susceptibility of the aggregate to inactivation by metal ions and by the glutamine analog, 6-diazo-5-oxo-1-norleucine. Third, direct physical evidence for conformational changes was obtained using the fluorescent probe, 8-anilino-1-naphthalene sulfonate.

The activities of the aggregate are regulated through alterations in the equilibrium between activated and inhibited conformational states associated with ligand binding. Regulation is competitive because the binding of chorismate and tryptophan or glutamine and tryptophan is mutually exclusive for allosteric reasons.

Several independent kinetic and inactivation experiments indicate that glutamine binds to the enzyme in the absence of chorismate. However, glutamine hydrolysis does require that chorismate be present. The binding of chorismate is presumably associated with a conformational change required for the hydrolysis of glutamine. Anthranilate synthetase thus has an ordered reaction mechanism, but random subunit binding.

Several mutationally altered anthranilate aggregates, either resistant to feedback inhibition, or supersensitive to feedback inhibition, were examined. A double mutant containing both resistant and the supersensitive alleles was very similar in kinetic properties to wild type. Revertants of the supersensitive strain were isolated, and their anthranilate aggregates were analyzed. In one revertant, the co-operative, competitive pattern of inhibition found in wild type was altered to a noncooperative, noncompetitive pattern. In another, tryptophan inhibition was completely eliminated, but the catalytic functions were unimpaired. Therefore, the chorismate and tryptophan binding sites, though competitive, are separate.

All the mutations had parallel effects on both anthranilate synthetase and phosphoribosyltransferase, indicating that both activities of the aggregate are regulated by a common mechanism. It is clear that the equilibrium between active and inhibited conformational states of the aggregate is susceptible to mutational alteration. A mechanical model for the operation of the regulatory mechanism in the anthranilate aggregate is presented.

A tetrameric protein, containing two subunits each of anthranilate synthetase and phosphoribosyltransferase, catalyzes the first two reactions specific for tryptophan biosynthesis in *Escherichia coli* (Fig. 1) (1). Anthranilate is formed on one subunit from chorismate and glutamine in a complex reaction (2-4), then converted to phosphoribosyl anthranilate on a separate subunit (5). The name "anthranilate aggregate" is suggested for these two associated enzymes, whose functions are to produce and consume anthranilate.

Both activities of the aggregate are inhibited by the end product of the pathway, tryptophan (6). In the presence of the substrate, tryptophan can completely inhibit anthranilate synthetase, but the maximum inhibition of phosphoribosyltransferase is approximately 70%. Glutaminase, required in the anthranilate synthetase reaction, apparently resides on the phosphoribosyltransferase subunit. Chain termination mutations in the operator proximal 25$^+$ of the trpD gene (phosphoribosyltransferase) lead to uncomplexed anthranilate synthetase, since they are no longer able to use glutamine as the source of the amino group (1, 7). Further evidence locating the glutamine site on the phosphoribosyltransferase subunit comes from studies using the glutamine analog, DON. This compound inactivates glutamine synthetase-chorismate-5-phosphoribosylpyrophosphate phosphoribosyltransferase enzyme aggregate.

1 "Anthranilate aggregate" is a short name for the anthranilate synthetase-chorismate-5-phosphoribosylpyrophosphate phosphoribosyltransferase enzyme aggregate.

2 The abbreviations used are: DON, 6-diazo-5-oxo-1-norleucine; ANS, 8-anilino-1-naphthalene sulfonate; PRPP, 5-phosphoribosyl-1-pyrophosphate.
mine-dependent anthranilate synthetase because it covalently binds to the glutamine sites on the phosphoribosyltransferase subunits (8).

In the wild type enzyme, the subunit aggregation is extremely tight. At neutral pH, 10^{-11} M enzyme retains full glutamine-dependent anthranilate synthetase activity. The standard techniques of protein purification will not separate the subunits (6, 9, 10), but they can be irreversibly dissociated by denaturation in 8 M urea (8). Attempts at in vitro subunit complementation in systems requiring dissociation of the tetramer have been negative. When an extract containing a catalytically defective anthranilate synthetase aggregated to an intact phosphoribosyltransferase is mixed with active but unaggregated anthranilate synthetase, formation of a species capable of utilizing glutamine in the anthranilate synthetase reaction does not occur (7). Subunit exchange is very poor even in vivo. A recombination defective strain harboring a missense mutation in the trpE gene (anthranilate synthetase) on an episome and a nonsense mutation in the trpD gene (phosphoribosyltransferase) on the chromosome was synthesized. An extract of this merodiploid, when assayed for glutamine-dependent anthranilate synthetase, contained only 10% of the activity expected if complementation were taking place. This indicates that aggregation between subunits is irreversible, and takes place without an opportunity for equilibration with the total cytoplasmic protein.3 Tryptophan has little or no effect on the molecular weight of the aggregate. The elution profile on a Sephadex G-200 column is not affected by 100 nM tryptophan.3 It is therefore unlikely that association-dissociation phenomena play a role in the regulation of the aggregate.

Several lines of evidence suggest that the regulation of this aggregate probably involves changes in conformation. With the very similar enzyme from Salmonella typhimurium, equilibrium dialysis studies revealed two competitively related binding sites each for tryptophan and chorismate (11). Tryptophan inhibits both activities of the aggregate, so the two tryptophan-binding sites communicate with all four subunits. Chorismate is not only a substrate of the anthranilate synthetase reaction, but it also counteracts inhibition by tryptophan of phosphoribosyltransferase (12). Because uncomplexed phosphoribosyltransferase is not susceptible to tryptophan inhibition (6), regulatory stimuli must emanate from the anthranilate synthetase subunits. Finally, plots of the reciprocal of anthranilate synthetase velocity versus the reciprocal of the concentration of chorismate show, in the presence of tryptophan, an upward curvature (Hill number = 1.4), which is most readily interpreted in terms of cooperativity between binding sites (9).

The glutamine-binding sites are also part of the communication system between the subunits. Glutamine is hydrolyzed by the phosphoribosyltransferase subunits, but the ammonia produced is used by the anthranilate synthetase subunits. Therefore, the catalytic mechanism itself involves interaction between subunits. In addition, regulatory signals pass from the anthranilate synthetase subunits to the sites of glutamine hydrolysis on the phosphoribosyltransferase subunits. The hydrolysis of glutamine, measured independently from the anthranilate synthetase reaction, requires chorismate and is inhibited by tryptophan (13, 14). Tryptophan is noncompetitive with glutamine in inhibiting the anthranilate synthetase reaction (9), suggesting that the binding of tryptophan alters that proportion of the enzyme that is able to hydrolyze glutamine.

There is evidence suggesting communication between sites on a single subunit in addition to communication between subunits. Tryptophan inhibition of anthranilate synthetase is competitive with chorismate (9), and indeed the binding of tryptophan is competitive with chorismate (11). Yet, it is unlikely that tryptophan and chorismate share the same sites. They are certainly not, in any obvious chemical sense, analogs. The tryptophan sites are quite specific; 

\[ \text{Trp} \]

is the mutant MTanya number 2 (15).

The present investigation was undertaken in order to extend our understanding of regulation of both activities in which the binding of the ligands chorismate, glutamine, and tryptophan control catalysis.

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1 R. L. Somerville, unpublished experiments.
Chemicals—Chorismic acid was prepared by the method of Gibson (16) and assayed by enzymatic conversion to anthranilate. D- and L-Glutamine, D- and L-tryptophan, and PRPP dimagnesium salt, were purchased from Calbiochem. m5-Methyltryptophan was obtained from Cyclo Chemical Corp. ANS, purchased from Eastman as the sodium salt, was dissolved in absolute ethanol, filtered, and recrystallized from hot ethanol. DON was a gift of the Cancer Chemotherapy National Service Center, Bethesda, Maryland. Protamine sulfate was obtained from Elanco Products.

Enzyme Assays—Anthranilate synthetase was assayed at 25° by measuring product formation fluorimetrically (excitation 310 nm, emission 400 nm). Standard reaction mixtures contained 0.1 mM chorismic acid, 25 mM glutamine, 2 mM magnesium chloride, and 70 mM Tris-chloride buffer, pH 7.8, in a total volume of 1.0 ml. Phosphoribosyltransferase was assayed at 25° by measuring the consumption of anthranilate fluorimetrically. This reaction mixture contained 0.01 mM anthranilate, 0.1 mM PRPP, 2 mM magnesium chloride, and 70 mM Tris in 1.0 ml. A unit of enzyme is that amount which catalyzes the formation or utilization of 1 pmole of anthranilate per min.

Growth of Cells and Enzyme Purification—The aggregate was purified from strain A2/F'.A2 (9). Cells were grown at 37° with shaking in 1-liter volumes of Vogel and Bonner (17) minimal medium type E with 0.2% glucose and limiting indole (2 mg per liter). Upon exhaustion of the indole, the tryptophan enzymes derepress, resulting in greatly elevated production of anthranilate synthetase-phosphoribosyltransferase. Derepression was routinely checked by testing for the accumulation of indoleglycerol phosphate with ferric chloride reagent (18). The yield of cells was approximately 1 g per liter.

The aggregate was purified by the procedure of Henderson et al. (10). In a typical preparation, cells (30 g wet weight) were suspended in 150 ml of Buffer A (0.05 M potassium phosphate buffer, pH 7.4, containing 0.2 mM EDTA and 1 mM dithiothreitol) and disrupted ultrasonically. The crude extract was treated with 0.17 mg of protamine sulfate per mg of protein. The precipitate was removed by centrifugation and discarded. The enzyme was precipitated at 30% saturation with ammonium sulfate, resuspended in Buffer A, centrifuged 2 hours at 175,000 × g, and the supernatant dialyzed extensively against Buffer B (Buffer A plus 30% glycerol). The dialyzed enzyme was applied to a DEAE-cellulose column (Bio-Rad Cellex D, 40 cm × 3 cm) and eluted with a linear gradient of ammonium chloride (0 to 0.4 M) in Buffer B (600 ml total eluent volume). The most active fractions were pooled, concentrated by ammonium sulfate precipitation, redissolved in Buffer B, applied to a Bio-Gel Agarose A-5m column (60 cm × 1.5 cm) and eluted with Buffer B. The fractions having the highest specific activity were pooled, concentrated by ultrafiltration, then refractionated on an Agarose column. The fractions of constant specific activity were pooled and concentrated by ultrafiltration. All column steps were performed with a pressure head of 1 m in order to provide reasonable flow rates with the viscous glycerol buffer. All steps were performed at 0-4°.

This procedure yielded approximately 25 mg of a preparation with a specific activity of 4100 units of anthranilate produced per min per mg of protein in the standard anthranilate synthetase reaction (glutamine as the amino donor). Upon polyacrylamide disc gel electrophoresis and staining, a single major band containing more than 95% of the protein was observed.

ANS Fluorescence—Fluorescence measurements were made on an Amino-Bowman spectrophotofluorimeter using quartz cuvettes having a 1 cm light path. The cuvette temperature was maintained at 25°. Emission spectra were not corrected for variation in the sensitivity of the detection system at different wave lengths.

When ANS fluorescence was measured, it was necessary to correct for the self-absorbance by ANS of the 370 nm excitation light. Absorbance of the 480 nm emitted light was negligible. The correction was made according to the relationship:

\[ I_{\text{corrected}} = I_{\text{observed}} \times \frac{2.303 \Delta y}{e^y - 1} \]

where \( \Delta y \) is the molar extinction coefficient of ANS at wave length \( \lambda \), \( c \) is the concentration of ANS in moles per liter, \( \Delta y \) is the emission slit width, and \( y \) is the distance the exciting light must travel from entering the cuvette until it reaches the plane parallel to the near side of the emission slit. (This expression is equivalent to Equation 24 in Reference 19.) In our case \( \Delta y = 0.30 \text{ cm} \) and \( y = 0.35 \text{ cm} \). The extinction coefficient for ANS determined under our conditions was 6300 M⁻¹ cm⁻¹ at 370 nm.

The correction factor has an approximate value of 1.0 at a concentration of ANS less than 10 µM. At 100 µM the correction factor is approximately 2; at 200 µM the factor is 4, and so on, increasing exponentially at higher concentrations.

In cases where the fluorescence of anthranilate was measured in the presence of ANS, both the exciting (310 nm) and the emitted (400 nm) light were absorbed by ANS. An approximate correction for this effect is given by the expression:

\[ I_{\text{corrected}} = I_{\text{observed}} \left( \frac{e^{2.303 \Delta y c}}{e^{2.303 \Delta y c} - 1} \right) \]

where \( e_\lambda \) is the molar extinction coefficient of ANS at the indicated wave length, \( c \) is the molar concentration of ANS, and \( I \) is the light path of the cuvette in centimeters. This expression assumes that, on the average, exciting light is absorbed from the wall of the cuvette to the center, and that emitted light is absorbed from the center to the perpendicular wall. These assumptions are appropriate in the case of the standard Amino-Bowman geometry, in which the slits are narrow relative to the light path of the cuvette, and in which the slits open onto the center of the cuvette. The extinction coefficients for ANS were \( e_{303} = 3500 \) and \( e_{100} = 2100 \). Again, the correction factor is approximately 1.0 when ANS concentration is less than 10 µM, increasing to 1.9 at 100 µM, and rising exponentially thereafter.

The parameters of ANS binding were determined by the procedure of Wang and Edelman (20). The maximum fluorescence intensity of a fixed amount of enzyme in the presence of an infinite amount of ANS (\( I_{\text{max}} \)), and the dissociation constant for the enzyme-ANS complex (\( K_d \)) was obtained from the intercept and slope of a double reciprocal plot of fluorescence intensity versus ANS concentration, according to the equation:

\[ \frac{1}{I} = \frac{1}{I_{\text{max}}} + \frac{K_d}{I_{\text{max}}} \times \frac{1}{\text{ANS}} \]

where \( I \) is the observed fluorescence intensity, and \( \text{ANS} \) is the total concentration of ANS. This equation applies under the condition that \( \text{ANS} \) is much greater than \( K_d \). In these experiments \( K_d = 0.05 \mu M \) (assuming mol wt 250,000) and \( \text{ANS} \) varied from 1 µM to 50 µM. Fluorescence intensities were corrected for self-absorbance by ANS as described above.

The number of ANS binding sites on the aggregate, \( n \), was determined by a double reciprocal plot of fluorescence intensity versus enzyme concentration, according to the equation:
\[
\frac{\text{ANS}_0}{I} = \frac{1}{\psi} + \frac{K_A}{\psi} \times \frac{1}{E_0}
\]

where \(\psi\) is the fluorescence intensity per unit concentration of the (enzyme-ANS) complex, obtained from the y-intercept. This equation holds under the condition that \(nE_0\) is much greater than \((E-\text{ANS})\). In these experiments, \(\text{ANS}_0 = 0.1 \mu\text{M}\), and \(E_0\) varied from 0.6 \(\mu\text{M}\) to 6.0 \(\mu\text{M}\).

**Mutant Strains**—All bacterial mutants were substrains of *E. coli* K-12. Mutant MTR, selected on the basis of its resistance to inhibition of growth by 5-methyltryptophan, is strain MTR number 2 of Somerville and Yanofsky (15). Strain MT8, selected by the penicillin technique for extreme sensitivity to 5-methyltryptophan, is strain 6B of Kuhn et al. (21).

The recombinant MT8/S was prepared by P1 transduction. A lysate prepared on strain MT8 was used to infect an MTR trpB double mutant. Elimination of trpB was selected by plating the infected cells on indole agar plates. The trpB+ recombinants fell into four classes based on sensitivity to 5-methyltryptophan: MTR, MT8, wild type, and a fourth class, the putative MT8/S recombinants. The identity of the MT8/S class was confirmed by recovering the MTR allele by transduction.

Revertants of MT8 were selected following treatment with 2-aminopurine, ethyl methanesulfonate, or nitrosoguanidine and selection for reversal of 5-methyltryptophan supersensitivity, i.e., ability to grow on plates containing 2 \(\mu\text{g}\) per ml of 5-methyltryptophan. Thirty-three revertants were picked and grouped into five classes on the basis of their sensitivity to 5-methyltryptophan, as established by a zone of inhibition test (21). In this test, a Petri dish is seeded with a culture of the strain to be examined. A filter paper disc (12.7 mm) is placed in the center of the dish and 5-methyltryptophan (20 \(\mu\text{g}\)) is placed on the disc. After incubation, a circular zone of growth inhibition, having a sharp boundary, appears around the disc. The diameter of this zone of inhibition was reproducible for the strain in question. The validity of this procedure was confirmed by recovering the MTR allele by transduction of the other strains discussed in this paper are also given. A preliminary kinetic analysis indicated that the classes represented by EMS4 and AP4 were of particular interest, and these two revertants were analyzed further. In a separate experiment, two spontaneous revertants were obtained. They were identical with EMS0.

**Preparation of Mutant Enzymes**—MT8 and MT8/S type enzyme aggregates were obtained from derived strains MT8A2/FMT8A2 and MT8/A2/FMT8A2, as described for the wild type enzyme. Similarly, MTR enzyme aggregate was obtained from strain MTRB311/FMTRB311. These mutant enzymes were partially purified exactly as described for wild type, up to and including dialysis against Buffer B.

The revertants of MT8 were grown directly, without recourse to the genetic manipulations employed to produce mutant derivatives capable of high enzyme yields. These cells were grown overnight with shaking at 37°C in type E minimal medium (17) plus 0.5% glucose. Before use, the crude extracts were dialyzed 16 hours against 100 volumes of Buffer D, changing the buffer every 4 hours.

### RESULTS

**Steady State Kinetic Analysis of Wild Type Aggregate: Competition between Tryptophan and Chorismate**—As first shown by Baker and Crawford (9), tryptophan inhibition of anthranilate synthetase is competitive with the substrate chorismate (Fig. 2A). In the absence of tryptophan, one obtains linear double reciprocal plots; the apparent \(K_m\) for chorismate is 5 \(\mu\text{M}\). In the presence of tryptophan, such double reciprocal plots show an upward curvature, suggesting a low level of cooperativity. The Hill number (22) in the presence of tryptophan is 1.4. Because of the curvature, the determination of \(K_m\) for chorismate from this type of plot is difficult. A more useful plot is that of Dixon (23), shown in Fig. 2B. The point of intersection of the lines, extended to the y axis, gives \(-K_m\), which in this case is 1 \(\mu\text{M}\). Again the lines exhibit curvature, corresponding to a Hill number of 1.5.

Values for the apparent \(K_m\) for chorismate and for the Hill numbers have been determined previously. However, plots of data determined under our conditions are given here, as they are the basis of comparison for similar plots for the phosphoribosyltransferase activity and for several mutant enzyme aggregates. This type of kinetics will be referred to as "pseudo-competitive," to convey the idea that while the basic pattern of inhibition is competitive, a simple model in which chorismate and tryptophan bind to the same site is not likely to be appropriate for this enzyme. Tryptophan also inhibits the phosphoribosyltransferase activity of the aggregate, as first shown by Ito and Yanofsky (6). Looking along the y-axis in Fig. 3A, if the velocity is 100% of the \(V_{\text{max}}\) in the absence of tryptophan, in the presence of 0.5 \(\mu\text{M}\) tryptophan the velocity is 50%, and at 5.0 \(\mu\text{M}\) tryptophan the velocity is 30%. The maximum inhibition that can be obtained is approximately 70%. Chorismate counters the inhibitory effect of tryptophan; the velocity of tryptophan-inhibited phosphoribosyltransferase increases with increasing concentrations of 5-methyltryptophan.

<table>
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<th>Class</th>
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<th>Number found</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>AP2</td>
<td>3.5</td>
<td>5</td>
</tr>
<tr>
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<td>5</td>
</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>AP4</td>
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<td>1</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>MT8/S</td>
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chorismate. Chorismate has no effect on the velocity in the absence of tryptophan (uppermost line).

These data yield additional important information. In Fig. 3B, the reciprocal of the velocity in the absence of the effector, tryptophan, minus the velocity in the presence of the effector \((1/V_0 - v)\) was plotted as a function of the reciprocal of the effector concentration. This plot is formally analogous to the standard double reciprocal plot of substrate saturation (24, 25). In this case, extent of inhibition rather than velocity is plotted versus ligand added. The y-intercept gives the maximum inhibition \((V_0 - v)_{\text{max}}\), and the x-intercept gives \(-1/K_i\) for tryptophan, in this case, is a \(K_i\). The \(K_i\) obtained from this plot is 0.5 \(\mu\)M, which is essentially identical to the \(K_i\) for tryptophan inhibition of anthranilate synthetase. In the absence of chorismate, inhibitor saturation is linear.

Chorismate does not alter the maximum inhibition obtained at high tryptophan concentrations (y-intercept), but does alter the \(K_i\) for tryptophan (x-intercept). The similarity to standard competitive kinetic plots is apparent; chorismate "competitively" reverses tryptophan inhibition of phosphoribosyltransferase. Though not usually expressed in this way, this statement is also true for the anthranilate synthetase reaction. The curvature, previously observed in the anthranilate synthetase reaction, is present here too (Hill number = 2.0). When plotted as \((1/V_0 - v)\) versus \((1/[\text{Trp}]_0)\), a pattern of straight lines is observed, and their common intersection on the y-axis is readily apparent (Fig. 3C). The Hill number of 2.0 is in excellent agreement with the number of tryptophan and chorismate binding sites on the aggregate (two of each) (11).

In Fig. 3D, the reciprocal of \(V_0 - v\) is plotted versus the concentration of chorismate at various levels of tryptophan, in a manner analogous to Fig. 2B. The intersection of the curves, when extended to the x-axis, gives a constant for the reversal by chorismate of tryptophan inhibition. This constant, which we designate \(k''_{i\text{Ch}}\) (for inhibition of inhibition), has a value of approximately 5 \(\mu\)M, which is identical to the \(K_m\) for chorismate in the anthranilate synthetase reaction.

While plots such as Fig. 3, B and D do not permit one to distinguish, for example, 5 \(\mu\)M from 3 \(\mu\)M, especially because of the curvature problem, the constants for the two activities are very similar. In addition, the over-all pseudocompetitive pattern of inhibition is identical with respect to both activities. Therefore, tryptophan and chorismate regulate both activities of the aggregate in a similar fashion.

Kinetics of Glutamine-Tryptophan Interactions in Wild Type Aggregate—Tryptophan has been shown to be strictly noncompetitive with glutamine in the anthranilate synthetase reaction, i.e. tryptophan affects only the \(V_{\text{max}}\) of anthranilate synthetase, and has no effect on the \(K_m\) for glutamine, which is approximately 1 \(\mu\)M (9). The simplest interpretation of this result is that the binding of glutamine requires the prior binding of chorismate, which tryptophan competitively prevents. This interpretation is, however, incorrect. Several lines of evidence show that the binding of glutamine may take place in the absence of chorismate. The first, to be discussed in detail in the next section, is that glutamine alone protects the enzyme against Zn*+ inactivation. The second is that glutamine reactivates tryptophan-inhibited phosphoribosyltransferase in the absence of any chorismate (Fig. 4A). For example, in the presence of 0.5 \(\mu\)M tryptophan and no glutamine, the phosphoribosyltransferase activity is 62% of the uninhibited level. Addition of 10 mM glutamine restores phosphoribosyltransferase activity to 93% of the uninhibited level. When these data are plotted in the double reciprocal form for an allosteric activator, a pattern of competitive interaction between glutamine and tryptophan emerges (Fig. 4B). Like the chorismate reactivation of phosphoribosyltransferase, the lines are curved, indicating cooperativity. A replot against \((1/[\text{Trp}]_0)\) produced straight lines intersecting on the y-axis (Fig. 4C), as was observed in the case of the chorismate reactivation, again suggesting two interacting tryptophan and glutamine sites. The \(K_i\) for tryptophan was 0.5 \(\mu\)M; the \(K_i\) for glutamine was 1 \(\mu\)M, which is identical to the \(K_m\) for glutamine in the anthranilate synthetase reaction. These data demonstrate clearly that glutamine binds to the aggregate in the absence of chorismate; furthermore, glutamine participates in the regulatory processes of the aggregate.

Inactivation of Wild Type Aggregate by Zn*+ and by DON—All activities of the aggregate can be completely inactivated within...
FIG. 3. Inhibition by tryptophan of the phosphoribosyltransferase activity of wild type anthranilate aggregate and reversal of inhibition by chorismate. Standard enzyme assays (see "Materials and Methods") were performed, except that chorismate and tryptophan were present at the indicated concentrations. A, primary data at several levels of tryptophan and chorismate. The tryptophan concentrations used were: (●) zero; (▲) 0.5 µM; (○) 2.0 µM; (▲) 5.0 µM. B, double reciprocal plot for several tryptophan and chorismate levels. The chorismate concentrations used were: (●) zero; (▲) 2.0 µM; (○) 5.0 µM. C, replot of B. The chorismate concentrations were: (●) zero; (▲) 5.0 µM; (○) 20 µM. D, Dixon plot for several levels of tryptophan and chorismate. The tryptophan concentrations used were: (●) 0.5 µM; (▲) 2.0 µM; (○) 5.0 µM; (△) 2.0 µM; (□) 20 µM. E, Dixon plot for several levels of tryptophan and glutamine. The tryptophan concentrations used were: (●) zero; (▲) 0.5 µM; (○) 1.0 µM; (△) 2.0 µM; (□) 5.0 µM. F, Dixon plot for several glutamine and chorismate levels. The chorismate concentrations used were: (●) zero; (▲) 0.25 µM; (○) 0.5 µM; (△) 1.0 µM; (□) 2.0 µM; (◇) 5.0 µM.

Fig. 4. Glutamine reversal of the inhibition by tryptophan of phosphoribosyltransferase of wild type anthranilate aggregate. Standard enzyme assays (see "Materials and Methods") were carried out, except that glutamine and tryptophan were present at the indicated concentrations. A, primary data at several levels of glutamine and tryptophan. The tryptophan concentrations used were: (●) zero; (▲) 0.5 µM; (○) 1.0 µM; (△) 2.0 µM; (□) 5.0 µM. B, double reciprocal plot for several glutamine and tryptophan levels. The glutamine concentrations used were: (●) zero; (▲) 0.5 mM; (○) 1 mM; (△) 2 mM; (□) 5 mM; (◇) 10 mM. C, replot of B. The glutamine concentrations were: (●) zero; (○) 1 mM; (▲) 2 mM; (◇) 5 mM. D, Dixon plot for several levels of glutamine and chorismate. The tryptophan concentrations used were: (●) 0.5 µM; (▲) 5 µM; (○) 20 µM.
5 min by 1 μM Cu++, Ag++, Zn++, Cd++, or Hg++ in 0.1 M Tris-
chloride buffer, pH 7.8, at 25°. Many other metals (Mn++, Fe++, Co++, Ni++, Sn++, Pb++, and Ca++) were tested and
found to have no effect. Inactivation is considerably slower in
phosphate buffer than in Tris, owing perhaps to the reduction in
effective metal ion concentration through complexing with phos-
phate. Therefore, in experiments where rates of inactivation
were to be compared, phosphate buffer was used.

Metal ions are reversible inactivators. The inactivation
caused by any of the above metal ions was completely reversed
by dialyzing the aggregate against Buffer A. The Cd++-inacti-
vated aggregate, in particular, regains full activity within 20
min upon being added to the anthranilate synthetase standard
assay mixture, presumably because Mg++ present in the assay
mixture displaces Cd++ from the enzyme surface. Unfortunately,
one cannot specify the nature of the metal-sensitive
protein just by knowing which metal ions are
effective (26).

Chlorismate, glutamine, and tryptophan modify the rate of
inactivation by metal ions. Fig. 5 gives the time course of the
inactivation of anthranilate synthetase activity by 5 μM Zn++
in phosphate buffer. The lower curve shows the inactivation in
the absence of any ligand. Chlorismate (100 μM) offers substan-
tial protection against inactivation. When the enzyme is
exposed to Zn++ in the presence of 100 μM chlorismate plus 1 mM
tryptophan, the protective effect of chlorismate is lost. Because
tryptophan reverses chlorismate protection, the modification of
the rate of inactivation must reflect specific binding of the ligand
to the enzyme rather than to a nonspecific chelation by trypto-
phane of the metal ion. L-Glutamine (25 mM) completely pro-
tected the enzyme against inactivation, whereas D-glutamine at
the same concentration had no effect. Tryptophan partially
reversed the protection offered by L-glutamine. Thus, the sub-
strates protect against Zn++, but tryptophan overcomes sub-
strate protection.

A similar situation appears when anthranilate synthetase is
inactivated by the glutamine analog DON (27). The enzyme is
rather insensitive to low concentrations of DON alone. After
5-min incubation with 20 μM DON, 80% of the glutamine-de-
dendent activity remains (Table II). If chlorismate is added,
the enzyme is almost completely inactivated. In the presence
of chlorismate plus tryptophan, the inactivation is similar to that
obtained with DON alone. Thus, tryptophan prevents choris-
minate-stimulated inactivation. However, chlorismate is not
absolutely required for inactivation. There is a certain amount
of inactivation with 20 μM DON alone. The extent of inactiva-
tion can be increased by raising the DON concentration to 200
μM.

**ANS Fluorescence—**To obtain direct physical evidence for
conformational changes, several techniques were explored.
Difference spectroscopy at a protein concentration of 0.45 mg
per ml (A280 = 0.5), revealed no changes in the absorption
spectrum (wave length range: 300 nm to 240 nm) of the aggre-
gate upon addition of chlorismate or tryptophan. Nor was it
possible to detect changes in the intrinsic fluorescence of the
protein upon adding ligands. (Excitation 280 nm, emission
scanned from 280 nm to 700 nm peak at 325 nm, protein con-
centration = 0.07 mg per ml.) These results suggest that
whatever conformational changes occur do not involve major
alterations in the environment of aromatic amino acids in the
protein.

The fluorescent probe ANS (28, 29) was a useful indicator of
conformational changes in the aggregate. The fluorescence
emission spectrum of ANS, noncovalently adsorbed onto the
aggregate, is given in Fig. 6 (middle curve). Several other
fluorescent probes (6- or 8-p-toluidino-2-naphthalenesulfonate,
and N phenyl-1-naphthylamine) were tested, but only ANS
produced a detectable fluorescence. Presumably, only ANS has
the proper structure to fit between subunits or into some other
appropriate type of hydrophobic cleft of this aggregate.

The upper curve shows the effect of Cd++ on the emission spec-
trum of ANS bound to the aggregate. There is a dramatic in-
crease in the intensity of emission, accompanied by a clear de-
crease of 10 nm in the wave length of maximum emission. This
suggests that when the aggregate is inactivated by divalent
ions such as Cd++, the conformation is altered in such a way
that the environment of the bound ANS becomes more nonpolar.

The effect of added regulatory ligands on ANS fluorescence is
less dramatic. Tryptophan, for example, produces a 30% de-

![Fig. 5. Inactivation of wild-type anthranilate aggregate by Zn++. Enzyme was incubated at 25° in the presence of 5 μM Zn++
plus ligands in 0.05 M potassium phosphate buffer, pH 7.4. At
time zero, a 5-μl sample was withdrawn from the incubation
mixture, and then the sample was added. Samples were then with-
drawn at various times thereafter and assayed in the standard
anthranilate synthetase reaction. (●) no additions; (□) 25 mM
D-glutamine; (○) 100 μM chlorismate; 1 mM L-tryptophan; (△)
100 μM chlorismate; ( Δ ) 25 mM L-glutamine, 1 mM L-tryptophan;
(▲) 25 mM L-glutamine. The effects of Zn++ and other metal ions
on phosphoribosyltransferase were qualitatively similar to those
shown for anthranilate synthetase.

### Table II

**Inactivation of wild-type aggregate by DON**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Remaining activity</th>
<th>Glutamine</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>20 μM DON</td>
<td>84</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>20 μM DON plus 1 mM chlorismate</td>
<td>7</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>20 μM DON plus 1 mM chlorismate plus 100 μM tryptophan</td>
<td>76</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>200 μM DON</td>
<td>56</td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>
crease in the emission intensity (lower curve). There may also be a few nanometers shift in the wave length of maximum emission; however, this shift was not studied in detail due to the broadness of the peaks. A similar situation exists with chorismate, which produces a 10% decrease in emission intensity (curve not given). The effect of the ligands on the height of the emission peak is summarized in the column headed "Relative Emission Intensity" in Table III. n-Tryptophan had no effect on the emission intensity.

There are two possible causes for a decrease in the intensity of the emission peak: either a conformational change altering the environment of the ANS or the affinity of the enzyme for ANS, or direct displacement of ANS from its binding sites by the ligands. To eliminate the latter possibility and prove conclusively the existence of conformational changes, we determined the parameters of ANS binding (Table III) by fluorescence measurements, as described under "Materials and Methods." Comparison of the parameters of ANS binding in the absence and in the presence of various ligands indicates that the addition of ligands does not alter the binding of ANS. The column headed "Kd" shows that the dissociation constant for the enzyme-ANS complex is not significantly altered by the presence of chorismate or tryptophan. The small increase in Kd in the presence of tryptophan (from 29 μM to 40 μM) would produce a difference of less than 10% in the amount of ANS bound, when the concentration of ANS is 100 μM. This small difference in binding could not account for a 30% difference in fluorescence intensity. The column headed "n" indicates that there are two binding sites for ANS, and that the number of binding sites does not change upon adding tryptophan. Because of the large amounts of pure enzyme required for such determinations, the number of binding sites in the presence of the substrates was not determined. However, it is clear from these data that the ligands do not displace ANS from its binding sites. Therefore, the decrease in ANS emission intensity must be due to conformational changes which increase the polarity of the ANS environment. (This type of analysis has been applied previously in other systems; see, for example, Reference 20.)

That ANS does not bind at chorismate binding sites has been confirmed by adding 100 μM chorismate last to an otherwise complete anthranilate synthetase standard assay mixture containing 100 μM ANS, and measuring the ANS fluorescence before and after the addition of chorismate. The addition of chorismate results in only a 10% reduction in ANS fluorescence; however, the velocity of the anthranilate synthetase reaction is 90% as great as that in an identical assay without ANS, proving that ANS does not bind at the chorismate sites. (The fluorescence of anthranilate was corrected for absorbance by ANS as described under "Materials and Methods.")

Kinetic Analysis of Mutant Aggregates—Both the anthranilate synthetase and the phosphoribosyltransferase activities of each mutant enzyme aggregate were examined kinetically. The results are summarized in Table IV. Taking the first row of figures for the wild type (W3110) as an example, the table is arranged as follows. The first column, headed Km, gives the apparent Michaelis constant for chorismate saturation of anthranilate synthetase. This number (5 PM) is derived from Fig. 2A. The next column gives the Kd (1 PM) for tryptophan inhibition of anthranilate synthetase (Fig. 2B). The third column gives the ratio of the maximum velocity of the anthranilate synthetase reaction divided by that of tryptophan synthetase in a crude extract of a haploid organism harboring the trpE (anthranilate synthetase) mutation. These two activities are coordinately regulated in the trp operon (30), so their ratio provides a rough measure of the catalytic efficiency of the anthranilate synthetase. The ratio is essentially the same for all of the mutants as for

![Fig. 6. ANS fluorescence spectra. ANS was added to 100 μg of enzyme aggregate in a total volume of 1 ml, and the emission spectrum was scanned. Aggregate alone had no fluorescence. At the instrument sensitivity used, ANS alone had a barely perceptible fluorescence in the region of 310 nm. Either Cd²⁺ or tryptophan was then added in a negligible volume to the enzyme-ANS mixture, and the spectrum scanned again. Final concentration of ANS in the cuvette was 100 μM, of Cd²⁺ 100 μM, and of tryptophan 10 μM.](http://www.jbc.org/)

**Table III**

Fluorescence of bound ANS in presence of ligands

<table>
<thead>
<tr>
<th>Added ligand</th>
<th>Relative emission intensity</th>
<th>Parameters of binding</th>
<th><strong>Relative</strong></th>
<th><strong>Kd</strong></th>
<th><strong>n</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme alone</td>
<td></td>
<td></td>
<td>1.0</td>
<td>1.00</td>
<td>25</td>
</tr>
<tr>
<td>E plus chorismate (0.1 mm)</td>
<td></td>
<td></td>
<td>0.9</td>
<td>0.68</td>
<td>22</td>
</tr>
<tr>
<td>E plus glutamine (25 μM)</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.53</td>
<td>22</td>
</tr>
<tr>
<td>E plus PRPP (0.1 mm)</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.69</td>
<td>21</td>
</tr>
<tr>
<td>E plus anthranilate (0.1 mm)</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.85</td>
<td>31</td>
</tr>
<tr>
<td>E plus Mg²⁺ (2 mM)</td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E plus L-tryptophan</td>
<td></td>
<td></td>
<td>0.7</td>
<td>0.51</td>
<td>40</td>
</tr>
<tr>
<td>E plus n-tryptophan</td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table IV**

Kinetic parameters of mutant enzymes

See text for a description of the table. Tryptophan synthetase (TSase) activity in the conversion of indole to tryptophan was measured by the method of Crawford and Yanofsky (18).
wild type, so the catalytic efficiency of the anthranilate synthetase is presumably unaffected. Thus, these mutations may rightly be called regulatory, as opposed to catalytic. The fourth column gives the Hill number for tryptophan, which is an indication of the curvature of the kinetic plots, and is taken to represent cooperativity between binding sites.

The right half of Table IV concerns phosphoribosyltransferase. The first column in this section is $K_{tr}$, the kinetic constant for the reversal of tryptophan inhibition by chorismate determined according to Fig. 3D; the next column gives the $K_i$ for tryptophan inhibition (Fig. 3B). As pointed out in the analysis of the kinetics of the wild type aggregate, similarity of the kinetic parameters for the two activities: $K_m = K_{tr}$ and $K_i = K_i$, is consistent with the operation of a common regulatory mechanism. The next column gives the ratio of phosphoribosyltransferase to anthranilate synthetase. This ratio is essentially the same for all strains, whether mutant or wild type. Taken together with the information on the ratio of anthranilate synthetase to tryptophan synthetase, these data indicate that phosphoribosyltransferase in the mutant strains is also catalytically normal. The last column, headed $K_m$, gives the constant for the activation of phosphoribosyltransferase by chorismate in the absence of tryptophan. There is no such activation in the wild type aggregate (Fig. 3A). However, in some of the mutants, chorismate activates phosphoribosyltransferase in the absence of added tryptophan.

Before discussing the mutants, it should be pointed out that their kinetic parameters were obtained from plots in exactly the same way as has been described for wild type. To limit the number of figures, not all of these plots are presented. (For additional plots see Reference 31.) Except where indicated, the mutants give typical curved, competitive plots as previously found for wild type.

All the kinetic data for anthranilate synthetase were obtained with glutamine as amino group donor. No significant differences were found when ammonia was used in place of glutamine, e.g. the aggregate from MTR is highly resistant to tryptophan whether ammonia or glutamine is used.

**Mutant MTR**—Strain MTR has a single site mutation in the trpE gene; its phenotype is resistance to high levels of 5-methyltryptophan. The $K_m$ for chorismate in this strain is identical to that of wild type (Table IV). The double reciprocal plot for the anthranilate synthetase activity in this strain displayed the upwardly curved lines (see Hill number in Table IV), intersecting at a common $V_{max}$, demonstrating that the pseudocompetitive pattern of inhibition obtained with wild type is retained by this mutant. The $K_i$ for tryptophan in MTR was found to be 150 $\mu$M, as compared with 1 $\mu$M for wild type (Table IV).

The phosphoribosyltransferase of mutant MTR is also feedback resistant, with a $K_i$ for tryptophan inhibition of 45 $\mu$M. The two tryptophan $K_i$ values in this case are not quite equal, but they are both much higher than the $K_i$ values for wild type, indicating that a single trpE mutation affects both activities in parallel—further evidence that a common regulatory system affects both activities. The $K_i$ (chorismate reversal of tryptophan inhibition), like the $K_m$, remains unchanged from wild type.

MTR also differs from wild type in its sensitivity to inactivation by DON (Table V). For example, 200 $\mu$M DON inactivates about 50% of the wild type anthranilate synthetase activity in 15 min, while 95% of the activity of MTR is lost. MTR is about as sensitive to inactivation by Zn$^{2+}$ as wild type; like wild type, MTR is protected by chorismate. However, in MTR, tryptophan (1 mM) does not counteract the protection afforded by chorismate. Thus, in MTR, residues are exposed under conditions in which they would be hidden in wild type, and vice versa. This suggests that for a given set of ligand concentrations, MTR tends to reside in a conformation which is different from that of wild type. Because MTR and wild type are so similar kinetically, it is probable that the conformational states available to the mutant are not grossly restricted compared to wild type.

**Mutant MTS**—Both the $K_m$ for chorismate and the $K_i$ for tryptophan of anthranilate synthetase are altered in the enzyme aggregate from mutant MTS. The $K_m$ has increased from 5 $\mu$M to 100 $\mu$M and the $K_i$ has decreased from 1 $\mu$M to 25 nM (Table IV). The phosphoribosyltransferase of mutant MTS was also feedback resistant, with a $K_i$ for tryptophan inhibition of 45 $\mu$M. The two tryptophan $K_i$ values in this case are not quite equal, but they are both much higher than the $K_i$ values for wild type, indicating that a single trpE mutation affects both activities in parallel—further evidence that a common regulatory system affects both activities. The $K_i$ (chorismate reversal of tryptophan inhibition), like the $K_m$, remains unchanged from wild type.

Drastic as the alterations in the regulatory properties of this mutant protein are, it still retains many of the characteristics of wild type. This protein and all the others listed in Table IV are fully competent catalytically, as indicated in the $V_{max}$ column. MTS also retains the wild type pseudocompetitive pattern of inhibition. The Hill coefficient for chorismate was 1.3. Thus, the basic mechanism regulating anthranilate synthetase appears to be intact.

**Table V**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additions</th>
<th>$%$ Remaining activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn$^{2+}$ (5 $\mu$M)</td>
<td>DON (50 $\mu$M)</td>
</tr>
<tr>
<td>W3110</td>
<td>None</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M chorismate</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>10 $\mu$M chorismate plus 100 $\mu$M Trp</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M chorismate plus 100 $\mu$M Trp</td>
<td>29</td>
</tr>
<tr>
<td>MTR$^R$</td>
<td>None</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M chorismate</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M chorismate plus 1 $\mu$M Trp</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>10 $\mu$M chorismate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10 $\mu$M chorismate plus 100 $\mu$M Trp</td>
<td>100</td>
</tr>
<tr>
<td>MTS$^R$</td>
<td>None</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>1 $\mu$M chorismate</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>1 $\mu$M chorismate plus 100 $\mu$M Trp</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1 $\mu$M chorismate plus 100 $\mu$M Trp</td>
<td>55</td>
</tr>
</tbody>
</table>
Phosphoribosyltransferase from MT$^8$ was also supersensitive to tryptophan. The $K_i$ for tryptophan inhibition was 20 nm, identical to the $K_i$ for anthranilate synthetase, and 40-fold lower than wild type. The $K_m$ of 50 $\mu$M was also much higher than wild type, and comparable to the $K_m$. The phosphoribosyltransferase of MT$^8$ differs strikingly from wild type in one respect: chorismate activates the phosphoribosyltransferase of MT$^8$ in the absence of tryptophan. This activation, of about 2-fold, is shown in Fig. 7A, uppermost curve. Replotting this activation as the reciprocal of $v - V_0$ versus the reciprocal of chorismate concentration gives a $K$ for activation of 200 $\mu$M and a value for $V_{max}$ which was used instead of $V_0$ in determining the $K_i$ and the $K_m$. The $K_a$ and the $K_i$ are probably two ways of looking at the same basic process, and, indeed, both $K$ values agree closely with the $K_m$. (Differences of 2-fold in a table of constants spanning five orders of magnitude are probably not significant.) Thus, apart from the additional feature that chorismate activates in the absence of tryptophan, MT$^8$ retains the wild type pattern of regulation. A single mutation in $trpE$ has altered the effectiveness of the regulatory ligands in parallel with respect to both activities.

Phosphoribosyltransferase of MT$^8$, unlike that of wild type, can also be activated by glutamine in the absence of tryptophan. The $K$ for this effect, obtained from a reciprocal plot ($1/v - V_0$ versus $1/[Gin]$), was 5 mM. This is about 5-fold higher than the wild type $K_m$ for glutamine in the anthranilate synthetase reaction, suggesting that MT$^8$ has a decreased affinity for glutamine, as well as for chorismate, relative to wild type.

A further difference between MT$^8$ and wild type proteins is that MT$^8$ is completely unaffected by DON, even in the presence of a high concentration of chorismate (1 mm) (Table V). $Zn^{2+}$ inactivation of MT$^8$ seems to be normal. Chorismate (1 mM) even protects against $Zn^{2+}$ inactivation to some degree.

Double Mutants MT$^{R/S}$—A double mutant containing both the MT$^R$ and MT$^S$ alleles was obtained in experiments designed to prove that the two mutations had occurred in separate codons. By constructing this strain, we were able to investigate the possibility that combining these two mutations would lead to the formation of an aggregate with properties resembling wild type. The recombinant was remarkably similar to wild type in many respects. The $K_i$ for tryptophan for both activities returned to the extremes of 130 $\mu$M and 25 $\mu$M to 1 $\mu$M, as found in wild type. The $K_m$ for chorismate (35 $\mu$M), although higher than wild type, was appreciably lower than the value of 100 $\mu$M found for MT$^S$. The pseudocompetitive pattern of inhibition was unchanged.

The MT$^{R/S}$ enzyme no longer exhibits the properties characteristic of that from strain MT$^S$. There is no activation of phosphoribosyltransferase by chorismate in the absence of tryptophan. The MT$^{R/S}$ enzyme is subject to inactivation by DON, although it is less sensitive than wild type (Table V). The MT$^{R/S}$ enzyme appears to be somewhat more sensitive to inactivation by $Zn^{2+}$ than wild type, but the effects of chorismate and tryptophan are normal.

MT$^S$ Revertant EMS4—To investigate MT$^S$ further, a number of phenotypic revertants were selected and classified (Table I). Revertants AP2 had kinetic parameters characteristic of wild type ($K_m = 5 \mu$M; $K_i = 1 \mu$M). A variety of other mutational changes can also counteract MT$^S$. Some of those appeared to be similar to MT$^{R/S}$; that is, the regular pseudocompetitive pattern of inhibition was maintained, but the $K_m$ values and $K_i$ values were nearer to wild type than to MT$^S$. A preliminary kinetic analysis of the class represented by EMS9 showed that it had a $K_m = 30 \mu$M and a $K_i = 10 \mu$M, much like MT$^{R/S}$. The class represented by EMS2 had a $K_m = 110 \mu$M and a $K_i = 250 \mu$M. The high $K_i$ is approximately equal to the $K_i$ for MT$^S$, but apparently the high $K_m$ dominates. This strain is still somewhat sensitive phenotypically, as indicated by a zone of inhibition larger than that of wild type. In addition to these revertants, two highly interesting classes of revertants were obtained. One of these is represented by revertant EMS4.

EMS4, like MT$^S$, has a relatively high $K_m$ but it is completely resistant to feedback inhibition by tryptophan (i.e. the $K_i$ was much higher than 1 mM). There is, of course, no Hill number for tryptophan with this strain. Phosphoribosyltransferase is also unaffected by tryptophan or chorismate.

MT$^S$ Revertant AP4—The revertant AP4 was the only member of its class to be isolated. The apparent $K_m$ and the $K_i$ for anthranilate synthetase of this revertant were unchanged from MT$^S$. However, in AP4 the typical pseudocompetitive pattern of inhibition was abolished. The Hill number (1.0) indicates that the cooperativity, characteristic of all other mutants examined, has vanished. The double reciprocal plot for the anthranilate synthetase of AP4 is shown in Fig. 7B. Not only are all the lines straight, but they also no longer intersect on the y-
was used except that the concentration of chorismate was 10 μM, and tryptophan was added at the concentrations indicated.

The phenotypes of the MT<sup>8</sup>, MT<sup>9</sup>, and MT<sup>R,8</sup> strains are readily explained by the observed changes in the binding constants of the regulatory ligands. In AP4 the constants are not significantly different from those of MT<sup>8</sup>; rather it is the pattern of inhibition which has changed. Can this account for the phenotype of AP4 as a revertant from MT<sup>8</sup>? At high levels of chorismate (100 μM, ~K<sub>m</sub> for MT<sup>8</sup>) and low levels of tryptophan (0.1 μM, ~K<sub>i</sub> for MT<sup>8</sup>) the extent of inhibition of the anthranilate synthetase activity of MT<sup>8</sup> is 68% and of AP4 is 60% (data taken from kinetic plots). This difference is insignificant and reflects the fact that the K<sub>m</sub> and K<sub>i</sub> are essentially the same for the two strains. On the other hand, Fig. 8 compares the inhibition of MT<sup>8</sup> and AP4 at “normal” levels of chorismate and tryptophan. Normal means chorismate at a concentration of 10 μM (2 × wild type K<sub>m</sub> for MT<sup>8</sup>) and tryptophan up to 1 μM (wild type K<sub>i</sub>). Presumably, these wild type constants reflect physiological levels of the regulators in all cells, wild type or mutant. At 10 μM chorismate and very low levels of tryptophan, AP4 is inhibited more rapidly than MT<sup>8</sup> (e.g. 81% versus 64% at 0.025 μM tryptophan). In both strains, 50% inhibition occurs at a tryptophan concentration of about 0.1 μM. At higher levels of tryptophan, however, the activity of MT<sup>8</sup> drops off considerably faster than the activity of AP4. At 10 μM tryptophan, AP4 is 21% active while MT<sup>8</sup> is only 3% active. This low activity of the anthranilate synthetase of MT<sup>8</sup> is presumably what limits its growth in the presence of 5-methyltryptophan. If AP4 has more activity than MT<sup>8</sup>, it might be expected to grow faster than MT<sup>8</sup> and have a smaller zone of inhibition. However, the difference between MT<sup>8</sup> and AP4 would be expected to be relatively small, and in fact, AP4 was the weakest revertant phenotypically, the zone decreasing only to 6.0 cm from 6.7 cm for MT<sup>8</sup> (Table I). Thus, AP4 is a unique second site mutation showing a revertant phenotype as the result of an alteration in the pattern of regulation from cooperative, competitive to linear, noncompetitive.

The noncompetitive inhibition in this mutant aggregate supports the idea that the competitive inhibition observed with wild type is not due to direct competition by chorismate and tryptophan for the same site or for overlapping sites. Noncompetitive inhibition requires that chorismate and tryptophan bind at separate sites.

A second implication of noncompetitive inhibition in AP4 is that this enzyme must occur in both active (E-S) and inactive (E-I) or less active (E-I-S) forms. While these forms need not correspond to conformational states allowable in wild type, it is tempting to speculate that AP4 is a distortion of the normal wild type regulatory system, which presumably involves conformational states corresponding to active and inactive forms of the enzyme, associated with the presence of appropriate regulatory ligands.

**DISCUSSION**

Three lines of evidence suggest that conformational changes, associated with the binding of the substrates and of the feedback inhibitor, take place in the anthranilate aggregate. Kinetic analysis indicates that the regulation of the aggregate involves communication between chorismate, glutamine, and tryptophan sites. Moreover, this communication is common to both activities and thus involves all four subunits of the aggregate. Barring association-dissociation, the regulation of the aggregate is most readily explained by conformational changes simultaneously influencing all four subunits, unless some complicated system of overlapping, interlocking sites were imagined.

Indirect evidence for conformational changes has been obtained by studying the relative rates of inactivation of the aggregate in the presence or absence of tryptophan, glutamine, and chorismate. There are two ways of interpreting these inactivation experiments. The first is to postulate that the chorismate, tryptophan, Zn<sup>2+</sup>, and DON sites all overlap in such way that the binding of chorismate exposes residues sensitive to DON and hides residues sensitive to Zn<sup>2+</sup>, and that the binding of tryptophan displaces chorismate directly, with the opposite effect on the sensitive residues. Alternatively, and more likely, the binding of chorismate and tryptophan could be associated with conformational changes in the aggregate, to expose or bury Zn<sup>2+</sup> or DON reactive residues.

Finally, upon addition of regulatory ligands, the fluorescence yield of the ANS-enzyme complex decreased, under conditions in which the binding of ANS was unaffected. These experiments provided direct physical proof that the environment of the ANS molecules was altered by conformational changes in the protein, associated with ligand binding.

The kinetic and inactivation experiments not only indicated the existence of conformational changes, but also provided other information relating to the regulation of the aggregate. For instance, since chorismate is a competitive reactivator of phosphoribosyltransferase, it is likely that chorismate is an activator as well as a substrate for anthranilate synthetase. There is only one binding site for chorismate on each anthranilate synthetase subunit; we postulate that both functions of chorismate are mediated via the same active site, in that the binding of...
Because glutamine protects against Zn$^{2+}$ inactivation and is an allosteric activator of the phosphoribosyltransferase activity, it must combine with the enzyme in the absence of chorismate, i.e. the binding of the substrates of the anthranilate synthetase reaction need not be ordered (8). However, it is probably accurate to say that glutamine hydrolysis requires the prior binding of chorismate. Glutaminase activity, measured by the production of γ-glutamylhydroxamate, requires a catalytic amount of chorismate (13, 14). Apparently, chorismate functions to maintain the enzyme in a conformation which is active in glutamine hydrolysis. The stimulation of DON inactivation by chorismate may be explained by suggesting that the (chorismate-enzyme) complex has a preferred conformation such that the residues responsible for glutamine hydrolysis are efficiently exposed and subject to attack by DON. Since glutamine hydrolysis, not merely glutamine binding, is an obligatory prerequisite for anthranilate synthetase activity, a kinetically ordered mechanism need not necessarily require ordered substrate binding. Chorismate and glutamine may bind in any order, but the liberation of NH$_3$ from glutamine probably requires a conformational change associated with the binding of chorismate. Thus, in the anthranilate synthetase reaction, it is really the hydrolysis of glutamine which is noncompetitive with tryptophan. The binding of glutamine, under the conditions of the anthranilate synthetase assay, might well be unaffected by tryptophan. In the presence of the phosphoribosyltransferase substrates, the binding of glutamine and tryptophan may become competitive; hence, glutamine can competitively reactivate tryptophan-inhibited phosphoribosyltransferase.

The information gained from studying the wild type enzyme is complemented by our analysis of regulatory mutants. We have found mutant aggregates with altered ligand binding, such that they are either supersensitive or resistant to tryptophan inhibition. In these mutants, the catalytic functions and the pattern of cooperative, competitive regulation remain intact. The altered ligand affinity is accompanied by changes in the conformation of the mutant enzymes such that their relative sensitivity to inactivation by Zn$^{2+}$ and DON is altered. The conformations of the MT$^\beta$ and MT$^\delta$ aggregates presumably resemble those of wild type in the presence of chorismate or tryptophan, respectively. In EMS4, the loss of tryptophan inhibition is accompanied by an alteration in conformation from its parent strain MT$^\delta$, as evidenced by its inability to be activated by chorismate. The diminution of cooperativity, and the alteration of the pattern of inhibition from competitive to noncompetitive in AP4 has two implications. The chorismate sites are separate from the tryptophan sites, and the enzyme exists in both active and inactive forms which may be related to the conformational states of wild type.

Based on these observations, we have drawn a simple analogy to illustrate some of the features of the regulation of the aggregate (Fig. 9). This model readily accommodates the results of kinetic analysis of tryptophan inhibition of anthranilate synthetase activity. Since chorismate and tryptophan cannot bind simultaneously, they are competitive. Depending on which is bound, the aggregate is either in an activated or in an inhibited form. Thus, with respect to all activities of the aggregate, tryptophan is competitive with chorismate.
regulatory machinery. (This effect, presumably conformational in nature, is indicated by the heavy double arrow in Fig. 9.) Our model postulates that this conformational change also engages the glutamine site into the tryptophan regulatory machinery. Thus, in the absence of PRPP or anthranilate or both, glutamine hydrolysis awaits upon the binding of chorismate to induce a specific conformational change. The observed kinetic pattern is that tryptophan is competitive with chorismate, and that chorismate and tryptophan competitively regulate glutamine hydrolysis, but that tryptophan binding and glutamine hydrolysis are noncompetitive, since only that proportion of the enzyme which is chorismate-activated can hydrolyze glutamine.

There is no means by which glutamine can change that proportion. However, once the phosphoribosyltransferase substrates are bound, a conformational change takes place which firmly engages the phosphoribosyltransferase subunit with the tryptophan regulatory system. Now chorismate and tryptophan competitively regulate the phosphoribosyltransferase activity, and the binding of glutamine and tryptophan become competitive. Glutamine is therefore able to competitively re-activate tryptophan-inhibited phosphoribosyltransferase.

Several observations lend credence to this theory. The requirement for the phosphoribosyltransferase substrates in order to observe maximum tryptophan inhibition implies that the substrates allosterically potentiate the effectiveness of the inhibition of phosphoribosyltransferase. Presumably the pertinent conformational change also affects the glutamine site, located on the same subunit as the phosphoribosyltransferase active site, and hence subject to the regulatory system. The observed 70% maximum inhibition by tryptophan may be explained by the requirement that the substrates be present in order for tryptophan to inhibit. If an excess of tryptophan begins to inhibit so effectively that the phosphoribosyltransferase substrates can no longer bind, then the phosphoribosyltransferase subunit becomes disengaged and is no longer subject to inhibition. The increase in tryptophan cooperativity from Hill number of about 1.4 in the anthranilate synthetase reaction to a Hill number of 2.0 in the phosphoribosyltransferase reaction may be a reflection of a tighter coupling between the subunits brought about by PRPP and anthranilate as they engage phosphoribosyltransferase into the regulatory system.

The decrease in the intensity of ANS fluorescence caused by both chorismate and tryptophan indicate that there are at least three conformational states available to the aggregate; a resting state in the absence of any ligand in which ANS fluorescence is at its maximum, an activated state in which ANS fluorescence is slightly decreased, but in which all activities may be fully active, and finally an inhibited state in which ANS fluorescence is considerably lessened and in which all the activities are inhibited.

The postulated multiple conformational states account nicely for the altered sensitivity of the aggregate to inactivation. If the activated conformation is the most sensitive to DON, we would expect chorismate to stimulate and tryptophan to prevent DON inactivation. The inhibited conformation and the unactivated conformation are more sensitive to DON inactivation. The inhibited conformation and the unactivated conformation are more sensitive to DON inactivation. The activated conformation are more sensitive to DON inactivation. Thus, chorismate partially protects against Zn2+-inactivation. The complete effectiveness of glutamine in protecting against Zn2+-inactivation suggests that glutamine binding might protect the Zn2+-sensitive residues sterically, rather than allosterically.

Assuming that the wild type aggregate in the absence of any ligand exists in a position intermediate between the chorismate-activated and the tryptophan-inhibited forms, the binding of either chorismate or tryptophan could stabilize a particular change in conformation. If we further assume that this rest position is somewhere midway between two extreme conformations, such that the wild type enzyme has an approximately equal affinity for both chorismate and tryptophan (K_res = 5 μM, K_i = 1 μM), the enzyme aggregate from mutant MTR may then be thought of as residing in a conformation resembling the chorismate-activated form of wild type. Therefore, it readily binds chorismate, but requires a high concentration of tryptophan to be forced into the inhibited conformation. Like the chorismate-activated form of the enzyme from wild type, the MTR aggregate exists in a conformation where residues sensitive to DON are exposed. These residues may be part of the site for glutamine hydrolysis, for example. The aggregate from mutant MT5 may reside in a conformation resembling the inhibited conformation of wild type. It readily binds tryptophan, but requires high chorismate to keep the active site open. MT5 is resistant to DON, because it resides in a conformation similar to the tryptophan-inhibited form of wild type in which the DON-sensitive residues are not exposed. If these residues were in the glutamine site, they would be buried. The two mutations combined in MT5,8 affect the equilibrium in opposite ways so that their contributions essentially cancel out, giving a wild type K_i. (The higher than wild type K_res of MT5,8 indicates that these mutations do have destructive effects, and are not purely effects on equilibrium.) Since, according to this theory, MTR and MT5 affect primarily the equilibrium between conformational states and do not destroy active sites or inhibitor binding sites or disrupt communication between subunits, catalysis and the basic pattern of cooperative, competitive regulation remain intact in these mutants.

In our model, the revertants EMS4 and AP4 counteract the effect of MT5, not by readjusting the equilibrium as in MTR and revertants like EMS9, but rather by altering the machinery of regulation. EMS4 modifies the machinery in MT5 such that the tryptophan inhibition sites become totally ineffective. AP4 causes a loss of cooperativity, and a change in the pattern of inhibition to noncompetitive. AP4 has warped the regulatory machinery. The gear teeth required for communication between identical subunits no longer mesh. Chorismate and tryptophan can now bind simultaneously. The binding of tryptophan not only discourages the binding of chorismate, but also interferes with catalysis (i.e. in AP4 there is a ternary E-chorismate-tryptophan complex with a lower turnover number than the E-chorismate complex). A high concentration of tryptophan cannot inhibit to the same extent in the presence of chorismate as in its absence, since some chorismate can bind even after the aggregate is saturated with tryptophan.

We have found that this mechanical model enables one to consolidate the observations reported in the “Results” section and in the earlier literature. Of course, we agree with Weber (32) who has stated the reasons for caution in ascribing to macromolecules in solution the mechanical properties of macroscopic objects. Our suspicion is that the binding of every ligand alters the properties of the protein in some way. Therefore, the model should be regarded as a description of one aspect of the functioning of the aggregate, but certainly the complete picture of ligand interactions and conformational changes in the in vivo catalytic and regulatory situation is far more complex than the simplified model presented here.

Correlations with Previous Work—Cordaro et al. (33) have shown that a single mutation in the trpA (anthranilate syn-
that the anthranilate synthetase subunit, regulating this earlier experiment, does not contradict the invent boxyltransferase subunit would seem to be excluded. Because a single mutation produces the same alteration in the feedback sensitivity for both activities, separate tryptophan sites on each subunit are unlikely. In strain MT$, phosphoribosyltransferase is, in fact, feedback resistant (Table II), so clearly the MT$ mutation affects both activities, not just anthranilate synthetase. How is it then possible to mix a feedback resistant anthranilate synthetase subunit and a wild type phosphoribosyltransferase subunit and get phosphoribosyltransferase activity showing wild type regulation? The explanation is that even MT$ is substantially inhibited by the high concentration of tryptophan used (50 μM) in the absence of chorismate. Thus, the erroneous conclusion was reached (6) that the regulation of phosphoribosyltransferase was like that of wild type. If 10 μM chorismate had been present in the phosphoribosyltransferase assay mixtures, as it was in the anthranilate synthetase assays, phosphoribosyltransferase would have shown the same resistance to feedback inhibition as did anthranilate synthetase. Thus, this earlier experiment does not contradict the present idea that the anthranilate synthetase subunit regulates phosphoribosyltransferase.

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Feedback Regulation in the Anthranilate Aggregate from Wild Type and Mutant Strains of *Escherichia coli*

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