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Substitution of Glutamic Acid 109 by Aspartic Acid Alters the Substrate Specificity and Catalytic Activity of the β-Subunit in the Tryptophan Synthase Bienzyme Complex from *Salmonella typhimurium*†

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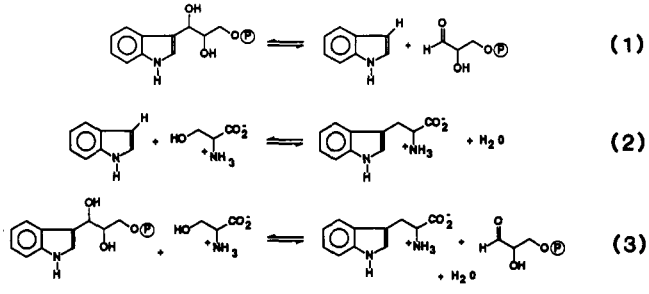
ABSTRACT: In an effort to understand the catalytic mechanism of the tryptophan synthase β-subunit from *Salmonella typhimurium*, possible functional active site residues have been identified (on the basis of the 3-D crystal structure of the bienzyme complex) and targeted for analysis utilizing site-directed mutagenesis. The chromophoric properties of the pyridoxal 5′-phosphate cofactor provide a particularly convenient and sensitive spectral probe to directly investigate changes in catalytic events which occur upon modification of the β-subunit. Substitution of Asp for Glu 109 in the β-subunit was found to alter both the catalytic activity and the substrate specificity of the β-reaction. Steady-state kinetic data reveal that the β-reaction catalyzed by the βE109D $\alpha_2\beta_2$ mutant enzyme complex is reduced 27-fold compared to the wild-type enzyme. Rapid-scanning stopped-flow (RSSF) UV–visible spectroscopy shows that the mutation does not seriously affect the pre-steady-state reaction of the βE109D mutant with L-serine to form the α-aminoacrylate intermediate, E(A-A). Binding of the α-subunit specific ligand, α-glycerol phosphate (GP) to the $\alpha_2\beta_2$ complex exerts the same allosteric effects on the β-subunit as observed with the wild-type enzyme. However, the pre-steady-state spectral changes for the reaction of indole with E(A-A) show that the formation of the L-tryptophan quinonoid, $E(Q_3)$, is drastically altered. Discrimination against $E(Q_3)$ formation is also observed for the binding of L-tryptophan to the mutant $\alpha_2\beta_2$ complex in the reverse reaction. In contrast, substitution of Asp for Glu 109 increases the apparent affinity of the βE109D α-aminoacrylate complex for the indole analogue indoline and results in the increased rate of synthesis of the amino acid product dihydroiso-L-tryptophan. Thus, the mutation affects the covalent bond forming addition reactions and the nucleophile specificity of the β-reaction catalyzed by the bienzyme complex.

The bacterial $\alpha_2\beta_2$ tryptophan synthase bienzyme complex catalyzes the final two reactions in the biosynthesis of L-tryptophan (Yanofsky & Crawford, 1972; Miles 1979, 1991). The α-subunit catalyzes the reversible aldolytic cleavage of 3-indole-D-glycerol 3′-phosphate (IGP) to D-glyceraldehyde 3-phosphate (G3P)[1] and indole (α-reaction). The β-subunit catalyzes the essentially irreversible condensation of indole with L-serine (L-Ser) to form L-tryptophan (L-Trp) (β-reaction). The physiological αβ-reaction is the sum of the individual α- and β-reactions linked via the common intermediate indole.



Crystallographic studies of the $\alpha_2\beta_2$ complex from *Salmonella typhimurium* have revealed that the α and β active sites are separated by nearly 30 Å but are directly connected by a tunnel of sufficient size to accommodate indole (Hyde et al., 1988; Hyde & Miles, 1990). Therefore, indole may be directly channeled between catalytic centers. Rapid kinetic studies have confirmed that the tunnel is the preferred route of entry for indole into the β active site in the β-reaction (Dunn et al., 1987b, 1990) and indole appears to be channeled between the α- and β-sites in the αβ-reaction (Lane & Kirschner, 1991; Anderson et al., 1991).

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[1] Abbreviations: PLP, pyridoxal phosphate; L-Ser, L-serine; L-Trp, L-tryptophan; DIT, dihydroiso-L-tryptophan; IGP, 3-indole-D-glycerol 3′-phosphate; IPP, 3-indolylpropanol 3-phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerol phosphate; 3-Cl-Ala, 3-chloro-L-alanine; BZI, benzimidazole; βE109D, $\alpha_2\beta_2$ complex of tryptophan synthase in which glutamate 109 in the β-subunit has been replaced by aspartate; RSSF, rapid scanning stopped flow; SWSF, single wavelength stopped flow; NMR, nuclear magnetic resonance; GPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactic dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate-*2,2,3,3-d*$_4$; $\alpha_2\beta_2$, native tryptophan synthase from *S. typhimurium*; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; $E(Q_1)$, $E(Q_2)$, or $E(Q_3)$, quinonoidal intermediates formed in the conversion of L-Ser and indole to L-Trp; $E(A_{ex})$, aldimine intermediates formed between the substrate amino acids and the PLP cofactor; E(GD), geminal diamine intermediate formed between the PLP cofactor, the amino group of the substrate, and the ε-amino group of Lys 87.

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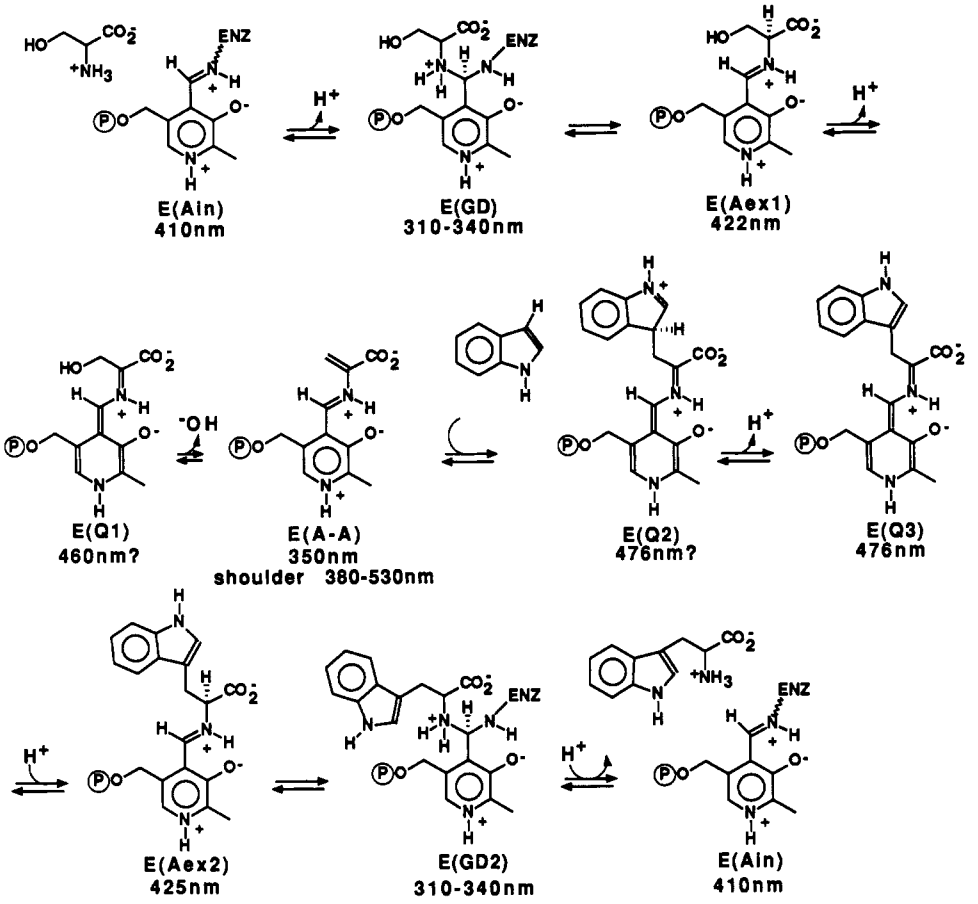
Scheme I: Mechanism of the PLP-Phosphate-Dependent β-Reaction[a]



[a] Changes in covalent bonding during the course of the reaction result in the production of reaction intermediates with distinct UV–visible properties. This allows direct observation of the reaction occurring at the β active site. The symbols denoting specific reaction intermediates are referred to throughout the text.

A number of studies have been focused on the characterization of the PLP-dependent β-reaction [for a recent review, see Miles (1991)]. The mechanism of the tryptophan synthase catalyzed β-replacement reaction may be divided into two sequential half-reactions (Scheme I). In the first stage of the β-reaction, L-Ser reacts with the PLP cofactor to form the quasistable electrophilic α-aminoacrylate intermediate, E(A-A). The second stage is initiated by the formation of a C–C bond between the C-3 carbon of indole and the β-carbon of the E(A-A) to give a quinonoidal species $E(Q_2)$. This species is rapidly deprotonated to give a second quinonoid, $E(Q_3)$, which then undergoes further conversion to L-Trp. Although a large number of nucleophiles will react with E(A-A) to form quinonoid intermediates (Dunn et al., 1987a,b; Roy et al., 1988a; Goldberg & Baldwin, 1967), only a small subset of these molecules have been shown to continue along the reaction pathway to yield new amino acid products (Roy et al., 1988a; Kayastha & Miles, 1990), and none of these work as well as the comparatively weak nucleophile indole. Thus, the enzyme displays a large degree of substrate specificity for the chemical and structural characteristics of indole.

The determination of the X-ray crystal structure has been instrumental in the identification of those amino acid residues which are likely either to play important roles in catalysis or in the allosteric function of the $\alpha_2\beta_2$ bienzyme complex (Hyde et al., 1988; Hyde & Miles, 1990). Use of this information to select α-subunit residues for site-directed mutagenesis has resulted in the identification of catalytic residues within the α-active site (Nagata et al., 1989; Miles et al., 1988; Yutani et al., 1987). Furthermore, mutation of certain α-subunit residues has been shown to alter the accumulation and distribution of intermediates covalently bound at the β active site (Kawasaki et al., 1987; Brzovic et al., 1992).

Recent work has focused on identifying active-site residues of the β-subunit which are important for catalysis (Miles et al., 1989; Kayastha et al., 1991). The position of glutamic acid 109 within the β-subunit suggests it may function as a possible catalytic residue. Glu 109 is located in a region adjacent to the covalently bound PLP cofactor and may form part of the putative indole-binding site of the β-subunit. In order to further understand the functional role of this residue in catalysis, Glu 109 has been converted to Asp by site-directed mutagenesis. The effects of this mutation on the reactivity of the β-subunit have been studied by exploiting the well-defined UV–visible properties of the system. The PLP cofactor provides a convenient spectroscopic probe for the direct observation and characterization of reaction intermediates (see Scheme I). In this study, the influence of the βE109D mutation on catalytic events has been examined and compared to the behavior of the wild-type enzyme. The results presented herein reveal that this mutation greatly reduces the reactivity of the enzyme complex toward indole yet enhances the specificity of the β active site for the indole analogue indoline.

Materials and Methods

Materials. L-Ser, L-Trp, indole, DSS, G3P, NADH, and Bicine were purchased from Sigma. Indoline, BZI, *N*-methylhydroxylamine, and D_2O were purchased from Aldrich. IGP was synthesized as previously described (Kawasaki et al., 1987). DIT was synthesized as previously described (Roy et al., 1988a). All reactions were studied in 50 mM Bicine buffer containing 1 mM EDTA at pH 7.8 unless otherwise indicated.

Enzymes. Wild-type and mutant forms of tryptophan synthase from *S. typhimurium* were purified and crystallized from extracts of a host strain, *Escherichia coli* (CB149), that lacks the *Trp* operon and harbors a high-copy plasmid (C51B7) carrying the wild-type or mutant β-subunit gene from *S. typhimurium.* Each liter of culture yielded approximately 20 mg of protein. Procedures used for the purification of wild-type and βE109D $\alpha_2\beta_2$ tryptophan synthase, determination of protein concentrations, and measurement of enzyme activity have been previously described (Miles et al., 1987, 1989). LDH and GPDH were purchased from Sigma.

Oligonucleotide-Directed Mutagenesis of TrpB from S. typhimurium. The *trpB* glutamic acid 109 codon (GAA) was changed to the aspartic acid codon (GAT) by general procedures described previously (Nagata et al., 1989). The mutagenic oligomer employed was a 21-mer complementary to nucleotides 311–334 in the mutant *trpB* gene with the sequence 5′-CCGGCT*CCGGTA*TCAGCGATA-3′. The complementary codon is underlined; the asterisks follow bases that differ in the complementary wild-type DNA. The sixth base, C, was replaced by T to remove one *Ban*I site to facilitate screening. The desired mutation was confirmed by dideoxy-DNA sequencing using a primer complementary to nucleotides 370–390 in the *trpB* gene as described (Nagata et al., 1989). The oligonucleotide was synthesized and purified as described (Kawasaki et al., 1987).

Enzyme Assays. One unit of activity in any steady-state reaction is the formation of 0.1 μM product in 20 min at 37 °C. The activity of the $\alpha_2\beta_2$ complex in the α- and αβ-reactions monitored the rate of G3P release in a coupled reaction with GPDH (Creighton, 1970). In order to test for the accumulation of free indole in solution under steady-state conditions, the progress of the α-reaction was monitored at 25 °C by a direct spectrophotometric assay for the synthesis of L-Trp from IGP and L-Ser. This assay exploits the differences in absorbance at 290 nm between IGP, indole, and L-Trp. The cleavage of IGP to indole and G3P is characterized by a decrease in absorbance ($\Delta\epsilon_{290}$ = 1.39 mM^{-1} cm^{-1}; Weischet & Kirschner, 1976b), while the conversion of IGP and L-Ser to L-Trp is characterized by a net increase in absorbance ($\Delta\epsilon_{290}$ = 0.50 mM^{-1} cm^{-1}). The β-reaction was measured by a direct spectrophotometric assay (Miles et al., 1987). The activity of the $\alpha_2\beta_2$ complex in reaction 4 was measured by a direct spectrophotometric assay at 306 nm where $\Delta\epsilon_{306nm}$ = 0.1 mM^{-1} cm^{-1} (Roy et al., 1988a). Pyruvate-forming reactions were monitored in a coupled assay with LDH (Crawford & Ito, 1964).

Static Spectrophotometric Measurements. All static UV–visible absorption spectra were collected with a Hewlett-Packard 8450A diode-array spectrophotometer. Fluorescence spectra were made using a Perkin-Elmer model MPF-44B fluorimeter.

^1H FT NMR Spectroscopy. All ^1H NMR spectra were recorded on a Nicolet 300-MHz spectrophotometer. Reactions were carried out in D_2O with 40 mM L-Trp and 10 mM Bicine buffer at 25 °C and pH* 7.8.

Rapid-Scanning Stopped-Flow (RSSF) Spectrophotometry. The RSSF spectrophotometer used in these studies employed elements of the Durrum D-110 rapid-mixing stopped-flow spectrometer and a Princeton Applied Research (PAR) OMA-III multichannel analyzer with a 1463 detector-controller card and a 1214 photodiode array detector. The RSSF system has been described in detail elsewhere (Koerber et al., 1983). For a typical experiment, a 100% transmission spectrum (defined as the light transmitted through the buffer solution used) and the diode array dark-current spectrum are first collected and stored. By use of these spectra, spectra collected at programmed intervals after mixing are converted to absorbance and stored on floppy disk. The experiments reported herein used 512 pixels for a repetitive scan time of 8.528 ms and a wavelength resolution of 0.5 nm. All concentrations refer to reaction conditions immediately after mixing.

RSSF data presented for the following experiments were collected utilizing various computer programmed timing sequences. Data collection was initiated after mixing was complete and flow had stopped. Timing sequence no. 1 collected spectra at 8.53, 17.1, 25.6, 34.1, 42.6, 59.7, 85.3, 170.6, 255.8, and 341.1 ms after mixing. Timing sequence no. 2 collected spectra at 8.53, 17.1, 25.6, 34.1, 42.6, 76.8, 127.9, 255.8, 426.4, and 639.6 ms after mixing. Timing sequence no. 3 collected spectra at 8.53, 17.1, 34.1, 42.6, 76.8, 127.9, 383.8, 852.8, and 1705.6 ms after mixing.

Results

In order to assess the effect of the βE109D mutation on the reactivity of the bienzyme enzyme complex, rapid-scanning stopped-flow (RSSF) transient kinetic techniques were applied to study the behavior of the mutant bienzyme complex for a number of well-characterized reactions. Significant alterations in the spectral properties of intermediates at the β active site or changes in the accumulation and decay of species as a result of the mutation may be directly observed via RSSF. RSSF studies in the 300–550-nm region of the UV–visible spectrum were undertaken to detect and characterize PLP intermediates along the reaction pathway (Drewe & Dunn, 1985, 1986; Roy et al., 1988b; Brzovic´ et al., 1990; Houben et al., 1989; Houben & Dunn, 1990). The reactions of L-Ser and L-Trp with βE109D and the reactions of indole and indoline with the E(A-A) complex, in the absence and presence of the α-subunit-specific ligand D,L-α-glycerol phosphate (GP), were monitored and compared with the reactivity of the wild-type enzyme.

Rapid-Scanning Stopped-Flow (RSSF) Spectra for the Reaction of L-Ser with Wild-Type and Mutant Enzyme Complexes. Figure 1 compares the reactions of L-Ser with the wild-type enzyme and the βE109D mutant in the absence and presence of GP. The spectral changes that accompany this reaction have been previously described for the wild-type enzyme from *E. coli* (Drewe & Dunn, 1985). The enzyme from *S. typhimurium* gives similar results (Figure 1A,B; Brzovic´ et al., manuscript in preparation). The spectrum of the unliganded native enzyme is characterized by an absorbance band centered at 410 nm arising from the internal aldimine Schiff base between the PLP cofactor and the ε-amino group of Lys 87. Rapid mixing with L-Ser results in the rapid formation of a new spectral band ($1/\tau_1$) with λ_{max} = 420 nm. Our previous work (Drewe & Dunn, 1985) and the findings of others (York, 1972; Miles & McPhie, 1974; Faeder & Hammes, 1971; Lane & Kirschner, 1983a) provide strong evidence in support of the assignment of this species as the external aldimine formed between L-Ser and the PLP cofactor [$E(A_{ex1})$, see Scheme I]. The 420-nm band subsequently decays in a biphasic process ($1/\tau_2 > 1/\tau_3$) (Lane & Kirschner, 1983a; Drewe & Dunn, 1985) to yield the final E(A-A) spectrum which is characterized by a peak at 350 nm with a broad shoulder extending from 380 to 530 nm (Figure 1A, spectrum 12). This spectrum likely consists of an equilibrating mixture of intermediates (Drewe & Dunn, 1985). In the presence of GP, the final spectrum displays greater absorbance

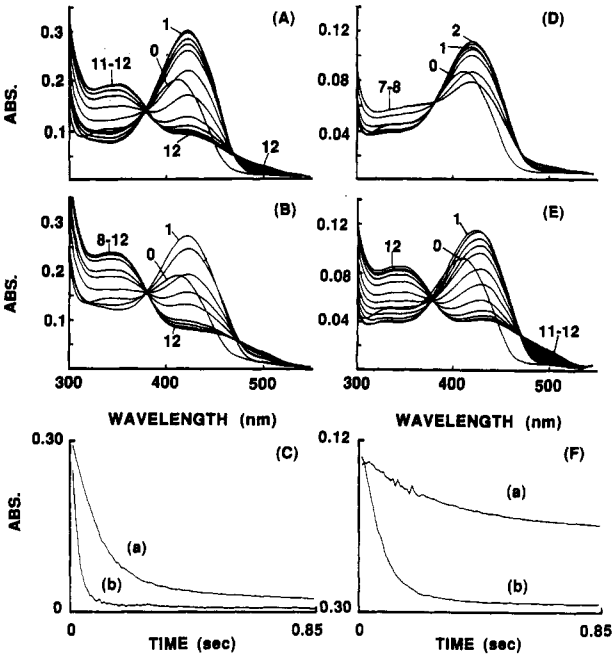


FIGURE 1: Comparison of the time-resolved pre-steady-state RSSF changes for the reaction of 40 mM L-Ser in the absence and presence of 100 mM D,L-GP for the wild-type and βE109D $\alpha_2\beta_2$ bienzyme complexes. L-Ser was in one syringe and enzyme in the other. GP, when present, was added to both syringes. (A) Reaction of 10 μM wild-type enzyme with L-Ser. Data were collected utilizing timing sequence no. 3 (see Materials and Methods). (B) Reaction of 10 μM wild-type enzyme with L-Ser in the presence of GP. Data were collected utilizing timing sequence no. 2 (see Materials and Methods). (C) Single-wavelength time courses at 420 nm for the reactions described in Figure 1A,B in (a) the absence and (b) the presence of D,L-GP. Time courses were derived from 100 consecutive rapid-scanning spectra collected at intervals of 8.54 ms for a total acquisition time of 0.854 s. (D) Reaction of 5 μM βE109D with L-Ser. Data were collected utilizing timing sequence no. 3 (see Materials and Methods). (E) Reaction of 5 μM βE109D with L-Ser in the presence of GP. Data were collected utilizing timing sequence no. 1. (F) Single-wavelength time courses at 420 nm for the reactions described for panels D and E in the (a) absence and (b) the presence of D,L-GP.

at 350 nm and less at 420 nm (compare Figure 1, panels A and B, spectrum 12).

The transient spectral changes which occur during the reactions of L-Ser with βE109D and the wild-type enzyme are qualitatively similar. In the absence of GP (Figure 1A,D), the same intermediates are observed with both the wild-type and mutant enzymes, but the spectra obtained at equilibrium (Figure 1D, spectrum 8) show that the final distribution of bound βE109D-serine intermediates has been altered relative to the wild-type enzyme (compare Figure 1A, spectrum 12 and Figure 1D, spectrum 8). The final spectrum (Figure 1D, spectrum 8) shows that a greater proportion of $E(A_{ex1})$ absorbing at 420 nm is present at equilibrium in the βE109D system. This intermediate is highly fluorescent (λ_{ex} = 420 nm, λ_{em} = 506 nm) (Goldberg et al., 1968), and the fluorescence of the reaction mixture is increased accordingly (data not shown). The equilibrium distribution of βE109D intermediates may be shifted either by varying the pH of the reaction mixture (data not shown) or by the addition of GP (compare Figure 1 panels B and E). This behavior is similar to that observed for the wild-type enzyme (Mozzarelli et al., 1991). There is very little difference between the equilibrium spectra of the wild-type and mutant enzymes in the presence of GP (compare Figure 1 panels B and E).

Figure 1 panels C and F compare the time courses for the decay of the 420-nm band in both the absence (trace a) and presence (trace b) of GP for the wild-type and βE109D bi-

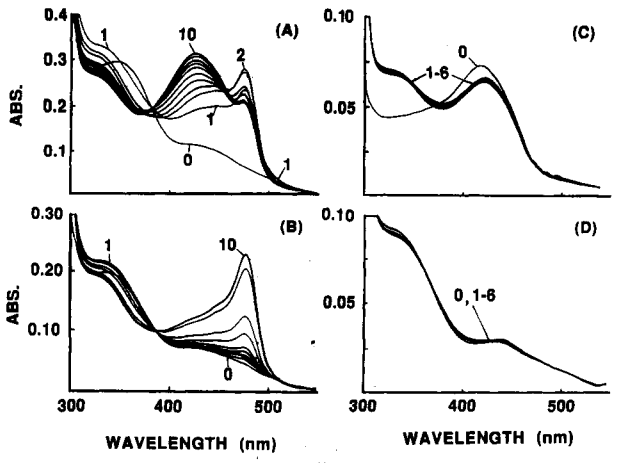


FIGURE 2: RSSF spectra for the reaction of the wild-type and βE109D E(A-A) complexes with 1 mM indole in the absence and presence of GP. Enzyme and L-Ser were in one syringe and indole and L-Ser were in the other. (A) Reaction of 10 μM wild-type E(A-A) complex with indole. Data were collected utilizing timing sequence no. 1 (see Materials and Methods). (B) Reaction of the wild-type E(A-A) complex with indole in the presence of GP. Data were collected utilizing timing sequence no. 3 (C and D). Reaction of 5 μM βE109D E(A-A) complex, in the absence (C) and presence (D) of GP, with indole. Data were collected utilizing timing sequence no. 2.

enzyme complexes, respectively. The time courses for both the wild-type and βE109D enzymes are well described by the sum of two exponential curves with $1/\tau_2 > 1/\tau_3$ (the time scales used in Figure 1 C,F emphasize $1/\tau_2$). The initial phase in the decay of the 420-nm band ($1/\tau_2$), which also exhibits the largest amplitude change, has also been shown to correspond to the rate of E(A-A) formation (Drewe & Dunn, 1986; York, 1972). For the wild-type enzyme, addition of GP to the reaction mixture increases the rate of $1/\tau_2$ from 10 s^{-1} to over 70 s^{-1} (Figure 1C). The corresponding relaxations observed during $1/\tau_2$ in the βE109D reaction with L-Ser are 3- and 4-fold slower, respectively, than the wild-type reactions (compare Figure 1 panels C and F).

Comparison of the Reactions of Wild-Type and βE109D E(A-A) with Indole and Indoline. Time-resolved spectra collected via RSSF spectroscopy for the reaction of indole with the E(A-A) complex of the wild-type enzyme are shown in Figure 2A. In this reaction, covalent bond formation results in the rapid accumulation of a quinonoid intermediate (Scheme I) absorbing at 476 nm (Goldberg & Baldwin, 1967; Lane & Kirschner 1983b). Under the experimental conditions used in Figure 2A, the apparent first-order rate constant for the appearance of the quinonoid[2] approaches 200 s^{-1}. Quinonoid formation is followed by the accumulation of an intermediate absorbing at 425 nm. The spectral properties of this species are consistent with the accumulation of the L-Trp external aldimine [$E(A_{ex2})$, Scheme I] (Metzler et al., 1973; Kallen et al., 1985; Drewe & Dunn, 1986). This assignment is in agreement with the finding that the release of L-Trp appears to be the rate-limiting step in the β-reaction (Lane & Kirschner, 1981, 1983b). In the presence of GP, the rate of quinonoid formation is inhibited nearly 15-fold, yet the total amount of quinonoid that is formed and persists into the steady-state phase of the reaction is substantially greater when

[2] Assuming an extinction coefficient of at least 50000 M^{-1} cm^{-1} (Karube & Matsushima, 1977; Ulevitch & Kallen, 1977; Roy et al., 1988b) for the indoline quinonoid, the final spectrum indicates that nearly all the enzyme active sites exist in the form of the DIT quinonoid. A smaller extinction coefficient is not compatible with the enzyme concentration.

Table I: Comparison of the Specific Activities of Reactions Catalyzed by the *S. typhimurium* Wild-Type and βE109D $\alpha_2\beta_2$ Bienzyme Complexes[a]

reaction	substrates	subunit site	GP	wt $\alpha_2\beta_2$	E109D
1	IGP → G3P + indole	α	−	30	20
2	L-Ser + indole → L-Trp	β	−	1080	40
			+	270	70
3	IGP + L-Ser → L-Trp	α,β	−	300	70
4	L-Ser + indoline → DIT	β	−	10	40
			+	0	26
5	3-Cl-Ala + indole → L-Trp	β	−	269	67
			+	31	50
6	3-Cl-Ala + indole → DIT	β	−	12	56
			+	2	12

[a] Assays were carried out at 37 °C as described under Materials and Methods. Specific activity is defined as units of enzyme activity per milligram of $\alpha_2\beta_2$ bienzyme complex. Subunit site refers to the subunit of the $\alpha_2\beta_2$ complex involved in the catalytic reaction. All enzyme activities were monitored in both the absence and presence of 100 mM α-subunit-specific ligand D,L-GP.

GP is present. GP has been shown to stabilize a variety of quinonoidal species (Dunn et al., 1987a,b; Roy et al., 1988a; Nagata et al., 1989; Houben et al., 1989; Houben & Dunn, 1990; Kirschner et al., 1991).

In marked contrast to the behavior of the wild-type enzyme, the reaction of the βE109D E(A-A) with indole, either in the absence or in the presence of GP, gives no detectable spectral changes that correspond to the accumulation of intermediates occurring during the second stage of the β-reaction. The addition of GP to the reaction mixture is no longer sufficient to promote the accumulation of a quinonoid species [$E(Q_2)$ or $E(Q_3)$] derived from indole. In the absence of GP, the RSSF data show that indole slightly perturbs the spectrum of the L-Ser complex with βE109D (Figure 2C). Since no subsequent spectral changes are detectable (the increase below 380 nm is due to free indole), this change is essentially complete within the mixing dead time of the instrument (3–4 ms). Benzimidazole (BZI), a close structural analogue of indole, also binds to the L-Ser complex with βE109D, shifting the equilibrium distribution of intermediates toward the formation of E(A-A) (data not shown). Although BZI is an inherently better nucleophile than indole, it does not appear to react with the wild-type E(A-A), and no amino acid products have been detected (Roy et al., 1988a). These observations are also true for the βE109D mutant (Leja and Dunn, unpublished results).

RSSF spectra collected during the reaction of indoline with E(A-A), both in the absence and presence of GP, are shown in Figure 3. In contrast to the reaction of indole with the E(A-A) of βE109D, the reaction of indoline is very similar to that of the wild-type enzyme (compare Figure 3A,B with Figure 3D,E). In both cases, the formation of the quinonoidal intermediate is a multiphasic process with a very rapid initial phase. The quinonoid band dominates the final spectrum at equilibrium. GP significantly inhibits the rate of quinonoid formation in the reaction of indoline with E(A-A) for both the wild-type and βE109D bienzyme complexes. Although the rate of quinonoid formation is decreased, under the experimental conditions described, GP increases the total yield of quinonoidal intermediate which accumulates at equilibrium for both wild-type and βE109D enzyme complexes. These findings are in agreement with the results reported for the *E. coli* enzyme (Dunn et al., 1990).

Specific Activities for Reactions Catalyzed by the Wild-Type and βE109D Bienzyme Complexes. Examination of Table I shows that the rate of the α-subunit-catalyzed cleavage of IGP to indole and G3P is similar to that of the wild-type enzyme. Secondly, L-Ser bound to the β active site stimulates the rate of the α-reaction in the synthesis of L-Trp from the

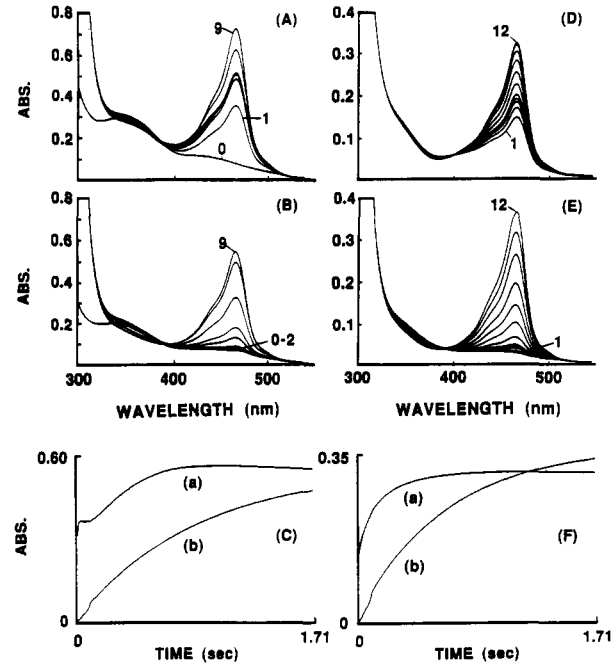


FIGURE 3: RSSF spectra for the reaction of the wild-type and βE109D E(A-A) complexes with 1 mM indoline in the absence and presence of 100 mM D,L-GP. Enzyme and 40 mM L-Ser were in one syringe, while indoline and 40 mM L-Ser were in the other. (A) Reaction of 10 μM wild-type E(A-A) with indoline. Spectra were collected utilizing timing sequence no. 3 (see Materials and Methods). (B) Reaction of the wild-type E(A-A) complex with indoline in the presence of GP. Spectra were collected using timing sequence no. 4. The total acquisition time was 4.28 s. (C) Single-wavelength time courses at 466 nm derived from 200 successive RSSF spectra collected at intervals of 8.54 ms for a total acquisition time of 1.71 s. Trace a is for the reaction in the absence of GP and trace b denotes the presence of GP. (D) Reaction of 5 μM βE109D E(A-A) complex with indoline. Spectra were collected using timing sequence no. 3. (E) Reaction of βE109D E(A-A) complex with indoline in the presence of GP. Spectra were collected utilizing timing sequence no. 3. (F) Single-wavelength time courses at 466 nm for the reactions described in panels E and F. The total acquisition time was 1.71 s.

physiological substrates (αβ-reaction). Nevertheless, the overall rate of L-Trp synthesis in the αβ-reaction is slower in the βE109D mutant. Direct assays of L-Trp formation in the αβ-reaction, which exploit the difference in absorbance between L-Trp, indole, and IGP at 290 nm, show that indole accumulates in solution during the course of the αβ-reaction catalyzed by the βE109D mutant at 25 °C. The steady-state time course is biphasic; an initial phase of decreasing absorbance that corresponds to the cleavage of IGP to G3P and

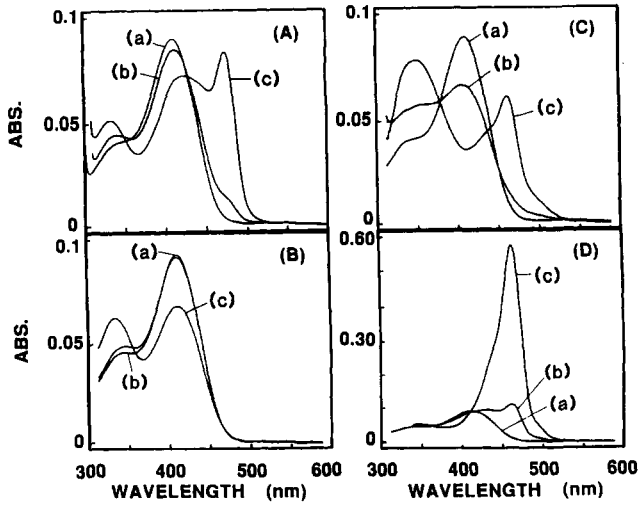
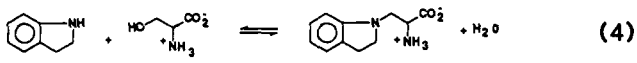


FIGURE 4: UV–visible spectra of wild-type $\alpha_2\beta_2$ and βE109D enzyme complexes with L-Trp and DIT in both the absence and presence of GP. (A) Reaction of 7.5 μM wild-type $\alpha_2\beta_2$ enzyme (spectrum a) with 2.5 mM L-Trp (spectrum b) and with 100 mM D,L-GP (spectrum c). (B) Reaction of 7.5 μM βE109D (spectrum a) with 2.5 mM L-Trp (spectrum b) and in the presence of 50 mM D,L-GP (spectrum c). (C) Reaction of 7.5 μM wild-type enzyme (spectrum a) with 1.1 mM DIT (spectrum b) and in the presence of GP (spectrum c). (D) Reaction of βE109D (spectrum a) with DIT (spectrum b) and in the presence of 50 mM GP (spectrum c).

indole, and a second phase of increasing absorbance corresponding to the formation of L-Trp. This behavior is not observed for the wild-type-catalyzed $\alpha\beta$-reaction. This is consistent with the finding that free indole is not detected in the reaction catalyzed by the wild-type enzyme (Yanofsky & Rachmeler, 1958).

The synthesis of L-Trp by the mutant enzyme in the β-reaction is decreased 27-fold relative to the wild-type enzyme. Although GP inhibits this reaction by nearly 4-fold for the wild-type enzyme, it has a slight stimulatory effect on the reactivity of the mutant enzyme. Similar rate effects have also been observed in the presence of IPP (data not shown). When indoline is substituted for indole [reaction 4, Roy et al. (1988a)], the synthesis of dihydroiso-L-tryptophan (DIT) is catalyzed very slowly by the wild-type enzyme. The steady-state rate of DIT synthesis catalyzed by the βE109D mutant is 5-fold faster. Table I shows that the mutant enzyme catalyzes the formation of DIT at a rate which exceeds that of L-Trp synthesis.



(4)

The $\alpha_2\beta_2$ catalyzed syntheses of L-Trp and DIT were also monitored with β-Cl-L-Ala as substrate (reactions 5 and 6, Table I). Although the β-substituent on the substrate has been changed, the same pattern of steady-state reactivities is observed for the reactions utilizing the L-Ser analogue β-Cl-L-Ala in place of L-Ser.

Reaction of Wild-Type and βE109D $\alpha_2\beta_2$ with L-Trp and DIT. The equilibrium spectra that occur upon the binding of L-Trp to the wild-type enzyme in both the absence and presence of GP are shown in Figure 4A. As evidenced by the small shoulder at 476 nm, only a very small amount of quinonoid accumulates at equilibrium. When GP is bound at the α-active site, the amount of quinonoidal species present at the β-active site is enhanced (Figure 4A, spectrum c) (Dunn et al., 1987a,b 1990; Houben & Dunn, 1990; Kirschner et al., 1991). Addition of a 5-fold larger concentration of L-Trp to the βE109D mutant does not produce any significant spectral

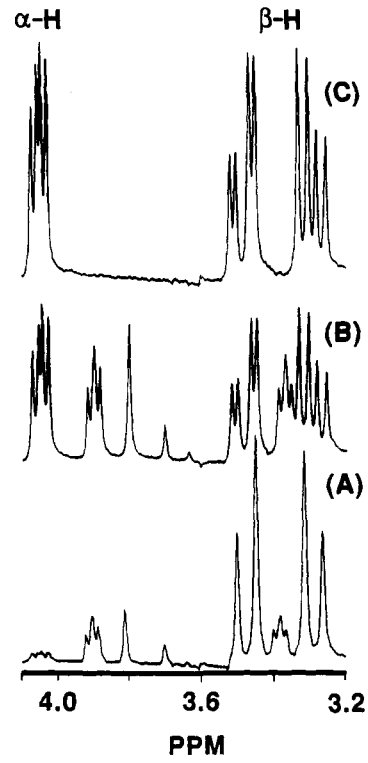


FIGURE 5: 300-MHz ^{1}H NMR spectra of the reaction of 40 mM L-Trp with (A) 1 μM wild-type and (B) 1 μM βE109D enzyme complexes at pH 7.8. Spectrum C is of 40 mM L-Trp in D_2O only. Chemical shifts determined relative to DSS. The α-H resonance of L-Trp is centered at 4.05 ppm. The β-proton resonances comprise a complex multiplet centered around 3.4 ppm. Resonances centered at 3.9, 3.8, 3.7, and 3.35 ppm that are observed in panels A and B arise from buffer salts present in the reaction mixture.

changes (Figure 4B, spectrum c). The addition of GP promotes the accumulation of a 330-nm band with a corresponding decrease in absorbance at 410 nm (Figure 4B, spectrum c). No quinonoidal intermediate accumulates in the reaction of βE109D with L-Trp. The same discrimination against quinonoid formation is observed for the reaction of indole with the E(A-A) complex (compare Figure 4B with Figure 2C,D).

The inability of the mutant enzyme to form detectable amounts of quinonoidal species is further illustrated in Figure 5. The wild-type enzyme readily catalyzes the exchange of the α-hydrogen of L-Trp for deuterium in D_2O (Tsai et al., 1978). Under experimental conditions where the wild-type reaction is essentially complete within 200 min, the βE109D mutant was found to catalyze no detectable proton exchange in L-Trp after 240 min of incubation.

In contrast to L-Trp, DIT reacts readily with the βE109D mutant to form a mixture of species, including a quinonoid intermediate absorbing at 465 nm (Figure 4D, spectrum b). Addition of GP to the reaction mixture increases the amount of quinonoid present by over 6-fold (Figure 4D, spectrum c). The wild-type enzyme binds and reacts with DIT, but only a very small amount of the quinonoidal intermediate accumulates. Addition of GP produces only a minor enhancement in the amount of quinonoid which accumulates at equilibrium (Figure 4C). The most noticeable spectral change is the appearance of a broad spectral band centered at 350 nm (Figure 4D, spectrum c).

Comparison of Quinonoid Spectral Bands Formed in the Reactions of Indoline and N-Methylhydroxylamine with the Wild-Type and βE109D Enzymes. Direct comparison of the quinonoid band formed in the reactions of indoline with the

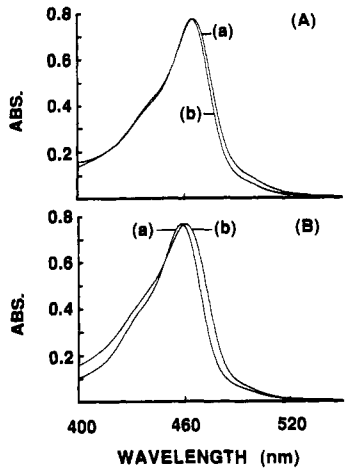


FIGURE 6: Comparison of wild-type and βE109D quinonoid spectral bands derived from (A) indoline and (B) *N*-methylhydroxylamine. For both panels, trace a is the wild-type enzyme and trace b is the βE109D enzyme. Spectra were normalized to the same maximum absorbance for the purposes of comparison.

wild-type and βE109D E(A-A) species shows that the βE109D quinonoidal band is slightly blue-shifted (from 466 to 465 nm) and the band shape is altered (Figure 6A). Addition of GP to the reaction mixture alters the amount of quinonoid at 465 nm but does not perturb the position of the absorbance maximum or the band shape. When *N*-methylhydroxylamine is the reacting nucleophile, the position of the βE109D quinonoid band is red-shifted by nearly 2.5 nm compared to that of the wild-type enzyme (Figure 6B).

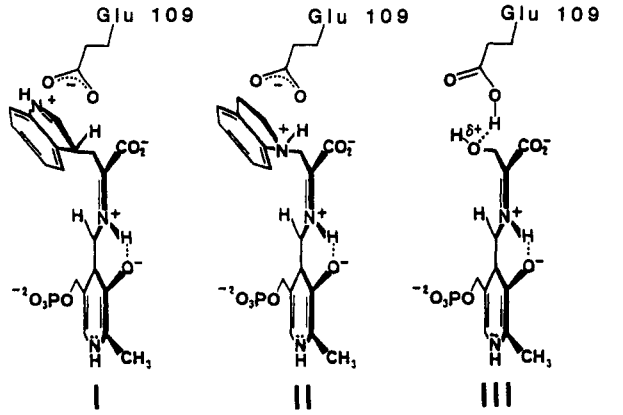
DISCUSSION

The X-ray structure of the tryptophan synthase bienzyme complex provides a useful framework for exploring structure function relationships (Hyde et al., 1988; Hyde & Miles, 1990). Residues with potential functional significance may be identified on the basis of the crystal structure and targeted for modification by site-directed mutagenesis. The effects of the mutation on the catalytic function of the bienzyme complex then can be probed by exploiting the chromophoric properties of the PLP cofactor bound to the β-subunit. Transient kinetic techniques give direct information about catalytic events occurring at the active site. Changes in the formation and decay of reaction intermediates relative to those observed for the wild-type enzyme may be directly detected and analyzed with respect to the mutation.

Glutamate 109 lies in an open region within the β-site adjacent to the bound PLP-cofactor in an area thought to comprise the putative indole binding site (Hyde et al., 1988; Hyde & Miles, 1990). The position of this residue suggests it may function as a catalytic residue in the activation of indole as a nucleophile and/or in the activation of the β-hydroxyl of L-Ser as a leaving group (Scheme II, structures I and III). Mutation of Glu 109 to Asp results in kinetic behavior which is consistent with the idea that it plays a catalytic role and is an important functional residue for determining the nucleophile specificity of the β-replacement reaction.

Conformational Integrity of the βE109D Bienzyme Complex. The reactivities of the individual enzyme components of the wild-type tryptophan synthase bienzyme complex are dependent upon interactions between the separate subunits in the $\alpha_2\beta_2$ complex. Formation of the $\alpha_2\beta_2$ complex increases the reactivity of the α- and β-subunits by 100- and 50-fold, respectively (Yanofsky & Crawford, 1972). Subunit interactions profoundly affect the accumulation of intermediates

Scheme II: Possible Catalytic Roles of Glutamate 109 at the β Active Site[a]



[a] (I) Glu 109 may play an important role in the activation of indole by promoting the formation of the iminium tautomer of indole or, as depicted here, in the stabilization of the resulting positive charge on the indole ring. (II) Glu 109 may interact with the indoline quinonoid to stabilize the positive charge on the indolinium nitrogen atom. Changing Glu 109 to Asp results in the enhanced stabilization of this intermediate (see text). (III) Glu 109 may also form important interactions with the β-hydroxyl group of L-Ser, facilitating the elimination of this substituent.

in the β-reaction (Miles & McPhie, 1974; Drewe & Dunn, 1985) and create an enzyme complex in which reciprocal communication of allosteric information between heterologous enzymes is an important aspect of catalytic function (Kawasaki et al., 1987; Houben & Dunn, 1990; Dunn et al., 1987a,b, 1990; Kirschner et al., 1991; Brzovic´ & Dunn, 1992). Any gross conformational change in the $\alpha_2\beta_2$ complex caused by the alteration of amino acid residues almost certainly will be reflected as a large perturbation in one or more of these characteristics.

Table I shows that the specific activity of the βE109D-catalyzed cleavage of IGP to indole and G3P is very similar to that of the wild-type enzyme, indicating that the α-subunit is in an active conformation and that interactions between the α- and β-subunits are essentially intact. The transient spectral changes that are observed for the reactions of L-Ser demonstrate that the same intermediates are detected for the reactions of both the βE109D and the wild-type bienzyme complexes (Figure 1). Therefore, the chemistry of E(A-A) formation catalyzed at the β active site is unaltered by the mutation. At pH 7.8, however, the final equilibrium distribution of intermediates is changed. Since the equilibrium may be shifted to the characteristic E(A-A) spectrum, either by the addition of GP to the reaction mixture (Figure 1D) or by modulation of the pH of the medium (Mozzarelli et al., 1991; data not shown), it is likely that the perturbation reflects a modified pK_a for the pH-induced allosteric transition rather than a gross conformational change in the β-subunit.

The ability of the α-specific ligand GP to accelerate the reaction of L-Ser at the β active site (Figure 1) and to enhance the accumulation of the indoline quinonoid (Figure 4D) coupled with the ability of L-Ser to stimulate the cleavage of IGP (Table I) establishes that the reciprocal transmission of allosteric information between heterologous active sites is still functional in the βE109D bienzyme complex. Therefore, on the basis of the reactivity of the mutant enzyme, we conclude that the overall conformation of the $\alpha_2\beta_2$ complex is very similar to that of the wild-type enzyme.

Substrate Specificity of the βE109D β-Subunit. Table I and Figure 2 show that the replacement of Glu 109 by Asp significantly changes the reactivity of the βE109D bienzyme

complex toward indole. The specific activity of the βE109D-catalyzed β-reaction has been reduced by 27-fold as compared to the β-reaction catalyzed by the wild-type enzyme. The large reduction in the rate of the β-reaction catalyzed by the βE109 mutant cannot be attributed to changes in the first stage of the β-reaction in which L-Ser reacts to form E(A-A). The rate of E(A-A) formation observed in transient kinetic studies with the βE109D mutant (Figure 1F) is only 3-fold slower than that observed for the wild-type enzyme. Substitution of L-Ser with β-Cl-Ala decreases the rate of the wild-type-catalyzed β-reaction by a factor of 4 (Table I). However, utilization of β-Cl-Ala in place of L-Ser in reactions catalyzed by the βE109D mutant produces only minor changes in steady reactivity (Table I). Therefore, the hydroxyl or chloride β-substituent, which must be eliminated in order to produce E(A-A), has only a minor influence on the course of the β-reaction. Some other step occurring after E(A-A) formation has become rate-limiting for the β-reaction catalyzed by the βE109D mutant.

The effects on the steady-state catalytic rate caused by the mutation are mirrored in the RSSF spectra shown in Figure 2C. Under reaction conditions where the rate of quinonoid formation is nearly 200 s^{-1} for the wild-type enzyme (Lane & Kirschner, 1983b; Dunn et al., 1990), no quinonoidal or subsequent reaction intermediates are detectable for the β-reaction catalyzed by the βE109D mutant (compare Figure 2 panels A and C). Whereas the release of L-Trp is the rate-limiting step in the wild-type β-reaction (Lane & Kirschner, 1981, 1983b), the observed spectral changes suggest that the formation of a covalent bond between indole and the βE109D E(A-A) has become the rate-determining step. This conclusion is consistent with the recent findings of Anderson et al. (1991). Utilizing rapid quench techniques, they concluded that some step in the chemistry of the β-reaction has become rate-determining in the reaction catalyzed by the βE109D mutant as opposed to the rate-limiting release of L-Trp for the wild-type enzyme. The binding of GP to the α-subunit, a process that stabilizes quinonoidal species formed during the second stage of the β-reaction (Houben & Dunn, 1990), has no effect on the accumulation of intermediates (compare Figure 2 panels B and D).

The mutation appears to have decreased the apparent binding affinity of indole at the β active site. Direct assays for the synthesis of L-Trp in the βE109D-catalyzed αβ-reaction (see Materials and Methods) show that indole accumulates in solution. The accumulation of indole in solution is not observed for the αβ-reaction catalyzed by the wild-type enzyme (Yanofsky & Rachmeler, 1958). The mutation may affect the intrinsic binding affinity of the βE109D E(A-A) complex for indole. Alternatively, the inability to accumulate covalent reaction intermediates would significantly increase the observed K_m, and thus decrease the apparent affinity, of indole in the β-reaction. In either case, indole binding to the βE109D E(A-A) complex appears to be very rapid. The small spectral changes induced by indole were found to be complete within the mixing dead time of the RSSF instrument. Indole has been shown to cause very rapid spectral changes in the wild-type E(A-A) spectrum prior to covalent bond formation and the accumulation of quinonoidal intermediates [$E(Q_2)$ and/or $E(Q_3)$] (Figure 2A, spectrum 1; Drewe & Dunn, 1986). These spectral changes are thought to be the result of a rapid perturbation in the equilibrium concentrations of the $\alpha_2\beta_2$–serine mixture, increasing the amount of reactive E(A-A) (Drewe & Dunn, 1986). BZI, a nonreactive structural analogue of indole (Roy et al., 1988a), binds tightly to the $\alpha_2\beta_2$–serine complex and causes a redistribution of bound intermediates at the β-site (Roy et al., 1988a; Houben & Dunn, 1990). The βE109D E(A-A) complex exhibits the same behavior. The final spectrum obtained when BZI is present at saturating concentrations (data not shown) is similar to the characteristic E(A-A) spectrum of the wild-type enzyme (see Figure 1A, spectrum 10).

In contrast to the βE109D-catalyzed β-reaction with indole, DIT (reaction 4) is synthesized 5 times faster by the βE109D enzyme than by the wild-type enzyme. The spectral changes and pre-steady-state kinetic behavior (both in the absence and presence of GP) are very similar to those observed for the wild-type enzyme. Binding studies (Kayastha & Miles, 1990, 1991) indicate that indoline binds more tightly to the βE109D E(A-A) complex than to the wild-type E(A-A), particularly in the presence of GP [$K_d(\text{wild-type})_{APP}$ + GP = 1.0 mM; $K_d(\beta E109D)_{APP}$ + GP = 0.12 mM]. These values represent apparent dissociation constants since indoline forms a covalent bond when bound to the enzyme–E(A-A) complex. The mutation has altered some aspect of the β active site that significantly increases the stability of the indoline quinonoid.

Specificity for the indoline ring system versus indole is also observed for the reactions of the product amino acids, L-Trp and DIT, with the wild-type and mutant enzymes. DIT rapidly forms a quinonoid when bound to βE109D. The addition of GP causes a 6-fold increase in the amount of quinonoid present.[3] However, binding of DIT to the wild-type enzyme results in a very different distribution of covalent intermediates. GP induces a small amount of quinonoid formation, but the predominant spectral feature is a broad absorbance band centered at 350 nm. This band may represent an E(GD) intermediate (Scheme I), an enolimine Schiff base between DIT and the PLP cofactor, or an equilibrating mixture of both (Metzler, 1979; Kallen et al., 1985; Houben et al., 1989).

The wild-type and mutant enzymes also show large differences in the equilibrium distribution of intermediates when L-Trp is bound. In the presence of GP, the spectrum of the wild-type L-Trp complex is characterized by three predominant spectral bands, one species absorbing at 330 nm, a quinonoid band [$E(Q_3)$] at 476 nm, and a third band at 425 nm (Figure 4A). Even though the βE109D mutant rapidly forms a quinonoid when DIT is bound, no quinonoid is observed to accumulate when L-Trp is bound to the βE109D enzyme, even in the presence of GP. The predominant species at equilibrium is an intermediate absorbing at 330 nm (Figure 4B, spectrum c). The spectral characteristics of the 330-nm species are consistent with the accumulation of a geminal diamine intermediate [$E(GD_2)$], although the enolimine tautomer of the external aldimine formed between L-Trp and the cofactor may also absorb in this region of the spectrum (Metzler, 1979; Tobias & Kallen, 1975). In these reactions, quinonoid formation from L-Trp is predicated on the removal of a proton from the α-carbon of the amino acid substrate. The βE109D enzyme has relatively little trouble removing the corresponding proton from L-Ser during reaction to form E(A-A) (Figure 1D,E) or in the reaction to form a quinonoid from DIT (Figure 4D). However, ^1H NMR experiments show no detectable βE109D-catalyzed α-proton exchange in L-Trp (Figure 5). These observations are consistent with the idea that quinonoid

[3] The addition of GP to the equilibrium mixture affects only the relative amounts of quinonoid present, not the position of the spectral bands. This finding is consistent with the idea that GP binding to the α-subunit alters the relative distribution of species by stabilizing specific preexisting conformations of the β-subunit which bind structurally different intermediates (Houben & Dunn, 1990).

formation has become the rate-limiting step for the βE109D-catalyzed β-reaction with indole (see above).

Origins of Substrate Specificity. Although substitution of Asp for Glu is generally considered a conservative replacement, removal of a methylene group would shift the position of the carboxylate by approximately 1.5 Å and alter the orientation of the carboxylate within the β active site. This substitution may affect either the electrostatic environment and/or the geometry of the β active site. A change in environment is demonstrated by the observed shift in the position of the absorbance maxima of the βE109D quinonoid bands relative to that of wild-type enzyme (Figure 6). This shift need not necessarily imply a global change throughout the entire active site region. The peak positions and band shapes of intermediates observed during the reaction of L-Ser appear to be unaffected. Instead, the spectral shifts may simply indicate a change in the environment surrounding the bound nucleophile in the quinonoid complex. Though separated by a methylene group from the highly conjugated π-system, the λ_{max} of quinonoid absorption bands are very sensitive to the structure of the β-substituent, ranging from 450 nm (CN^-) (Brzovic´ and Dunn, unpublished results) to 464 nm (β-mercaptoethanol; Goldberg & Baldwin, 1967) to 476 nm (indole; Drewe & Dunn, 1986) and 494 nm (2,3-dihydro-L-Trp; Roy et al., 1988b; Phillips et al., 1984). Thus, the effect of the mutation could be localized to the nucleophile-binding region of the β active site. This suggestion is consistent with the hypothesis that Glu 109 forms a part of the indole-binding site (Hyde & Miles, 1990).

As described above, the most striking effect of the βE109D mutation, as shown in Figures 2, 4, and 5, is the destabilization of the L-Trp quinonoidal intermediate [$E(Q_2)$ or $E(Q_3)$, Scheme I]. Significant destabilization of this species, according to the Hammond postulate (Jencks, 1969), would also alter the structure of the transition states for both the formation and breakdown of the L-Trp quinonoid. On the other hand, the DIT quinonoid is stabilized by the βE109D mutation and is readily formed in both the forward (Figure 3D,E) and reverse (Figure 4D) reactions. The enhanced stability of the DIT quinonoid indicates that a step occurring after quinonoid formation is rate-determining in the βE109D-catalyzed synthesis of DIT. Thus, the relative changes in nucleophile specificity caused by the mutation are manifest in different rate-determining steps in the reaction mechanism of the βE109D-catalyzed synthesis of L-Trp and DIT. One possible structural explanation for the changes in substrate specificity involves the enzymatic activation of indole as a nucleophile. The β-subunit of tryptophan synthase may activate indole as a nucleophile by facilitating the formation of the indole iminium tautomer, a species with significant electron density on the C 3-carbon of indole (Walsh, 1979; Phillips et al., 1984). This mechanism predicts the development of positive charge on the ring nitrogen of indole in the transition state and in the immediate product $E(Q_2)$. Therefore, electrostatic interactions, which include hydrogen-bonding interactions, would be of fundamental importance in the catalysis of L-Trp synthesis. These interactions are depicted in Scheme II (structure I). The electrostatic force between two charged particles is inversely related to the distance between them. If Glu 109 is directly involved in the stabilization of the iminium tautomer of indole, then changing the distance between charges could seriously affect the ability of the enzyme to activate indole as a nucleophile. Furthermore, it is conceivable that Asp may form interactions within the active site that are unavailable to Glu, thus causing further distortions of the electrostatic environment within the β active site. Indoline, a much better nucleophile than indole, does not require the same type of electrostatic activation in order to react and form a covalent bond with the electrophilic E(A-A). Thus, the mutation would not affect the ability of indoline to react as a nucleophile.

The replacement of Glu by Asp at position 109 also could alter the structure of the β-subunit nucleophile-binding site, leading either to the nonproductive binding of indole or to a change in the orientation of indole when bound in the β active site. Stereoelectronic control of the nucleophilic addition reaction is an important feature of tryptophan synthase catalysis (Drewe & Dunn, 1986; Roy et al., 1988a). Changes in the active site which affect the position (or orientation) of bound indole with respect to the E(A-A) π-system would affect the rate of covalent bond formation.

The mutation βE109D introduces structural changes within the β-subunit active site which favor the binding of indoline and facilitate the enzymatic synthesis of DIT. The origins of this difference may be the inherent structural difference between the indole- and indoline-derived quinonoids. The nucleophilic and hydrogen-bonding properties of the indoline nitrogen may form hydrogen-bonding or ion pair interactions with active site residues, significantly stabilizing the indoline quinonoid (Scheme II, structure II). Secondly, nucleophilic attack by indole on E(A-A) results in a quinonoid with tetrahedral geometry at the C-3 position of indole [$E(Q_2)$], which then is rapidly deprotonated to yield the enamine moiety with sp^2 hybridization at the C-3 carbon. Since this deprotonation step is not rate-determining for quinonoid formation (Lane & Kirschner, 1983b), the 476-nm quinonoid species which accumulates most likely represents the $E(Q_3)$ species, which is destabilized in the βE109D system. On the other hand, the nucleophilic atom of indoline is the ring nitrogen which occupies the position analogous to the C-3 carbon of indole. This nitrogen atom retains sp^3-like hybridization throughout the reaction sequence. In the wild-type enzyme, this structural feature may hinder quinonoid formation when the amino acid DIT reacts at the β active site (Figure 4C, spectra b and c) and, in the reverse reaction, may hinder the breakdown of the indoline quinonoid to products. It is interesting to note that 2,3-dihydro-L-Trp and oxyindolyl-L-Ala, both of which have sp^3 geometry at a position corresponding to the C-3 position of the indole ring in L-Trp, bind tightly to the *E. coli* enzyme primarily as the geminal diamine species (Roy et al., 1988b) and only form small amounts quinonoids.

An interesting, but unexplained, observation is the large disparity in the amount of indoline quinonoid formed depending on whether indoline reacts with E(A-A) or whether DIT is the substrate in the reactions catalyzed by the wild-type enzyme (compare Figure 3A,B with Figure 4C). In either case, the position of the quinonoid band is the same, and the structures (and the relative free energies) of the quinonoidal intermediates are assumed to be identical. Lane and Kirschner (1983b) have proposed that the PLP cofactor undergoes a reorientation within the β-site at the L-Trp quinonoid stage of the β-reaction. It is likely that the DIT quinonoid would have to undergo a similar rearrangement. Such a reorientation of the PLP cofactor would almost certainly be accompanied by a change in the conformation of the β active site. From this point of view, indoline, in the wild-type-catalyzed reaction, cannot easily trigger the reorientation of the cofactor. However, the βE109D mutation may facilitate this transition, or the mutation may have rendered such a conformational change unnecessary.

The preceding discussion has focused on the relative specificity of the wild-type and mutant enzymes for nucleophilic substrates. However, these data do not preclude a catalytic role for Glu 109 in the elimination of the β-hydroxyl from L-Ser. The altered equilibrium distribution of L-Ser-derived intermediates and the behavior of a second mutant at position 109 (data to be presented elsewhere) suggest that this residue also interacts with the β-hydroxyl group of L-Ser (Scheme II, structure III). The results presented herein emphasize the importance of Glu 109 for the structural integrity and, therefore, the reaction specificity of the β active site. A single conservative mutation affects nucleophile specificity and the stability of catalytic intermediates. Further characterization of this and other mutants at the β active site should provide significant new insights into the catalytic mechanism of tryptophan synthase.

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Artificial Pigments of Halorhodopsin and Their Chloride Pumping Activities

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ABSTRACT: Halorhodopsin (HR), the light-driven chloride pump of *Halobacterium halobium*, was bleached with hydroxylamine and regenerated with *all-trans*-retinal under several different conditions. The largest recovery of the pigment was found with apoprotein obtained from detergent-free HR [HR(BB)]. To compare the chloride-pumping mechanism of HR with that of bacteriorhodopsin (BR; the light-driven proton pump of the same bacteria), HR pigment analogues were reconstituted with the bleached HR(BB) and retinal analogues. The corresponding BR pigment analogues have previously been shown to have little or no proton-pumping activity, except for retinal$_2$ (3,4-dehydroretinal). Pigment analogues with 13-demethylretinal or retinal$_2$ showed an "opsin shift" similar to that of the *all-trans*-retinal pigment of both HR and BR. Opsin shifts of the pigments of 9-12-phenylretinal and 3,7-dimethyl-2,4,6,8-decatetraenal and haloopsin are slightly different from those of the corresponding BR pigment analogues, presumably reflecting differences of the chromophoric structures in HR and BR. In addition to the spectral properties, the effect of chloride ion on deprotonation of the Schiff base was measured. These pigment analogues showed the "chloride effect" (a shift of the pK value for deprotonation of the Schiff base), but a smaller one than that seen in HR. For a measurement of the chloride-pumping activity, each retinal analogue was added to a culture of L07 cells (BOP^-, HOP^+, Ret^-), and the activity was measured with the cell suspension. Only cultures with retinal or retinal$_2$ showed chloride-pumping activity, as is true for proton pumping by BR. This suggests that a similar retinal–protein interaction is necessary for both ion pumps.

Halorhodopsin (HR)[1] is a light-driven chloride pump found in the cytoplasmic membrane of *Halobacterium halobium* (Lanyi, 1986; Oesterhelt & Tittor, 1989). Like bacteriorhodopsin (BR), the light-driven proton pump found in the purple membrane of the same bacteria, HR has a retinal bound to a Lys residue via a protonated Schiff base as a chromophore. HR has been purified by several groups (Steiner & Oesterhelt, 1983; Taylor et al., 1983; Ogurusu et al., 1984; Sugiyama & Mukohata, 1984). The amino acid sequence has been deduced from the nucleotide analysis of the haloopsin gene (Blanck & Oesterhelt, 1987). From comparison of the primary sequences of both light-driven pumps, about 35% of the total amino acids seem to be similar in the putative membrane domain. This should imply a similar mechanism for converting light energy into chemical energy.

Recently, several single amino acid mutants of BR were made in an *Escherichia coli* expression system and were investigated by Khorana's group (Khorana, 1988). By the mutagenesis of *Halobacterium* GRB strain, several mutants defective in the proton-pumping activity were obtained (Soppa & Oesterhelt, 1989). Both studies indicate the importance of Asp85 and Asp96 in the proton-pumping mechanism (Mogi et al., 1988; Butt et al., 1989). Henderson et al. (1990) reported the structural model for BR based on high-resolution cryomicroscopy, which shows seven membrane-spanning α-helices, bulky aromatic side chains, and the β-ionone ring of retinal. Thus, it is possible to assign a role to certain amino acids in the proton-pumping mechanism and in chromophoric structure on the basis of the molecular structure. In the case of HR, however, such mutants have not been reported yet. The naturally occurring alternative of HR was found in *Natoronobacterium pharaonis*, an alkaline-halophilic bacterium (Bivin & Stoeckenius, 1986) whose gene has been cloned and

[1] Abbreviations: HR, halorhodopsin; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; BR, bacteriorhodopsin; BOP, bacterioopsin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HOP, haloopsin; HR(OG), halorhodopsin solubilized in a 1% octyl glucoside solution; HR(BB), halorhodopsin incubated with Biobeads SM2; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ret, retinal; SOP, sensoryopsin; SR, sensoryrhodopsin; λ_{max}, the wavelength of the maximum absorbance.

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