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Identification of Amino Acid Residues Involved in Feedback Regulation of the Anthranilate Synthase Complex from *Salmonella typhimurium*

EVIDENCE FOR AN AMINO-TERMINAL REGULATORY SITE*

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The anthranilate synthase-phosphoribosyl transferase complex, a heterotetrameric enzyme made up of the TrpE and TrpD polypeptides, catalyzes three reactions comprising the first two steps of tryptophan biosynthesis in *Salmonella typhimurium*. All three activities of the complex are subject to feedback inhibition by tryptophan, which results from allosteric effects associated with the binding of one molecule of inhibitor to each of the TrpE subunits of the complex. Random *in vitro* chemical mutagenesis of the *trpE* gene was used to generate a collection of mutant forms of the complex which displayed varying degrees of resistance to feedback inhibition. Single amino acid substitutions, identified by DNA sequencing, were found at 14 different residues within the TrpE polypeptide. The residues were distributed throughout TrpE, but those that appeared to be most critical for regulation were found in two clusters, one at the extreme amino-terminal end, including residues Glu-39, Ser-40, and Ala-41, and the other in the middle of the polypeptide, including residues Asn-288, Pro-289, Met-293, Phe-294, and Gly-305. Kinetic and binding studies of the purified mutant complexes demonstrated that 9 of the 14 had a marked decrease in affinity for tryptophan with little or no change in substrate affinity or catalytic capacity. The remaining five enzymes exhibited more subtle changes, having small decreases in inhibitor affinity coupled with small increases in substrate affinity. Mutant enzymes that were not totally feedback-resistant had a decreased kinetic response to tryptophan binding. All enzymes exhibited alterations in tryptophan-induced conformational changes as monitored by dye-ligand chromatography.

Carbon flow through the tryptophan biosynthetic pathway in bacteria and fungi is controlled by negative feedback regulation of the first enzyme of the pathway, anthranilate synthase (EC 4.1.3.27). This enzyme catalyzes the formation of anthranilate in a bisubstrate reaction utilizing glutamine and chorismate, the branch point precursor for all aromatic compounds in the cell (Reaction 1).

$$\text{Chorismate} + \text{glutamine} \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{glutamate} + \text{pyruvate} + H_2O \quad (1)$$

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

$$\text{Anthranilate} + \text{PRPP} \xrightarrow{Mg^{2+}} \textit{N}\text{-phosphoribosyl anthranilate} + \text{pyrophosphate} \quad (3)$$

The inhibition of anthranilate synthase by the end product of the pathway, L-tryptophan, in combination with analogous feedback regulation of the initial enzymes of the phenylalanine and tyrosine pathways, ensures the appropriate partitioning of chorismate into the three amino acid pathways in response to the needs of the cell (1).

The anthranilate synthase from *Salmonella typhimurium* is part of a multifunctional, tetrameric complex made up of two molecules each of the TrpE and TrpD polypeptides, the products of the first two genes of the *trp* operon (2, 3). In addition to synthesizing anthranilate, the complex also catalyzes the second step of the pathway, the transfer of the phosphoribosyl group of 5-phosphorylribose-1-pyrophosphate (PRPP) to anthranilate (phosphoribosyl transferase activity, EC 2.4.2.18) (Reaction 3). The TrpD subunit is a bifunctional molecule with independent structural domains (4). Its amino-terminal domain functions as a glutamine amidotransferase, releasing the amide group of glutamine for the amination of chorismate by the TrpE subunit, while the carboxyl-terminal domain carries out the phosphoribosyl transferase activity of the complex. Glutamine hydrolysis by the TrpD subunit is greatly enhanced by the binding of chorismate to the TrpE subunit (5). Thus, glutamine amidotransferase activity and glutamine-dependent anthranilate synthase activity are exclusive properties of the complex. In contrast, both the complex and the free TrpE subunit are capable of ammonia-dependent anthranilate synthase activity (Reaction 2) (6). Similarly, both the complex and the free TrpD subunit possess phosphoribosyl transferase activity (7, 8).

The glutamine-dependent and ammonia-dependent anthranilate synthase, glutamine amidotransferase, and phosphoribosyl transferase activities of the complex, as well as the ammonia-dependent anthranilate synthase activity of the TrpE subunit, are all subject to feedback inhibition, resulting from the binding of one molecule of tryptophan to the TrpE subunit (9). In each case, inhibition is competitive with respect to chorismate and noncompetitive with respect to the other substrates. It appears likely that tryptophan and chorismate bind to distinct sites on the TrpE subunit of the complex rather than competing for the same site, since the

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This paper is dedicated to the memory of Irving Crawford who made so many outstanding contributions to our knowledge of the genes and enzymes of tryptophan biosynthesis.

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enzyme can be desensitized with respect to tryptophan inhibition both *in vivo* by mutations in the TrpE subunit (5, 10) and *in vitro* by high concentrations of Mg^{2+} (11). Thus conformational changes within the enzyme associated with the binding of tryptophan to the TrpE subunit appear to be responsible for inhibition of the various activities of the complex.

In this report, we present the results of a mutational analysis of the feedback site of the TrpE subunit of the complex from *S. typhimurium*. This has led to the identification of amino acid residues critical for tryptophan binding and has provided evidence for the existence of an amino-terminal regulatory domain in the TrpE subunit.

MATERIALS AND METHODS[1]

RESULTS

Isolation of Feedback-resistant trpE Mutants—Mutations leading to the loss of feedback inhibition in the anthranilate synthase of *S. typhimurium* were sought by isolating strains resistant to 5-methyltryptophan (MTR),[2] an effective false feedback inhibitor, after random *in vitro* mutagenesis of the cloned *trpE* gene. The vector used for the mutagenesis was plasmid pSTG3, which carries the *S. typhimurium trpE* gene under the control of its own promoter (Fig. 1). The host used for the selection of mutant plasmids was *Escherichia coli* strain CB90, which is deleted for *trpE* but has *trpD* and the remaining genes of the operon intact. The use of a single copy *trpD* host was necessitated by the fact that the growth on minimal medium of strains with a multicopy plasmid carrying both *trpE* and *trpD* is essentially unaffected by 5-methyltryptophan, even at concentrations as high as 200 μg/ml. In contrast, the growth of CB90/pSTG3 is completely suppressed on minimal medium by moderate concentrations of 5-methyltryptophan (50 μg/ml), allowing the selection of MTR derivatives. *E. coli* CB90 was chosen as host, rather than a *S. typhimurium* strain of similar genotype, because of the superior transformability of *E. coli* strains. It has been shown that the *S. typhimurium* TrpE and the *E. coli* TrpD subunits assemble *in vivo*, forming a heterologous complex with close to normal catalytic and feedback properties (12).

By using the CB90/pSTG3 host-vector system, 45 prototrophic Amp^r MTR strains were isolated after *in vitro* treatment of *trpE* DNA with either nitrous acid or hydroxylamine. The sensitivity of the heterologous complexes of the MTR isolates to feedback inhibition by tryptophan was assessed in the standard anthranilate synthase assay using crude extracts of cells grown in minimal medium + ampicillin. Each of the mutant enzymes exhibited a loss of feedback control relative to the wild type enzyme, the magnitude of which varied widely (data not shown). Thus, these preliminary results indicated that the stringency of the selective screen used was appropriate for the recovery of mutants with diverse alterations in feedback regulation.

The nature and location of the mutational change in the *trpE* gene of each of the 45 MTR plasmids was then determined by DNA sequence analysis. This led to the identification of 14 different residues within the TrpE polypeptide at which amino acid substitutions resulted in the feedback-resistant MTR phenotype (Table I). The identified residues were distributed throughout the gene, although two clusters were readily apparent. One was located at the extreme amino terminus of the polypeptide, specified by mutations at residues Glu-39, Ser-40, and Ala-41; the other was found in the middle of the polypeptide, specified by mutations at residues Asn-288, Pro-289, Met-293, Phe-294, and Gly-305. Most of the mutations were recovered multiple times. After discounting multiple isolations of the same mutation within a single mutagenesis experiment, it was concluded that the collection consisted of a minimum of 22 independent clones.

Kinetic Analysis of Wild Type and Mutant Anthranilate Synthases—Plasmids were constructed carrying the 14 mutant $trpE^{MTR}$ genes together with the translationally coupled, wild type *S. typhimurium* $trpD^+$ gene. These were transferred into *E. coli* strain CB25, which is deleted for the entire *trp* operon, thereby creating strains synthesizing homologous *S. typhimurium* anthranilate synthase complexes. The wild type and the 14 mutant enzymes were overexpressed in these constructs, purified to homogeneity, and characterized by steady state kinetic analysis.

The kinetic properties of the wild type enzyme (Table II) were essentially as reported in previous studies (5, 11). The apparent K_m for chorismate (K_m^{Chr}) was 2.3 μM, the K_i for tryptophan (K_i^{Trp}) was 1.3 μM, and the turnover number (k_{cat}) was 12 s^{-1}. Feedback inhibition by tryptophan was competitive with respect to chorismate, and positive cooperativity of chorismate binding was apparent at higher concentrations of tryptophan (Fig. 2*A*).

The mutant enzymes displayed a variety of changes in kinetic properties (Table II). Nine of the 14 had striking increases in K_i^{Trp}, with little or no change in K_m^{Chr}. Four of these, E39K, S40F, M293T, and C465Y, were completely insensitive to tryptophan inhibition under the conditions tested, indicating >300-fold increase in K_i^{Trp}. The other five, A41V, N288D, P289L, F294L, and G305S, exhibited moderate increases in K_i^{Trp}, ranging from 7 to 20 times that of wild type. It is noteworthy that all of the enzymes with TrpE mutations located in the two clusters mentioned above have robust phenotypes, *i.e.* either total feedback resistance or moderate increases in K_i^{Trp}. The remaining mutant enzymes, R128H, C174Y, R402W, G460D, and H515Y, displayed only subtle changes in apparent ligand affinities, characterized in most cases by a marginal increase in K_i^{Trp} and a marginal

TABLE I

Nature and location of 5-methyl tryptophan-resistant mutations in trpE

Mutant plasmid	Codon change	Amino acid change	Independent isolates
pSTG43	GAA → AAA	Glu-39 → Lys	1
pSTG39	TCC → TTC	Ser-40 → Phe	1
pSTG42	GCG → GTG	Ala-41 → Val	1
pSTG41	CGT → CAT	Arg-128 → His	1
pSTG35	TGC → TAC	Cys-174 → Tyr	2
pSTG4	AAC → GAC	Asn-288 → Asp	3
pSTG7	CCC → CTC	Pro-289 → Leu	4
pSTG20	ATG → ACG	Met-293 → Thr	2[a]
pSTG8	TTC → CTC	Phe-294 → Leu	1
pSTG11	GGC → AGC	Gly-305 → Ser	1
pSTG10	CGG → TGG	Arg-402 → Trp	1
pSTG29	GGC → GAC	Gly-460 → Asp	1
pSTG27	TGT → TAT	Cys-465 → Tyr	2
pSTG28	CAT → TAT	His-515 → Tyr	1

[a] Both isolates contained a second amino acid substitution, Val-248 → Ala. The two mutations were separated *in vitro* using standard molecular cloning methods; the MTR phenotype was found to be associated with the Met-293 → Thr change, while the Val-248 → Ala change was found to be silent.

[1] Portions of this paper (including "Materials and Methods" and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: MTR, resistance to 5-methyl-DL-tryptophan; Amp^r, resistance to ampicillin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

TABLE II

Kinetic constants of wild type and mutant anthranilate synthase complexes

Kinetic analysis of the glutamine-dependent anthranilate synthase activity of the complexes was carried out as described under "Materials and Methods." The values for K_m^{Chr}, K_i^{Trp} and k_{cat} are mean values from two or more independent determinations. Deviations from the mean in the individual experiments were less than or equal to ±25% for K_m^{Chr} and K_i^{Trp} and less than or equal to ±30% for k_{cat}. Mutant enzymes are designated by the wild type residue followed by the position of the residue and the mutant residue, using the single amino acid code.

AS-PRT complex	K_m^{Chr}	K_i^{Trp}	k_{cat}
	μM	μM	s^{-1}
Wild type	2.3	1.3	12
E39K	3.9	>350	6.2
S40F	2.4	>450	12
A41V	3.7	21	13
R128H	1.6	3.3	11
C174Y	1.5	5.0	5.5
N288D	3.1	10	3.7
P289L	5.0	26	11
M293T	2.9	>450	11
F294L	1.6	18	11
G305S	2.2	26	8.8
R402W	1.9	2.6	12
G460D	1.8	1.8	14
C465Y	8.1	>450	10
H515Y	2.1	2.5	13

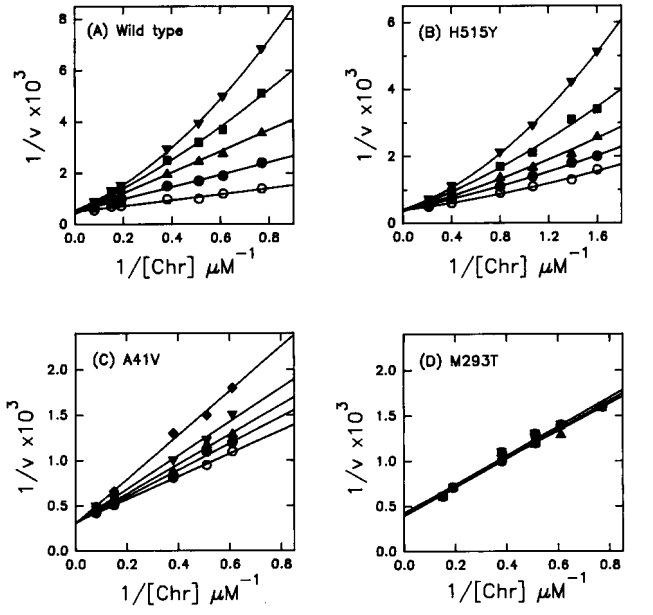

FIG. 2. **Lineweaver-Burk plots of steady state kinetic analysis of wild type and feedback-resistant mutant anthranilate synthase complexes.** Conditions were as described under "Materials and Methods." Mutant enzymes are designated as in Table II. Tryptophan concentrations were as follows: ○, none; ●, 2.5 μM; ▲, 5.0 μM; ■, 7.5 μM; ▼, 10 μM; ♦, 20 μM. The *curves* were drawn with either first order (*C* and *D*) or second order (*A* and *B*) regression using the Sigma Plot software package (Jandel Scientific).

decrease in K_m^{Chr}. The sensitivity of these mutant enzymes to feedback inhibition by 5-methyltryptophan relative to the wild type enzyme was found to parallel their sensitivity to tryptophan, indicating that differential sensitivity to 5-methyltryptophan did not account for their isolation (data not shown). Kinetic data for the wild type and for mutant enzymes representative of marginal, moderate, and complete loss of feedback control (H515Y, A41V, and M293T, respectively) are presented in Figs. 2 and 3.

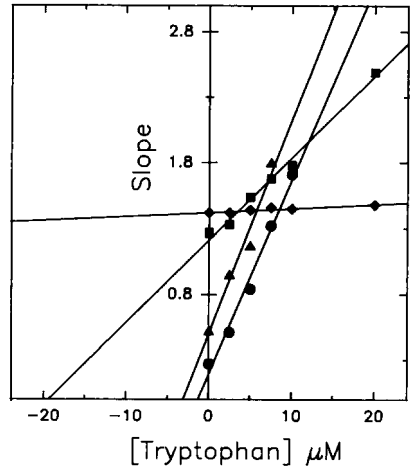

FIG. 3. **Secondary slope replots of steady state kinetic data.** The slopes of the lines from Fig. 2 were plotted *versus* the concentration of tryptophan. At concentrations of tryptophan where upward curvature was evident, slopes were approximated from a first order regression line drawn to the data obtained at chorismate concentrations ranging between 2 and 12.5 μM where the kinetics were essentially linear. Some data points in this range were omitted from the Lineweaver-Burk graphs for clarity.

The turnover number of most of the mutant enzymes varied only slightly (*i.e.* no greater than ±30%) from that of the wild type enzyme (Table II). Exceptions were E39K, C174Y, and N288D, which had reductions in k_{cat} ranging from 2- to 3-fold. Additional experiments (data not shown) indicated that the K_m for glutamine, the substrate ligand of the amidotransferase domain of the TrpD subunit of the complex, was not significantly altered in any of the mutant enzymes (data not shown).

Tryptophan Binding in Wild Type and Mutant Enzymes—In order to ascertain whether the increases in K_i^{Trp} of the mutant enzymes were correlated with decreases in their affinity for tryptophan, dissociation constants for tryptophan (K_d^{Trp}) were determined by direct binding analysis using ultrafiltration. A summary of the results for all the enzymes is presented in Table III. Langmuir double-reciprocal plots of the binding data obtained with the wild type enzyme and with two representative mutant enzymes that retain measurable binding activity (A41V and H515Y) are shown in Fig. 4. The K_d^{Trp} of the wild type enzyme was found to be 4 μM, in agreement with the value determined previously by equilibrium dialysis (9). There was no detectable tryptophan binding in the four mutant enzymes with complete loss of feedback inhibition (E39K, S40F, M293T, and C465Y). On the other hand, those enzymes with moderate increases in K_i^{Trp} (A41V, N288D, P289L, and G305S) showed moderate (*i.e.* 3–16-fold) increases in K_d^{Trp}, while those with subtle changes in K_i^{Trp} and K_m^{Chr} (namely R128H, C174Y, R402W, G460D, and H515Y) had K_d^{Trp} constants close to that of wild type. It is noteworthy that, in the partially feedback-resistant enzymes where K_i^{Trp} constants were measurable, the magnitude of the kinetic effect of tryptophan binding, as indicated by the K_d^{Trp}/K_i^{Trp} ratio, was not constant and in every case was less than that observed with the wild type enzyme (Table III). This difference was most pronounced in the enzymes with mutations in the middle region of the TrpE polypeptide.

The interaction coefficients (n_H) of the wild type and mutant complexes were determined from Hill plots of the binding data and are included in Table III. The n_H of the wild type enzyme was 1.2, indicating positive cooperativity between the tryptophan binding sites of its two TrpE subunits. Several of the mutant enzymes lost cooperativity for tryptophan binding,

TABLE III

Tryptophan dissociation constants and interaction coefficients of wild type and mutant anthranilate synthase complexes

Tryptophan dissociation constants (K_d^{Trp}) and interaction coefficients (n_H) were determined by ultrafiltration as described under "Materials and Methods." The values for K_d^{Trp} and n_H are the mean of two or more independent determinations. Deviations from the mean in the individual experiments were less than or equal to ±25% for K_d^{Trp} and less than or equal to ±10% for n_H. K_d^{Trp}/K_i^{Trp} ratios were calculated using the K_i^{Trp} values from Table II. Designations for the mutant enzymes are as in Table II.

AS-PRT complex	K_d^{Trp}	K_d^{Trp}/K_i^{Trp}	n_H
	μM		
Wild type	4.1	3.15	1.2
E39K	>200		
S40F	>200		
A41V	29	1.38	1.1
R128H	5.3	1.61	1.0
C174Y	5.4	1.08	1.0
N288D	11	1.10	1.3
P289L	26	1.00	1.6
M293T	>200		
F294L	13	0.72	1.1
G305S	67	2.58	1.3
R402W	6.8	2.58	1.1
G460D	4.1	2.28	1.0
C465Y	>200		
H515Y	4.6	1.84	1.1

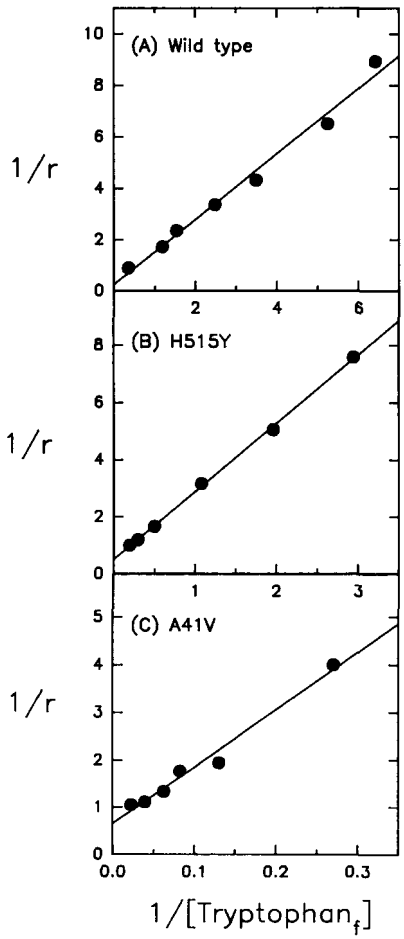

FIG. 4. **Tryptophan binding of wild type and mutant anthranilate synthase complexes.** Tryptophan binding was analyzed by ultrafiltration as described under "Materials and Methods." *r* represents moles of tryptophan bound per mol of enzyme and [*Tryptophan$_f$*] is the concentration of free tryptophan.

whereas others, in particular P289L (n_H = 1.6), had increased cooperativity.

Feedback Inhibition of Phosphoribosyl Transferase Activity—The phosphoribosyl transferase activity of the TrpD subunit of the complex is also subject to feedback inhibition by tryptophan. This regulation is the result of conformational effects associated with the binding of tryptophan to the TrpE subunit (13) and thus is a manifestation of communication between the regulatory site on TrpE and the catalytic site of the phosphoribosyl transferase domain of the TrpD subunit. In view of this, the feedback sensitivity of the phosphoribosyl transferase of the wild type and mutant enzymes was assessed by assaying activity under standard conditions in the presence of a range of concentrations of tryptophan.

As found previously (8, 11), inhibition of the phosphoribosyl transferase activity of the wild type enzyme was partial, reaching a maximum of about 70% at tryptophan concentrations of 5 μM and above; half-maximum inhibition ($Trp_{0.5}$) was at 1.4 μM tryptophan (Table IV). However, the sensitivity of the mutant enzymes varied widely, closely paralleling the affinity for tryptophan (*i.e.* the K_d^{Trp} values) of the TrpE subunit of each complex. Specifically, the transferase activity of the E39K, S40F, M293T, and C465Y enzymes was completely resistant to tryptophan inhibition, even at concentrations as high as 100 μM, while the activity of the other enzymes displayed slight to moderate resistance with $Trp_{0.5}$ values ranging from 1.6 to 21 μM (Table IV). The extent of inhibition in the latter group did not change significantly, in all cases attaining a maximum of 60–70% (data not shown).

Dye-Ligand Chromatographic Behavior of Mutant Anthranilate Synthase Complexes—Dye-ligand chromatography utilizing Matrex Gel Orange A has previously been shown to be an effective method for the purification of the wild type anthranilate synthase-phosphoribosyl transferase complex (3). While the nature of the binding of the enzyme to the dye-conjugated agarose gel is not fully understood, it is likely to involve both electrostatic and hydrophobic interactions between the enzyme and the procion yellow dye (14). It has been suggested that the tryptophan-dependent elution of the complex from the gel results from a conformational change associated with the binding of this ligand to the TrpE subunit (3). Thus, the tryptophan-induced conversion of the enzyme from

TABLE IV

Feedback inhibition of phosphoribosyl transferase activity of wild type and mutant anthranilate synthase complexes

Phosphoribosyl transferase activity was measured as described under "Materials and Methods" at tryptophan concentrations varying between 0 and 100 μM. $Trp_{0.5}$ is the concentration of tryptophan necessary to achieve half-maximal inhibition; NI indicates no inhibition.

AS-PRT Complex	$Trp_{0.5}$
	μM
Wild type	1.4
E39K	NI
S40F	NI
A41V	14
R128H	2.3
C174Y	5.2
N288D	5.4
P289L	12
M293T	NI
F294L	8.6
G305S	21
R402W	5.7
G460D	1.9
C465Y	NI
H515Y	1.6

a conformation with high affinity for the Orange A gel to one with low affinity may be a manifestation of the allosteric transition associated with ligand binding to TrpE. The behavior of the feedback-resistant mutant enzymes observed during their purification by dye-ligand chromatography supports this idea.

Like the wild type enzyme, each of the 14 mutant enzymes was found to bind tightly to the Orange A-agarose, with no more than 5–10% of the applied anthranilate synthase activity emerging in the wash fraction of the columns. However, the enzymes were differentiated into three groups on the basis of the elution profiles obtained upon development of the columns with an isocratic tryptophan gradient (Fig. 5). The enzymes of each group were related to one another by the magnitude of the alterations in their K_i^{Trp} and K_d^{Trp} constants.

The wild type enzyme eluted from the Orange A-agarose in typical fashion as a sharp peak between 25 and 50 μM tryptophan (Fig. 5). Very similar elution patterns were found with the mutant enzymes with subtle changes in K_i^{Trp} and K_d^{Trp} (*i.e.* N288D, R402W, G460D, and H515Y). The mutant enzymes with moderately elevated K_i^{Trp} and K_d^{Trp} constants (*i.e.* A41V, P289L, F294L, and G305S) were also eluted by tryptophan but not as effectively as the wild type enzyme. The broad elution pattern of the P289L enzyme shown in Fig. 5 is representative of this group. Finally, the mutant enzymes that were devoid of tryptophan inhibition and binding (*i.e.* E39K, S40F, M293T, and C465Y) were completely refractory to tryptophan elution; however, they were readily recovered in excellent yield by salt elution following the tryptophan gradient as exemplified in Fig. 5 by S40F.

To test further the conclusion that alterations in conformational changes induced by tryptophan binding were the basis of these chromatographic results, equivalent amounts of the purified wild type enzyme and feedback-resistant S40F mutant enzyme were brought to 0.5 mM tryptophan and then applied to Orange A gel columns that had been equilibrated with standard buffer containing 0.5 mM tryptophan. The columns were developed with the same buffer, and fractions were collected and assayed for anthranilate synthase activity. Unlike the tight binding of the enzyme observed in the absence of tryptophan, greater than 80% of the activity of the wild type enzyme emerged in the void volume of the column (data not shown), indicating that presaturation of the enzyme with tryptophan substantially decreased its affinity for the gel. In contrast, the same pretreatment of the enzyme and gel with tryptophan had no effect on the chromatographic behavior of the S40F enzyme. As in the absence of tryptophan, greater than 98% of the S40F activity was bound to the gel and was recovered upon subsequent salt elution.

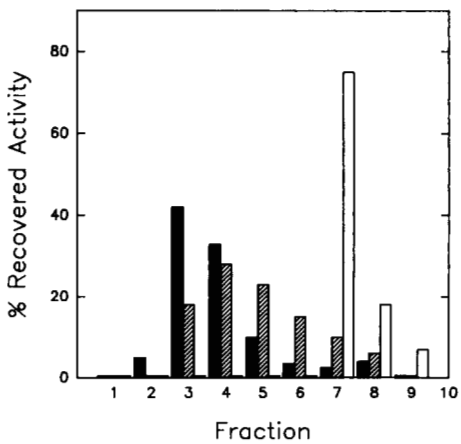

FIG. 5. **Fractionation of wild type and mutant anthranilate synthase complexes by dye-ligand chromatography on Matrex Gel Orange A.** Fraction numbers represent the following steps of an isocratic tryptophan gradient prepared in standard phosphate buffer: *1*, no tryptophan; *2*, 10 μM tryptophan; *3*, 25 μM tryptophan; *4*, 50 μM tryptophan; *5*, 100 μM tryptophan; *6*, 250 μM tryptophan; *7–9*, 1.5 M KCl. Fractions were assayed for glutamine-dependent anthranilate synthase activity. *Solid bars*, wild type complex; *diagonally filled bars*, P289L mutant complex; *open bars*, S40F mutant complex.

DISCUSSION

Kinetic characterization of the mutant anthranilate synthase complexes generated in this study has shown that feedback resistance can result either from moderate to large increases in K_i^{Trp} or from a combination of small increases in K_i^{Trp} and small decreases in K_m^{Chr} (Table II). Companion tryptophan binding studies with the mutant enzymes revealed increases in K_d^{Trp} that generally paralleled the increases in K_i^{Trp} (Table III). The majority of the enzymes were of the former type, with marked decreases in inhibitor affinity; a number of these were totally resistant to inhibition and had no detectable tryptophan binding. The mutational changes in this group are undoubtedly the best indicators of residues important for tryptophan binding and feedback regulation. The clustered arrangement of these mutations indicate that two noncontiguous regions of the TrpE polypeptide, *i.e.* residues 39–41 and 288–305, are components of the feedback site of the TrpE subunit. On the other hand, the residues identified by mutations having only marginal effects on the kinetic properties of the enzyme most likely contribute only indirectly to the structures involved in feedback regulation. These mutations may have been recovered by virtue of the amplification of their relatively weak effects by the multicopy state of the cloned *trpE* gene in the mutant strains, aided perhaps by reduced feedback sensitivity of the heterologous anthranilate synthase complexes present in the primary mutant isolates (12).

The properties of mutant enzymes such as E39K, S40F, and M293T, in which tryptophan binding was completely eliminated while the apparent affinity for chorismate remained essentially unaffected, indicate the existence of separate binding sites for substrate and inhibitor. This conclusion is supported by our finding that the feedback site mutations described here and the active site mutations previously characterized (3) are segregated to nonoverlapping regions of the TrpE polypeptide. All but one of the moderate to strong regulatory mutations are found in two clusters within the first 305 residues of the 520-residue polypeptide (Fig. 6). In contrast all of the six identified active site residues are located within the carboxyl-terminal third of the polypeptide, the most proximal one being residue Thr-329. It is significant that no inactivating missense mutations have yet been recovered in the amino-terminal two-thirds of TrpE, in spite of extensive random mutagenesis, and that nonconservative substitutions placed throughout this region by genetic suppression of nonsense mutations are without effect on catalytic activity.[3]

Taken together, these findings provide strong evidence that the feedback site and the active site of TrpE are not only distinct but also reside in separate structural domains. In such a structural model, the regulatory domain would be composed of the first 310–320 residues, while the catalytic domain would be made up of the remaining carboxyl-terminal segment of about 200 residues. It is also reasonable to speculate that the two segments of the regulatory domain encompassing residues 39–41 and 288–305 interact to form the tryptophan binding

[3] R. Bauerle, S. C. Hong, J. Sennewald, J. Hess, and T. Patterson, manuscript in preparation.

FIG. 6. **Amino acid sequence of *S. typhimurium* TrpE and the sequence relatedness of TrpE homologues.** The amino acid sequence of *S. typhimurium* TrpE was derived from the nucleotide sequence of the *trpE* gene, determined originally by Maxam-Gilbert sequencing (15) and later verified in this laboratory by the dideoxy chain-terminating method. The sequence presented includes corrections in the original sequence at residues 61, 70, 164, 187, 348, 359, 360, 368, 395, 397, and 481. The sequences of 12 TrpE homologues were compared with the *S. typhimurium* sequence using the alignment of Crawford (16). *Boldface capital letters,* using the single letter amino acid code, designate identity at that position in at least 11 of the 13 sequences; *lightface capital letters* designate identity or a conservative replacement in at least 9 of the 13 sequences; and *lower case letters* designate nonconserved residues in more than 5 of the 13 sequences. Conservative replacements are defined as follows: G = A, D = E, N = Q, S = T, H = K = R, I = L = M = V, F = Y = W. *Double arrows* (⇑) indicate the positions of feedback resistant mutations causing large increases in K_i^{Trp} and K_d^{Trp}; *single arrows* (↑) indicate the positions of feedback-resistant mutations having subtle effects on K_i^{Trp} and K_m^{Chr}; *shaded residues* indicate the positions of inactivating missense mutations.

site. This model is consistent with the results of the proteolytic probing of the native TrpE subunit, which revealed the existence of a large amino-terminal domain with molecular mass of about 30 kDa and two smaller carboxyl-terminal domains of 16 and 12 kDa (3). Furthermore, photoaffinity labeling of the native subunit with 6-azidotryptophan, an effective false feedback inhibitor of the enzyme, has localized the tryptophan binding site to the putative amino-terminal regulatory domain.[4]

It is interesting to consider these results in view of the unique amino acid sequence conservation that exists among TrpE homologues. The DNA sequences of the *trpE* genes of more than a dozen bacterial and yeast species have been determined, and the derived amino acid sequences of the polypeptides have been aligned (16). As summarized in Fig. 6, the TrpE polypeptides are highly conserved, as expected. However, the sequence conservation is found predominately in the carboxyl-terminal half of the molecule, where 55% of the residues are invariant or highly conserved in 13 homologues compared. In contrast, only 13% of the residues of the amino-terminal half are conserved, and these are mostly located in a few stretches of limited similarity. Significantly, the two segments of the polypeptide identified here to be important for feedback inhibition of the *S. typhimurium* enzyme, *i.e.* residues 39–41 and 288–305, are within highly conserved regions that are separated by a very large stretch with little sequence conservation. On the other hand, the active site residues in TrpE are found exclusively at invariant or highly conserved residues within the highly conserved carboxyl terminus of the polypeptide. It is curious that sequence divergence has been so much greater throughout most of the putative amino-terminal regulatory domain of the TrpE polypeptide than in the carboxyl-terminal catalytic domain; nevertheless, all of the homologues are feedback-regulated by tryptophan. Although no detailed mutational analysis of a TrpE homologue from another organism has yet been reported, a single feedback-resistant mutation in the TrpE subunit of *Brevibacterium lactofermentum* has been identified at Ser-38, which aligns with Ser-40 of the *S. typhimurium* enzyme (17).

One apparent discrepancy with respect to the domain model proposed here for TrpE is the strong feedback-resistant mutation at Cys-465 (Table II). However, several factors suggest that Cys-465 is not likely to be an essential residue within the feedback site. Unlike all the other residues altered in the feedback-resistant enzymes, residue 465 is not highly conserved (Fig. 6), with cysteine being replaced by alanine, glycine, or serine in 5 of the 13 TrpE homologues. Moreover, in chemical modification studies of the TrpE polypeptide from *Serratia marcescens* with the sulfhydryl reagent, 5,5′-dithiobis-2-nitrobenzoic acid (13), there was no indication that this or any other cysteine residue was protected by tryptophan. Lastly, aside from the loss of feedback inhibition, the C465Y enzyme also suffered a significant increase in K_m^{Chr} (Table II). Thus it may be that Cys-465 is positioned in the folded structure of TrpE such that the bulky aromatic substitution in the C465Y mutant enzyme perturbed both the feedback site and the active site of the TrpE subunit. Interactions between the putative regulatory and catalytic domains are also suggested by the fact that several of the regulatory mutations (*e.g.* E39K, C174Y, and N288D) had significant effects on k_{cat} (Table II) and by the finding that some inactivating active site mutations simultaneously altered the apparent affinity of the enzyme for tryptophan (as measured by the relative sensitivity of the PRT activity to feedback inhibition).[3]

There are indications that conformational properties and subunit interactions were also affected in the mutant complexes. Those that were not completely feedback-resistant had a decreased response to tryptophan binding, as indicated by the reduced K_d^{Trp}/K_i^{Trp} ratios, and some of these had changes in the apparent cooperativity of tryptophan binding (Table III). Also, the sensitivity of the phosphoribosyl transferase activity to tryptophan inhibition decreased in all the mutant enzymes in parallel with decreases in the apparent affinity for the inhibitor (Table IV). Similarly, the effectiveness of tryptophan elution of the mutant enzymes from Orange A-agarose declined in parallel with the decline in the avidity of tryptophan binding (Fig. 5).

The conformational properties of the anthranilate synthase complex are presently not well understood. However, the results of dye-ligand chromatography suggest that the native enzyme exists in a conformational state intermediate to the classical T and R states proposed for allosteric proteins (18). Whereas the unliganded enzyme has strong affinity for the Orange A-agarose, both the T state ligand, tryptophan (Fig. 5), and the R state ligand, chorismate,[5] effectively reverse this interaction. Mutant enzymes that no longer bind tryptophan

[4] J. Hess and R. Bauerle, manuscript in preparation.

[5] J. Sennewald and R. Bauerle, unpublished observations.

apparently do not undergo the transition to the T state in the presence of tryptophan, and those with reduced affinity for tryptophan require higher concentrations for the transition (Fig. 5). Similar alterations in the conformational response to chorismate have been observed in enzymes with mutations in the catalytic domain.[5] A model accommodating three conformational states has also been proposed for the *S. marcescens* anthranilate synthase on the basis of ligand-induced spectral changes of a nonspecific fluorescent probe bound to the enzyme (2, 19).

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SUPPLEMENTARY MATERIAL TO:

Identification of Amino Acid Residues Involved in Feedback Regulation of the Anthranilate Synthase Complex from *Salmonella typhimurium*. Evidence for an Amino-terminal Regulatory Site.

Maureen G. Caligiuri and Ronald Bauerle

MATERIALS AND METHODS

Chemicals- Ampicillin, L-amino acids, 5-methyl-DL-tryptophan, 5-phosphorylribose-1-pyrophosphate, Tris, N-[tris-(hydroxymethyl)methyl]glycine, dithiothreitol and bovine serum albumin were from Sigma Chemical Co. Chorismic acid was isolated from the culture medium of *Klebsiella pneumoniae* strain 62-1 (ATCC25306) (20), crystallized and assayed enzymatically by its conversion to anthranilic acid by homogeneous wild type *S. typhimurium* anthranilate synthase (3). Anthranilic acid was from Sigma Chemical Co. and was recrystallized three times from water. Bacto-agar, Bacto-tryptone and Bacto-yeast extract were from Difco Laboratories. Acid hydrolyzed casein was from United States Biochemical Corp. All other chemicals were analytical reagent grade.

Enzymes and oligonucleotides- Restriction endonucleases, Klenow DNA polymerase and T4 DNA ligase were purchased commercially. Sequenase DNA polymerase was from United States Biochemical Corp. Deoxyoligonucleotides were custom synthesized by either the University of Virginia Sequencing Center or Synthetic Genetics Inc.

Bacteriological media- Minimal medium contained 10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 0.1 g/l $MgSO_4$, 2.5 g/l glucose and 2 g/l acid hydrolyzed casein. L broth contained 10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract and 10 g/l sodium chloride. Solid media contained 15 g/l Bacto-agar. When used, ampicillin and 5-methyl-DL-tryptophan were at 50 μg/ml and thiamine was at 50 μM.

Construction of plasmids pSTG3 and pSTG9- Plasmid pSTG3 (6331 bp), which carries the wild type *S. typhimurium trpE* gene under transcriptional control of its own promoter and terminator (Fig. 1), was constructed by cloning the 3.7 kb *SalI-EcoRI trpE* restriction fragment of plasmid pSTH10 (21) into the *SalI* and *EcoRI* sites of plasmid pBR327 (22). Plasmid pSTG9 (7189 bp), a derivative of pSTG3 carrying the wild type *trpE* and *trpD* genes of *S. typhimurium* (Fig. 1), was constructed by cloning the 4.0 kb *BalI* fragment of pSTP36 (23), into the *SalI* and *HindIII* sites of pSTG3 after end filling of the *SalI* and *HindIII* overhangs with Klenow polymerase. All DNA manipulations were carried out using standard methods (24).

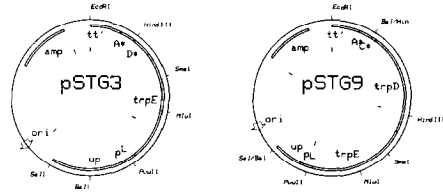

Fig. 1. Physical maps of plasmids pSTG3 and pSTG9. *p, L and tt'* represent, respectively, the *trp* promoter, leader region and transcriptional terminator; *A*, C** and *D** represent fragments of the *trpA, trpC* and *trpD* genes, respectively; *up* represents the chromosomal upstream flanking region of the *trp* operon.

In vitro mutagenesis of TrpE- For nitrous acid mutagenesis, the coding region of the *trpE* gene was first divided into three fragments by digesting plasmid pSTG3 with the following three pairs of restriction enzymes: (1) *PvuII* and *MluI*, (2) *MluI* and *SmaI* and (3) *SmaI* and *HindIII* (Fig. 1). The three restriction fragments were separated by agarose gel electrophoresis and recovered from the gel with Gene Clean (BIO 101). Approximately 100-200 ng of each of the three fragments was treated for one or two hours at room temperature in a mixture containing 250 mM sodium acetate buffer, pH 4.5, and freshly prepared 1.0 M sodium nitrite in a final volume of 100 μl (25). The reaction was terminated by the addition of 0.4 volumes of 1.0 M Tris-HCl, pH 8.0 (26). The DNA was precipitated twice with isopropanol and redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6).

Hydroxylamine mutagenesis (27) was carried out using intact pSTG3 plasmid DNA rather than restriction fragments, thereby avoiding the preferential attack of the single stranded ends of the DNA fragments by this mutagen (28). Approximately 1-2 μg of plasmid DNA was treated at 37 C for 12 or 24 h with 400 mM hydroxylamine hydrochloride, 1 mM EDTA, 200 mM potassium phosphate buffer, pH 6.0, in a final volume of 100 μl. The reaction was terminated by the removal of the DNA from the reaction mixture with Gene Clean. The treated plasmid DNA was divided and digested with the same three pairs of restriction endonucleases used in the nitrous acid mutagenesis. The three *trpE* fragments were then separated by gel electrophoresis in low melting temperature agarose and recovered in gel slices.

The various nitrous acid- and hydroxylamine-treated restriction fragments were ligated into plasmid pSTG3 from which the corresponding fragment had been removed, thereby reconstructing the *trpE* gene. The ligated DNA was transformed into *E. coli* strain CB90 (C600 *ΔtrpE5 hsdR leuB6 thi-1*). After DNA uptake, the cells were incubated with shaking in L broth for 30 min at 37 C, then collected by centrifugation and resuspended in 0.9% sodium chloride. Transformants simultaneously resistant to ampicillin (Amp^r)[2] and 5-methyl tryptophan (MTR) were selected by plating the cell suspensions on minimal agar containing ampicillin, 5-methyl-DL-tryptophan, and thiamine. Clones were purified by single colony isolation on the same medium and the MTR and Amp^r phenotypes were shown to be plasmid-associated by re-transformation of strain CB90. The $trpE^{MTR}$ mutations were then identified by DNA sequencing.

DNA sequencing- The nucleotide changes in the *trpE* genes of the MTR plasmids were determined by dideoxy DNA sequencing (29) using the Sequenase polymerase, following the protocol recommended by the manufacturer. The 2.0 kb *PvuII-HindIII* fragment of each mutant *trpE* plasmid was cloned into the *SmaI* and *Hin*dIII sites of the replicative form of bacteriophage M13mp19 and single stranded template DNA containing the non-coding strand of *trpE* was isolated from phage propagated in strain JM107 (24). A set of complementary hexadecameric deoxyoligonucleotides, spaced approximately 250 nucleotides apart along the gene, was used as sequencing primers. In most cases only that segment of the gene encompassing the restriction fragment targeted in the mutagenesis was sequenced in the parental MTR plasmid.

Construction of $trpE^{MTR}trpD^{+}$ ***plasmids***- Plasmids with $trpE^{MTR}trpD^{+}$ inserts were constructed from the parental $trpE^{MTR}$ plasmids by cloning the 2.0 kb *Hin*dIII-*Eco*RI fragment from pSTG9 (Fig. 1) into the *Hin*dIII and *Eco*RI sites of the $trpE^{MTR}$ plasmids. The plasmids were recovered by transformation of *E. coli* strain CB96 (C600 *ΔtrpED24 hsdR leuB6 thi-1*) with selection for Amp^r prototrophs on minimal agar + ampicillin. The MTR mutation of each was then localized by *in vitro* restriction fragment exchange mapping as follows. The *PvuII-MluI*, *MluI-SmaI* and *SmaI-Hin*dIII restriction fragments of each plasmid were used to replace the corresponding fragments of the $trpE^{+}$ plasmid, pSTG3. The resulting reconstructions were transformed into strain CB90 with selection on minimal agar + ampicillin. The Amp^R transformants were then replica plated onto minimal agar + 5-methyl-tryptophan to test for co-inheritance of the MTR phenotype. With this procedure, it was possible to establish that the mutations identified by DNA sequencing of the parental $trpE^{MTR}$ plasmids were solely responsible for the MTR phenotype and also to demonstrate plasmid homogenotization in the $trpE^{MTR}trpD^{+}$ strains. The mutations of the $trpE^{MTR}trpD^{+}$ plasmid constructs were also verified directly by DNA sequencing.

Purification of anthranilate synthase-phosphoribosyl transferase complexes- High level expression of the wild-type and mutant anthranilate synthase-phosphoribosyl transferase complexes was achieved using the $trpE^{+}trpD^{+}$ and $trpE^{MTR}trpD^{+}$ plasmids carried in *E. coli* host strain CB25 (W3110 *ΔtrpE-A2 tna-2 bglR*). A two liter baffled growth flask containing one liter minimal medium+ampicillin+L-tryptophan (3 μg/ml) was inoculated with 100 μl of an overnight L broth+ampicillin culture of the strain and incubated with vigorous shaking for 12 hours at 37 C. The cells were harvested by centrifugation and crude extracts were prepared by sonification (3).

The wild type and mutant complexes were purified to homogeneity using the method described previously (3), which features dye-ligand chromatography on Orange A agarose (Matrex Gel Orange A, Amicon Corp.). Standard phosphate buffer (100 mM potassium phosphate, pH 7.0, 10% glycerol, 1.0 mM dithiothreitol, 0.1 mM EDTA) was used throughout the purification and all manipulations were performed at 0-4 C. Crude extract was first fractionated by ammonium sulfate precipitation by the addition of a saturated (25 C) solution of enzyme grade NH_4SO_4 to 38% saturation. The precipitate was collected by centrifugation, dissolved in buffer and desalted by gel filtration over a column of Sephadex G-25. The desalted preparation, containing approximately 40-50 mg protein in 5 ml, was then applied to a 2 ml column of the Matrex Orange A gel. The column was washed with two volumes of buffer and then developed with buffer containing 50 μM tryptophan or with an isocratic tryptophan gradient (10 to 250 μM), as indicated in the text. Feedback resistant enzyme complexes which were not specifically eluted from the Matrex Orange A gel by the tryptophan buffer were eluted with buffer containing 1.5 M KCl. Tryptophan and KCl were removed from the purified enzymes by gel filtration over columns of Sephadex G-25.

The high level of expression of the *trpE* and *trpD* genes achieved by the host-vector system (>20% of total soluble protein), coupled with the selective protein-binding properties of the Matrex Orange A gel, led to the recovery of highly purified enzyme preparations with both methods of elution, as judged by SDS PAGE. The procedure routinely yielded about 15 mg of enzyme complex per liter of bacteria.

Assay and kinetic analysis of enzymatic activity- Anthranilate synthase and phosphoribosyl transferase activities were determined using continuous spectrofluorometric assays which monitor the rate of appearance and disappearance of anthranilic acid, respectively (3). Fluorescence changes were measured at 25 C using a Shimadzu model RF5000 spectrofluorophotometer operated at excitation wavelength of 308 nm and emission wavelength of 388 nm. The standard reaction mixture for the assay of glutamine-dependent anthranilate synthase activity contained 0.25 mM chorismic acid, 20 mM L-glutamine, 10 mM $MgCl_2$ and 100 mM potassium phosphate buffer, pH 7.0. The reaction mixture for the assay of phosphoribosyl transferase activity contained 10 μM anthranilic acid, 0.3 mM 5-phosphorylribose-1-pyrophosphate, 10 mM $MgCl_2$ and 100 mM N-[tris-(hydroxymethyl)-methyl] glycine, pH 7.6.

For kinetic analysis of anthranilate synthase activity, conditions were as in the standard assay except that chorismic acid was varied over a range of 0.5 to 25 μM. The concentration of glutamine, fixed at 20 mM, was saturating, being 20-fold K_m^{Gln} for the wild-type enzyme. The concentration of tryptophan was varied from 0 to 20 μM. Apparent K_m and k_{cat} constants were derived using the Enzfitter non-linear regression data analysis program (Elsevier Co., Amsterdam, The Netherlands). K_i values were determined graphically from secondary plots of the slopes of the lines of the Lineweaver-Burk plots of data versus the concentration of L-tryptophan, where K_i is equal to the negative intercept on the abscissa (30). For those curves where upward curvature in the Lineweaver-Burk plots appeared at low concentrations of chorismate, slopes were extrapolated from the linear portion of the curve corresponding to the higher chorismate concentrations.

Determination of protein concentration- The relative protein concentration of the purified wild type and mutant enzyme complexes was determined colorimetrically (31) utilizing the Bio-Rad protein reagent (Bio-Rad Laboratories) and bovine serum albumin as standard. The absolute protein concentration was calculated from the relative concentration by multiplying by 0.675, a factor which corrects for the overestimation of the concentration of the complex by the Bio-Rad method. This correction factor was derived from the results of the quantitative amino acid analysis of homogeneous wild type complex.

Tryptophan binding studies- Dissociation constants for L-tryptophan (K_i^{Trp}) of the wild-type and mutant anthranilate synthase-phosphoribosyl transferase complexes were determined by ultrafiltration (32) utilizing the Amicon Centrifree micropartition system (Amicon Corp.). Purified enzymes at a concentration of 2-5 μM were mixed with various concentrations of L-tryptophan, ranging from 0.5 to 200 μM containing 5 nCi L-[3-^{14}C]tryptophan (51.8 mCi/mmol) (New England Nuclear), in a final volume of 200 μl. The mixture was equilibrated at room temperature for 15 minutes and transferred to the micropartition unit. The cartridge was centrifuged at 1000 X g in a fixed angle rotor until approximately a third of the liquid volume passed into the lower chamber. The radioactivity of 50 μl of the ultrafiltrate was determined by liquid scintillation counting in 5.0 ml of Ready-Safe scintillation cocktail (Beckman Instruments) to determine the amount of free L-tryptophan. Dissociation constants were determined graphically from the double reciprocal plot of the Langmuir equation ($1/r = 1/(K_d/n[A] + n)$, where r is equal to moles of ligand bound per mole of protein, n is the number of ligand binding sites on the protein and [A] is the concentration of free ligand (33). The slope of the line is K_d/n and the intercept equals $1/n$. The binding data gave straight line curves with slight deviations from linearity only at very low free L-tryptophan concentrations. Interaction (Hill) coefficients, n_H, were determined graphically (34).