Articles

L-Serine Analogues Form Schiff Base and Quinonoidal Intermediates with *Escherichia coli* Tryptophan Synthase†

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ABSTRACT: Substrate analogues of L-serine have been found that react with the $\alpha_2\beta_2$ complex of *Escherichia coli* tryptophan synthase. Upon reaction with $\alpha_2\beta_2$, the analogues glycine, L-histidine, L-alanine, and D-histidine form chemical intermediates derived from reaction with enzyme-bound pyridoxal 5′-phosphate with characteristic UV–visible spectral bands. The spectra of the products of the glycine, L-histidine, and L-alanine reactions with $\alpha_2\beta_2$ contain contributions from the external aldimine, the quinonoid species, and other intermediates along the catalytic pathway. Just as previously reported for the reaction of L-serine with β_2 [Goldberg, M. E., York, S., & Stryer, L. (1968) *Biochemistry 7*, 3662–3667], the reactions of glycine, L-histidine, and L-alanine with the β_2 form of tryptophan synthase yield spectra with no contributions from catalytic intermediates beyond the external aldimine. The kinetics of intermediate formation and comparisons of the time courses for the exchange of α-^{1}H for solvent ^{2}H catalyzed by $\alpha_2\beta_2$ or β_2 were found to be consistent with these assignments. Intermediates further along the tryptophan synthase catalytic pathway are stabilized to a greater degree in the $\alpha_2\beta_2$ complex than in the β_2 species alone. This observation strongly suggests that the association of α and β subunits to form the native $\alpha_2\beta_2$ species lowers the activation energies for the interconversion of the external aldimine with chemical species further along the catalytic path. The spectral changes that accompany the reaction of D-histidine with $\alpha_2\beta_2$ indicate that this ligand forms an external aldimine ($\lambda \simeq 412$ nm) and one or more species absorbing in the 330-nm range. No evidence was found for the formation of a D-histidine quinonoid. Isotope exchange studies showed that the $\alpha_2\beta_2$-catalyzed rate of exchange of α-^{1}H is too slow to be measured.

The enzyme tryptophan synthase from *Escherichia coli* is a bienzyme complex that catalyzes the synthesis of L-tryptophan (L-Trp)[1] from 3-indole-D-glycerol 3′-phosphate (IGP) and L-serine (L-Ser) (Yanofsky & Crawford, 1972; Miles, 1979; Miles et al., 1987). The enzyme complex has an $\alpha_2\beta_2$ quarternary structure that can be assembled from the α subunit and the tightly associated β_2 dimer, yielding a complex that is indistinguishable from the native enzyme. Neither the α subunit nor the β_2 dimer is capable of catalyzing the complete physiological reaction:

$$\text{3-indole-D-glycerol 3′-phosphate} + \text{L-serine} \xrightleftharpoons{\alpha_2\beta_2} \text{L-tryptophan} + \text{D-glyceraldehyde 3-phosphate} \quad (1)$$

The α component contains the active site for the reversible aldolysis of IGP to indole and D-glyceraldehyde 3-phosphate (eq 2), and the purified species exists as a monomer (α) with

$$\text{3-indole-D-glycerol 3′-phosphate} \xrightleftharpoons{\alpha \text{ or } \alpha_2\beta_2} \text{indole} + \text{D-glyceraldehyde 3-phosphate} \quad (2)$$

a molecular weight of 29,000. The purified β component exists as a dimer (β_2) with a molecular weight of 90 000. The β_2 species catalyzes the practically irreversible condensation of indole with L-Ser to form L-Trp (eq 3) (Yanofsky & Crawford,

$$\text{indole} + \text{L-serine} \xrightarrow{\beta_2 \text{ or } \alpha_2\beta_2} \text{L-tryptophan} + H_2O \quad (3)$$

1972; Ahmed et al., 1986; Miles et al., 1987). Each β subunit contains one molecule of bound pyridoxal phosphate (PLP). The UV–visible spectral properties of PLP provide a sensitive probe for detecting chemical intermediates along the catalytic pathway. The turnover numbers for the reactions shown in eq 2 and 3 increase dramatically (100- and 50-fold, respectively) when the α and β subunits are associated into the native $\alpha_2\beta_2$ complex (Yanofsky & Crawford, 1972).

The partial reaction of L-Ser with the $\alpha_2\beta_2$ complex has been studied by several investigators (Faeder & Hammes, 1970, 1971; Lane & Kirschner, 1983a; Drewe & Dunn, 1985). The work of Drewe and Dunn (1985), which employed rapid-scanning stopped-flow (RSSF) UV–visible spectroscopy, showed that the reaction of L-Ser with $\alpha_2\beta_2$ rapidly forms two transient species identified as the external aldimine and the corresponding quinonoid. Scheme I extends the set of structures considered by Drewe and Dunn 1985) to include relevant tautomeric and ionization states of the internal and external

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; L-His, L-histidine; Gly, Glycine; D-His, D-histidine; L-Ala, L-alanine; L-Ser, L-serine; L-Trp, L-tryptophan; $\alpha_2\beta_2$ and β_2, native and β_2 forms of *E. coli* tryptophan synthase (EC 4.2.1.20), respectively; E(Ain-E) and E(Ain-K), internal ϵ-imine lysyl aldimine enolimine and ketoenamine tautomers of enzyme-bound PLP; E(GD), *gem*-diamine tetrahedral intermediates formed in the reactions of analogues with the internal aldimine form of enzyme-bound PLP; E(Aex-K) and E(Aex-E), external aldimine ketoenamine and enolimine tautomers formed with analogues; E(ex), external aldimine; A-A, α-aminoacrylate; E(Q), enzyme-bound quinonoid species formed with analogues; EDTA, ethylenediaminetetraacetate; RSSF, rapid-scanning stopped flow.

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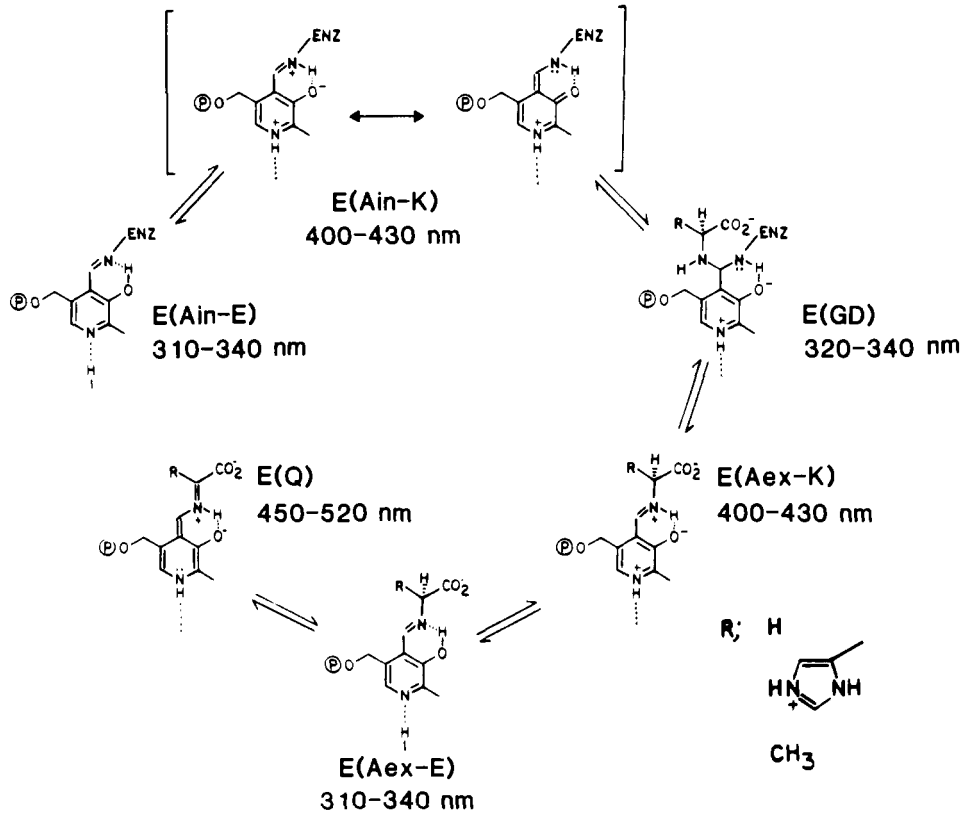
Scheme I: Organic Structures Showing the Expected Tautomeric States and Ionization States and the Expected Positions of UV–Visible Absorption Bands for the Species Formed in the Reactions of the L-Ser Analogues L-His, L-Ala, and Gly with PLP Bound to the β Subunits of Tryptophan Synthase[a]



[a] These analogues are chemically incapable of progressing beyond the quinonoid intermediate, E(Q). The structures shown are E(Ain-E) and E(Ain-K), the internal aldimine enolimine and ketoenamine tautomers, respectively; E(GD), the *gem*-diamine formed by reaction of the internal aldimine with L-Ser analogues; E(Aex-K) and E(Aex-E), the external aldimine ketoenamine and enolimine tautomers; and E(Q), the quinonoid.

aldimines and other probable intermediates along the pathway for the partial reactions of L-Ser and L-Ser analogues with the PLP moiety of $\alpha_2\beta_2$. In the L-Ser reaction, these species decay to a mixture composed primarily of the α-aminoacrylate Schiff base intermediate, whereas for the analogues presented in this study, reaction stops with the formation of the quinonoid intermediate.

Several amino acid analogues of L-Ser are known to react with the PLP moiety of the $\alpha_2\beta_2$ complex [see Crawford and Ito (1964), Miles (1978), Esaki et al. (1983), Kumagai and Miles (1971), Ahmed et al. (1986), Phillips et al. (1984), Miles et al. (1986), and Roy et al. (1988)]. L-Trp reacts with the $\alpha_2\beta_2$ complex to form a species with λ_{max} = 476 nm. This band has been ascribed to a quinonoid (an α-carbanionic intermediate) derived from the removal of the α-hydrogen of the corresponding external aldimine formed with the PLP moiety (Lane & Kirschner, 1981; Miles et al., 1986). The reactions of oxindolyl-L-alanine and 2,3-dihydro-L-Trp with $\alpha_2\beta_2$ yield species with absorption bands at 480 and 494 nm, respectively. These bands also have been ascribed to quinonoid intermediates (Phillips et al., 1984; Roy et al., 1988). These two compounds have strong affinities for tryptophan synthase with reported half-saturation values of 5 and 25 μM for oxindolyl-L-alanine and (3*S*)-2,3-dihydro-L-Trp, respectively. The L isomers of 5-fluoro-L-Trp and of (3*S*)-2,3-dihydro-5-fluorotryptophan form absorption bands with tryptophan synthase similar to those of the corresponding compounds lacking the 5-fluoro group (Miles et al., 1986). In the same study, Miles et al. found that the D and L isomers of these analogues and of tryptophan slowly interconvert via epimerization. The compound 6-azido-L-Trp has been produced enzymatically from 6-azidoindole and L-Ser (Miles & Phillips, 1985). This product also forms a quinonoid intermediate with spectroscopic properties essentially identical with those formed with L-Trp (λ_{max} = 476 nm).

This paper is the first of a series reporting the results of investigations with the L-Ser analogues glycine (Gly), L-histidine (L-His), D-histidine (D-His), and L-alanine (L-Ala). Herein, we show that these analogues react with the PLP chromophore of tryptophan synthase to yield equilibrating mixtures of the species depicted in Scheme I. Via titration, isotope exchange, rapid kinetic experiments, and a thorough analysis of spectra, these studies establish that the distribution of species formed at equilibrium depends both on the structure of the amino acid side chain and on α–β subunit interactions. This work forms the basis for manuscripts now in preparation (K. F. Houben and M. F. Dunn) wherein it will be shown that the distribution of intermediates formed at the β-site is modulated by the binding of effectors to tryptophan synthase.

Materials and Methods

Purification of $\alpha_2\beta_2$ *E. coli* tryptophan synthase and the β_2 dimer, determination of protein concentrations, and measurement of enzymatic activity have been described previously (Drewe & Dunn, 1985; Adachi et al., 1974; Miles & Moriguchi, 1977; Tschopp & Kirschner, 1980a). Reagent grade chemicals were purchased from Sigma and were used without further purification.

Static UV–Visible and Titration Measurements. All static UV–visible absorption spectra and spectrophotometric titration studies were carried out with a Hewlett-Packard 8450A spectrophotometer. The titrations were performed such that the concentration of all but one of the constituents of the

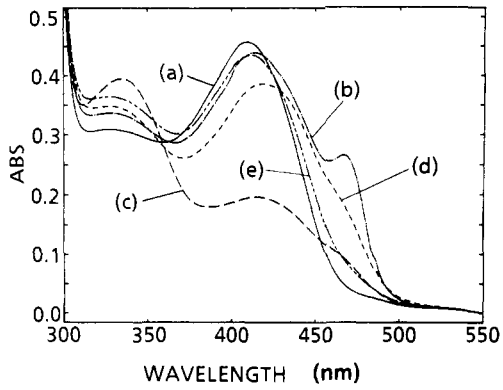


FIGURE 1: Absorption spectra of 38.5 μM $\alpha_2\beta_2$ in the presence of (a) no ligands (—), (b) 200 mM L-His (–·–), (c) 750 mM Gly (– –), (d) 750 mM L-Ala (– – –), and (e) 200 mM D-His (– – – –) in 0.05 M Bicine buffer and 1 mM EDTA, pH 7.8, 25 °C.

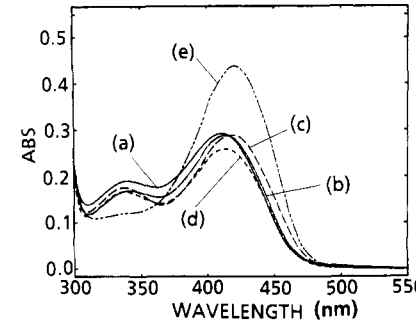


FIGURE 2: Absorption spectra of 23.5 μM β_2 in the presence of (a) no ligands (—), (b) 220 mM L-His (–·–), (c) 1.25 M Gly (– –), (d) 833 mM L-Ala (– – –), and (e) 24 mM L-Ser (– – – –) in 0.1 M potassium phosphate buffer and 1 mM EDTA, pH 7.8, 25 °C.

solution were kept constant. For example, a cuvette containing a predetermined concentration of enzyme and buffer at pH 7.8 was monitored spectroscopically while a second solution containing the same concentration of enzyme and buffer, but also the substrate analogue molecule, was added titrimetrically. All of the reported titration results were found to be satisfactorily fitted by the equation for a single hyperbolic isotherm as described by Dunn et al. (1979). The absorption spectrum shown in Figure 6 was corrected for 100% L-His saturation with $\alpha_2\beta_2$ (Houben, 1988) in order that the spectrum could be more easily fit to log-normal distribution curves.

Rapid-Scanning Stopped-Flow (RSSF) Spectrometry. The RSSF instrumentation used in this study is identical with that used in previous studies (Drewe & Dunn, 1985, 1986) except a later version of the multichannel analyzer is used. For spectra in Figure 3, the collection of the first scan relative to flow cessation is noted in the figure legends. The repetitive scan rate is 8.98 ms. The second through fourteenth scans in Figure 3A were collected at the following intervals after the first: (2) 8.9, (3) 17.8, (4) 26.7, (5) 35.6, (6) 44.5, (7) 53.4, (8) 62.3, (9) 80.1, (10), 97.9, (11) 133.5, (12) 160.2, (13) 195.8, and (14) 240.3 ms. The second through fourteenth scans in Figure 3B were collected at the following intervals after the first: (2) 8.9, (3) 17.8, (4) 26.7, (5) 35.6, (6) 44.5, (7) 53.4, (8) 62.3, (9) 80.1, (10) 97.9, (11) 106.8, (12) 195.8, and (13) 329.3 ms.

^{1}H FT NMR Spectroscopy. A Nicolet NT 300 spectrometer was used to obtain NMR spectra. The kinetic runs were performed in 0.1 M deuterated sodium phosphate buffer at pH* = 7.8 and 25 ± 1 °C. The concentrations of $\alpha_2\beta_2$ used were 125 and 25 μM, respectively, for panels A and B of Figure 5, and 100 mM substrate was used in both cases. A one-pulse sequence was used with a tip angle ~33° and a delay of 3 s. Kinetic runs were spaced as shown in Figure 5.

RESULTS

Reaction of $\alpha_2\beta_2$ and β_2 with Gly, L-His, D-His, and L-Ala. The internal aldimine form of PLP bound to the β subunits of the $\alpha_2\beta_2$ complex is characterized by at least two absorption bands: a prominent band with λ_{max} at 410 nm and a higher energy band with λ_{max} at approximately 330 nm (Figure 1, spectrum a). The spectral changes that take place when Gly (spectrum c), L-His (spectrum b), D-His (spectrum e), and L-Ala (spectrum d) react with the $\alpha_2\beta_2$ complex are also presented in Figure 1. Upon reaction with $\alpha_2\beta_2$, all of these substrate analogues (spectra b–e) undergo decreases in absorbance in the 400-nm region and bathochromic shifts in λ_{max}, whereas the 330- and 470-nm regions show significant increases in absorbance. The complex with L-His shows distinctive new bands with λ_{max} of 468, 413, and ~330 nm (spectrum b, Figure 1). In comparison to native $\alpha_2\beta_2$ (spectrum a), the 410-nm region of the Gly complex (spectrum c) is partially bleached and there is increased absorbance in the 340-nm region. The new bands formed with Gly exhibit apparent λ_{max} of ~460 (a shoulder), ~410, and ~333 nm. L-Ala also shows a slight bleaching of the spectrum in the 410-nm region (spectrum d). The new bands formed with L-Ala have apparent λ_{max} of ~465, ~416, and ~330 mn. D-His (spectrum e) gives new bands located at ~412 and ~330 mn. There is no indication of any new bands at longer wavelengths.

As is evident in the spectra shown in Figure 2, analogues Gly (spectrum c), L-His (spectrum b), and L-Ala (spectrum d) also react with the β_2 dimer of tryptophan synthase. These spectra are compared in Figure 2 with the spectrum of β_2 before reaction with these compounds (spectrum a) and with the spectrum of the external aldimine formed in the reaction of β_2 with L-Ser (spectrum e) (Goldberg et al., 1968).

The amino acids L-phenylalanine, L-lysine, L-aspartate, and L-glutamate also give evidence of reacting with the $\alpha_2\beta_2$ complex at high concentrations (data not shown) though these spectral changes are quite small.

Rapid-Scanning Stopped-Flow Studies. The time-resolved absorbance changes that occur in the reaction of $\alpha_2\beta_2$ with Gly and with L-His are shown in Figure 3. When the $\alpha_2\beta_2$ complex reacts with Gly (Figure 3A), the 410-nm band of the internal aldimine disappears in two phases with apparent first-order rate constants of 18 and 6.5 s^{-1} (viz. the single-wavelength time course shown in inset a) and is replaced by a broad, red-shifted absorption band with apparent λ_{max} at 412 nm and with a shoulder in the 460-nm region. The concentration dependencies of these relaxations indicate they are coupled to the Gly binding step (data not shown). The appearance of the 460-nm band is also biphasic. A second band with λ_{max} at 335 nm is clearly formed. When the $\alpha_2\beta_2$ complex reacts with L-His (Figure 3B), the major spectral change is the appearance of a band formed in two kinetic phases at 468 nm with apparent first-order rate constants of 36 and 17 s^{-1}. However, there are also small decreases in absorbance in the 330- and 410-nm regions and a slight bathochromic shift from the original λ_{max} at 410 nm to 413 nm. There are no isosbestic points present in the Gly and L-His reactions with $\alpha_2\beta_2$.

Detailed single-wavelength kinetic studies (data not shown) characterize the kinetic phases of the L-His time courses as essentially zero order with respect to the concentration of L-His. Kinetic studies (analogous to those shown in the insets to Figure 3) were carried out in 100 mM phosphate buffer, pH 7.8, to investigate kinetic isotope effects. α-^{2}H-substituted

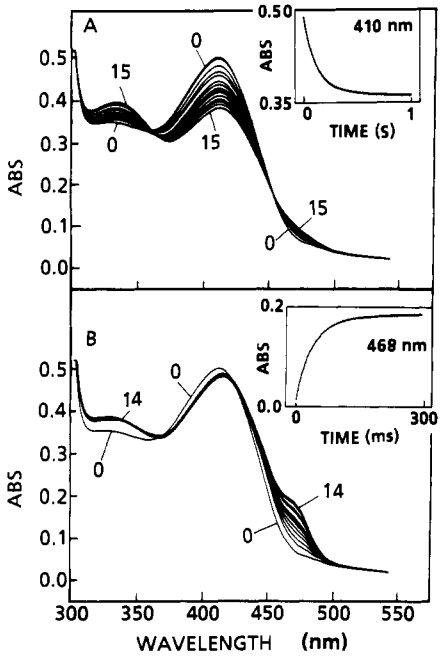


FIGURE 3: Rapid-scanning stopped-flow spectra showing the spectral changes for the reactions of $\alpha_2\beta_2$ with (A) Gly and (B) L-His. The insets are the single-wavelength time courses monitored at (A) 410 nm and (B) 468 nm. The initiation of scanning in (A) and (B) occurred ~1 ms after flow stopped. The traces designated 0 are the reconstructed spectra of the reactants. See Materials and Methods for the timing sequence. Conditions after mixing: (A) 40 μM $\alpha_2\beta_2$ and 50 mM Gly; (B) 40 μM $\alpha_2\beta_2$ and 134 mM L-His. Each experiment was carried out in 0.05 M Bicine-HCl buffer and 1 mM EDTA, pH 7.8, at 25 °C. Assuming a biphasic time course, the best fits of the data give the following values: (A) $A_1 = 0.095$, $1/\tau_1 = 18\ s^{-1}$, $A_2 = 0.030$, $1/\tau_2 = 6.5\ s^{-1}$; (B) $A_1 = 0.09$, $1/\tau_1 = 36\ s^{-1}$, $A_2 = 0.08$, $1/\tau_2 = 17\ s^{-1}$.

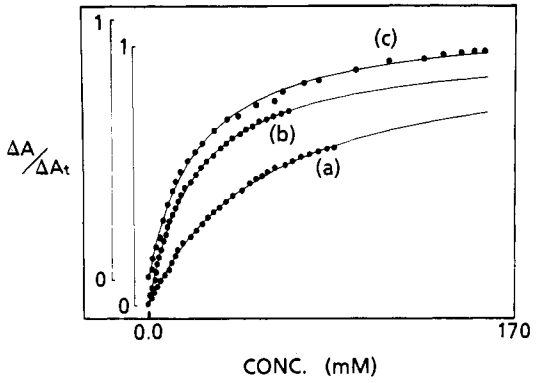


FIGURE 4: Titration data of ligand binding to $\alpha_2\beta_2$ by measuring the change in absorption (a) at 454 nm upon addition of D-His, (b) at 468 nm upon addition of L-His, and (c) at 410 nm upon addition of Gly. [Note: The data in (c) is offset to correspond to the scale at the far left.] Conditions were similar to those in Figure 1. The best-fit K values assuming eq 4 are (a) D-His, 54 mM, (b) L-His, 22 mM, and (c) Gly, 23 mM.

Gly with the isotopically normal compound showed no kinetic isotope effect.

Titration Studies. Titrations were carried out as described under Materials and Methods. Titration of $\alpha_2\beta_2$ with Gly (Figure 4a), L-His (Figure 4b), and D-His (Figure 4c) produces hyperbolic saturation curves that are satisfactorily fitted by the Langmuir isotherm:

$$\Delta A = \frac{\Delta A_T[S]}{K + [S]} \quad (4)$$

where ΔA is the observed change in absorbance at a given concentration of free substrate [S], ΔA_T is the change in absorbance at saturation, and K is the concentration of free ligand that gives $\Delta A_T/2$. The computer analysis best-fit K values are 23, 22, and 54 mM, respectively, for the reactions of Gly, L-His, or D-His with the $\alpha_2\beta_2$ complex.[2]

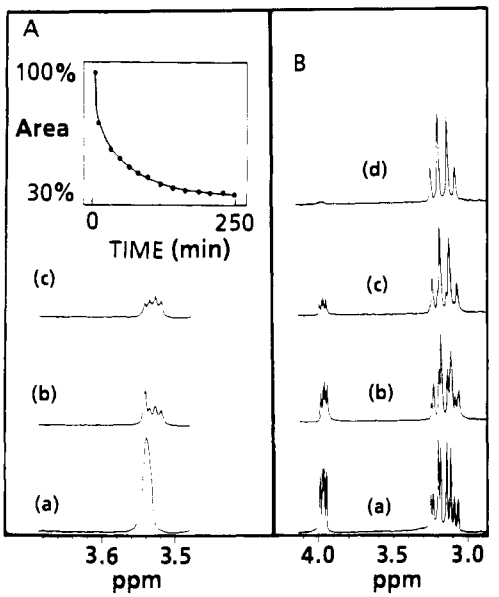


FIGURE 5: NMR spectra of the reaction of 100 μM $\alpha_2\beta_2$ with (A) Gly at (a) 0, (b) 30, and (c) 90 min and (B) L-His at (a) 0, (b) 30, (c) 90, and (d) 240 min. Inset in (A) is the integrated area of the Gly α-H resonance plotted versus time. The time course in the inset gives values of $k_1 = 1.6 \times 10^{-3}\ s^{-1}$ and $k_2 = 4.1 \times 10^{-5}\ s^{-1}$ when fitted to the equation $A_t = A_\infty + A_1 \exp(t/k_1) + A_2 \exp(t/k_2)$ (where A_t is the area at time t, A_∞ is the area at infinite time, and A_1 and A_2 are the amplitudes associated with k_1 and k_2, respectively).

Nuclear Magnetic Resonance Experiments. Nuclear magnetic resonance experiments show that the α protons of both Gly and L-His are abstracted during reaction with either the $\alpha_2\beta_2$ complex or the β_2 dimer. In the Gly reaction with $\alpha_2\beta_2$ in D_2O (Figure 5A), conversion of the singlet resonance to a triplet with half of the area of the singlet occurs as the C-α proton disappears. The triplet is shifted slightly upfield relative to the singlet. These findings provide conclusive evidence of a stereoselective replacement of one of the two α protons by deuterium. The splitting of the remaining C-α proton by the adjacent deuterium accounts for the triplet, while the slight upfield shift is a deuterium isotope effect on the remaining C-α proton. If exchange were completely stereospecific, then the decrease in area (viz., insert in Figure 5A) should approach 50% at $t = \infty$. Since the integrated areas of the resonances become significantly less than 50% of the initial integrated resonance area, it is clear that exchange of both α protons occurs, but the replacement is stereoselective for one of the C-α protons. The observed biphasic rates ($k_1 \sim 1.6 \times 10^{-3}\ s^{-1}$ and $k_2 \sim 4.1 \times 10^{-5}\ s^{-1}$) show there is considerable preference for the abstraction of one C-α proton over the other.

The time-dependent NMR spectra of the reaction of L-His and $\alpha_2\beta_2$ are shown in Figure 5B. The multiplet centered at 3.16 ppm is an AB quartet of the tightly coupled β protons split by the α proton. As the α proton is replaced by deuterium, the multiplet becomes a simple AB quartet. Initially

[2] We have discovered that phosphate ion is a strong allosteric effector that influences both the affinity and the equilibrium distribution of species formed in the reactions of amino acids with the $\alpha_2\beta_2$ complex (K.F. Houben and M.F. Dunn, unpublished results). Since these phosphate effects are complicated, the work described here is restricted to studies in Bicine, a buffer system that appears to exert no allosteric effects. However, because of our choice of buffers, the dissociation constants reported cannot be directly compared with values reported for other amino acids measured in phosphate buffer.

the α proton is split into a quartet by the two nonequivalent C-β protons. Over time, the α-proton resonance disappears due to the replacement by deuterium. These observations provide conclusive evidence that the α proton of L-His is abstracted and exchanged with solvent in a reversible reaction of L-His with $\alpha_2\beta_2$.

The relative efficiencies of α-^{1}H exchange for solvent ^{2}H catalyzed by $\alpha_2\beta_2$ and β_2 were compared for L-His and for Gly in experiments similar to those described in Figure 5. By use of enzyme solutions containing the same concentrations of β subunits (108–200 μM) and identical concentrations of either L-His (26.7 mM) or Gly (50 mM), it was found that the relative rate of exchange, $k(\alpha_2\beta_2)/k(\beta_2)$, for L-His is $\simeq$11 while for the faster phase of the Gly replacement reaction, it is $\sim$1.9. When incubated with 140 μM $\alpha_2\beta_2$, 35 mM D-His was found to undergo no detectable exchange of α-^{1}H for solvent ^{2}H during a 12-h period at 25 °C.

Discussion

Because, Gly, L-His, D-His, and L-Ala alter the UV-visible spectra of the PLP chromophore (Figures 1 and 3) and display saturable titration curves (Figure 4), it is reasonable to conclude that these molecules undergo covalent reaction with the PLP chromophore bound to the $\alpha_2\beta_2$ complex of tryptophan synthase. This is further substantiated by the finding that the C-α protons of Gly and L-His undergo a facile $\alpha_2\beta_2$-catalyzed exchange with D_2O. The titration data for three of the analogues (Figure 4), Gly, L-His, and D-His, are well described by the expression for a single hyperbolic isotherm (eq 4), indicating two identical and noncooperative sites. Assuming a site stoichiometry of 2.0, the best-fit values of K are 23, 22, and 54 mM, respectively, for Gly, L-His, and D-His. From these results, it is evident that Gly, L-His, and D-His have relatively weak affinities for the $\alpha_2\beta_2$ complex. The L and D isomers of Trp have reported K values of 125 μM and 15 μM, respectively (Lane & Kirschner, 1981).[2] Previous investigators have reported little or no reaction of L-Ala with the $\alpha_2\beta_2$ complex of tryptophan synthase (Goldberg et al., 1968; DeCastel & Goldberg, 1978). The *N*-phosphopyridoxal amino acid of L-Ala, *N*-phosphopyridoxal-L-alanine, also is reported not to bind to the $\alpha_2\beta_2$ complex (Tschopp & Kirschner, 1980a,b).

Correlation of Spectra with Structure. The organic structures and the expected positions of the absorption bands of internal and external aldimines and other intermediates detected in the reactions of L-Ser, L-His, Gly, and L-Ala are given in Scheme I. The positions of the spectral bands are largely dependent on the protonation states of the azomethine nitrogen, the phenolic oxygen, and the pyridine ring nitrogen and the number of double bonds in conjugation with the ring, while the phosphate protonation state is apparently of little consequence (Karube & Matsushima, 1977; Snell et al., 1968). Provided the extent of protonation of the azomethine nitrogen and/or phenolic oxygen is unchanged, it appears that protonation of the pyridine ring nitrogen produces only a small effect on the λ_{max} of the absorption spectra ($\Delta\lambda_{max} \simeq$ 5 mn) (Benecky et al., 1985; Karube et al., 1978; Metzler et al., 1980).

The PLP Schiff base structures believed to be relevant to tryptophan synthase catalysis are the ketoenamine [E(Ain-K) and E(Aex-K), λ_{max} 400–430 nm] and enolimine [E(Ain-E) and E(Aex-E), λ_{max} 310–340 nm] tautomers (Scheme I) (Martell, 1962; Inouye, 1967; Metzler et al., 1980; Karube & Matsushima, 1977; June et al., 1981; Kallen et al., 1985; Morozov, 1986; Matsushima & Martell, 1967; Karube et al., 1978). Hence, the tryptophan synthase absorption band with λ_{max} at $\simeq$410 nm is the E(Ain-K) tautomer, and the band with $\lambda_{max} \simeq$330 nm probably is the E(Ain-E) tautomer; however, adducts and *gem*-diamines with tetrahedral C-4′ atoms also are possibilities (see below). Absorption in the 350–380-nm region also may be due to minor amounts of species with either the azomethine nitrogen and phenolic oxygen protonated or unprotonated. We conclude that the E(Ain-K) and E(Ain-E) tautomers are the species that dominate the spectrum of the $\alpha_2\beta_2$ complex before reaction with substrate or analogue.

The initial chemical step in the catalytic mechanism of a PLP-dependent enzyme is transimination via a *gem*-diamine E(GD) intermediate with λ_{max} expected to be in the 320–340-nm region (see Scheme I) (Metzler, 1979; Ivanov & Karpeisky, 1969; Braunstein, 1985). Recently, Roy et al. (1988) have proposed that both oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan react with $\alpha_2\beta_2$ to form E(GD) species with $\lambda_{max} \simeq$340 and $\simeq$325 nm, respectively.

The absorption spectra of E(Q) species have unique characteristics; the long-wavelength bands are much narrower than other PLP absorption bands, and there is at least one shoulder located about 1200 cm^{-1} (27 nm) from the absorption maximum of the major peak on the high-energy side (Davis & Metzler, 1972). The extinction coefficients of PLP quinonoidal species are estimated to range from 28 000 to 40 000 M^{-1} cm^{-1} (Karube & Matsushima, 1977; Ulevitsch & Kallen, 1977). By use of computer modeling (Roy et al., 1988), an extinction coefficient of 37 000 M^{-1} cm^{-1} has been estimated for the $\alpha_2\beta_2$ dihydrotryptophan quinonoid.

The substrate analogues Gly, L-His, D-His, and L-Ala are unable to form intermediates with $\alpha_2\beta_2$ or β_2 beyond the E(Q). Gly lacks the β-carbon of L-serine and is thus unable to form an α-aminoacrylate intermediate, while elimination of hydride ion from the L-Ala quinonoid with PLP is chemically improbable. Although L-Trp can be cleaved to indole, pyruvate, and ammonia by tryptophan synthase (Ahmed et al., 1986), the rate of this reaction is practically undetectable. It is chemically unlikely that cleavage of the bond between C-β and the ring occurs with either D- or L-His. Indeed, the spectra of the complex of L-His with $\alpha_2\beta_2$ remains essentially unchanged over a 3-day period, a finding consistent with the absence of any significant slow reactions, and the NMR data (Figure 5B) give no evidence of C–C bond cleavage.

Interpretation of the Spectral Changes in the Reaction of L-His with $\alpha_2\beta_2$. A priori, the species with λ_{max} 413 nm could be either the Michaelis complex in which L-His is bound to the internal aldimine or the external aldimine resulting from transimination. Because the accompanying spectral changes (viz. Figures 1 and 3B) occur as the sum of two single exponentials that are both zero order with respect to the concentration of L-His, we conclude that formations of the 413- and 468-nm species are uncoupled from the bimolecular process of Michaelis complex formation. Michaelis complex formation must be a rapid process that is completed within the stopped-flow mixing dead time and occurs without detectable perturbation of the spectrum of E(Ain-K). We note that detailed kinetic studies of the reactions of L-Ser or L-Trp with $\alpha_2\beta_2$ have failed to detect Michaelis complexes (Lane & Kirschner, 1981, 1983a; Drewe & Dunn, 1985). Therefore, we assign the 413-nm band to the L-His external aldimine.

The log-normal distribution curve provides an excellent approximation to the shapes of the electronic transitions of organic compounds (Metzler et al., 1985). We have fitted the 400–550-nm region of the absorption spectrum of the L-His–$\alpha_2\beta_2$ system with log-normal distribution curves (Figure 6). The log-normal parameters (Table I) of the curves rep-

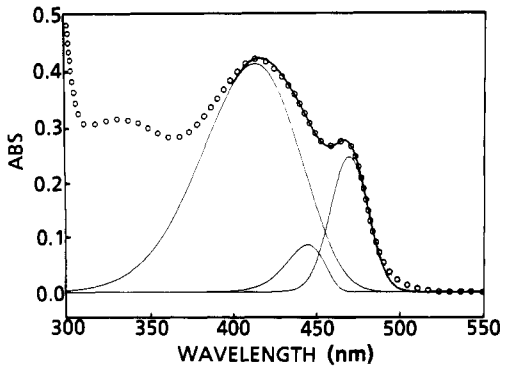


FIGURE 6: Log-normal distribution curve fitting for the absorption region 400–550 nm of the complex formed with 38.5 μM $\alpha_2\beta_2$ and 200 mM L-His in 0.05 M Bicine buffer and 1 mM EDTA, pH 7.8, 25 °C. The solid line through the experimental points (O) is the sum of the log-normal curves which are individually shown below the solid line. The individual log-normal parameters used are given in Table I. The absorption spectra were corrected for base-line errors and small amounts of turbidity as in Likos et al. (1982).

Table I: Parameters of the Log-Normal Distribution Curves Shown in Figure 6

species	λ_{max}	abs[a]	HW[b]	Ω[c]
E(Q)	469	0.24	1.2	1.1
	445	0.08	1.4	1.4
E(Aex-K)	412	0.41	4.1	1.4

[a] Absorbance at λ_{max}. [b] The width of the curve at half-height (in units of 10^3 cm^{-1}). [c] The skewness of the curve [$\Omega = (V_v - V_0)/(V_0 - V_r)$, where V_0 is the wavenumber corresponding to λ_{max} and V_v and V_r are the wavenumber values at half-height on the "violet" and "red" sides of the curve, respectively].

resenting E(Aex-K) and E(Q) agree well with known log-normal parameters for these structures (Kallen et al., 1985; Metzler et al., 1973). The major absorption band ($\lambda_{max} \simeq 468$ nm) of E(Q) has a height approximately 3 times the height of the band ($\lambda_{max} \simeq 445$ nm) on the short-wavelength side; this relationship is very characteristic of absorption bands corresponding to the quinonoid structure (Kallen et al., 1985). The E(Aex-K) absorption band is represented by a single log-normal distribution curve with the maximum absorption at $\simeq 413$ nm. The lack of spectral structure at wavelengths below this band (i.e., at $\lambda_{max} < 400$ nm) and the variety of structures that can give rise to absorption bands in this region preclude a detailed interpretation of the 300–400-nm region. Various C-4′ adducts E(GD), E(Ain-E), E(Aex-E), and the pyridoxamine ketimine structure with C-4′ protonated ($\lambda_{max} \simeq 340$ nm) (Metzler, 1979; Miles, 1987), are likely to give absorption bands with λ_{max} in the 310–360-nm region. The second electronic transition for certain quinonoid structures is expected to occur in the 330–360-nm range (Morozov et al., 1982).

We conclude that the chemical species formed in the reaction of L-His with the $\alpha_2\beta_2$ complex with $\lambda_{max} = 468$ nm must be E(Q) for the following reasons: (1) Structures such as E(Ain-K) or E(Aex-K) are unlikely to give absorption maxima above 435 nm (Karube et al., 1978; Davis & Metzler, 1972). (2) Elimination to form an α-aminoacrylate intermediate should give a spectrum identical with that obtained in the L-Ser reaction. (3) NMR results (Figure 5B) establish that the α proton is abstracted in the reaction of L-His with $\alpha_2\beta_2$. (4) The absorption bands located in the 440–480-nm region have the unique spectral features characteristic of E(Q) species.

Interpretation of the Spectral Changes in the Reaction of Gly with $\alpha_2\beta_2$. The amplitude of the absorption band corresponding to E(Aex-K) formed in the reaction of Gly with $\alpha_2\beta_2$ (Figure 1c) is significantly lower than are the amplitudes of the bands of E(Aex-K) species formed from the other analogues with $\alpha_2\beta_2$. Hence, it is likely that there is less E(Aex-K) present in the Gly–$\alpha_2\beta_2$ reaction mixture than in the reaction mixtures involving the other analogues. There is insufficient spectral structure and amplitude in the 335-nm range of the spectrum of the Gly–$\alpha_2\beta_2$ system to allow meaningful fitting of the 300–400-nm region. Fitting of the 335-nm region with a single log-normal distribution curve (data not shown) produced a curve with bandwidth too wide to represent a single chemical species (Metzler et al., 1980); consequently, at least two absorption bands corresponding to at least two distinct chemical species likely contribute to this region. A major contributor to absorption in that region almost certainly is E(Aex-E). The pK value of the azomethine nitrogen is lowered as the side chain becomes less bulky (Metzler et al., 1980); therefore, it is likely that the ratio of enolimine to ketoenamine tautomer is more favorable for Gly than for the other analogues. The identity of other chemical intermediates absorbing in the 335-nm region is unknown, but E(GD) is a likely candidate. The 460–470-nm band probably is due to a small amount of the Gly E(Q). The NMR experiment (Figure 5) establishes that abstraction of the α proton from the Gly E(Aex-E) to form the corresponding E(Q) is stereoselective. If an extinction coefficient of $\sim 40\,000$ M^{-1} cm^{-1} is assumed, then this quinonoid intermediate accounts for roughly 3% of the total fraction of enzyme-bound PLP present.

Interpretation of the Spectral Changes in the Reaction of L-Ala with $\alpha_2\beta_2$. The spectrum of the products formed in the reaction of L-Ala with $\alpha_2\beta_2$ (Figure 1d) shows bands with λ_{max} at approximately 416 and 460–470 nm and absorption in the 330-nm region. The intensity and width of the 416-nm peak indicate this band is the E(Aex-K) tautomer, while the identity of species absorbing in the 330-nm range is unknown. If one assumes an extinction coefficient of 40 000 M^{-1} cm^{-1} for this quinonoid, then the amplitude of the 460-nm band corresponds to roughly 6% of the total species present.

Interpretation of the Spectral Changes in the Reaction of D-His with $\alpha_2\beta_2$. The UV–visible spectrum of the products formed in the reaction of D-His with $\alpha_2\beta_2$ (Figure 1e) shows an absorption band with $\lambda_{max} \simeq 412$ nm and one or more bands in the 330-nm range. Absorption in the the 330-nm region probably results from at least two intermediates, while the 412-nm band no doubt corresponds to the E(Aex-K) tautomer. Evidence for a quinonoid intermediate in the reaction of $\alpha_2\beta_2$ with D-His is weak. Neither second nor fourth derivative spectra reveal the presence of additional absorption bands in the 440–480-nm region (data not shown). Furthermore, unlike L-His, L-Ser (Drewe & Dunn, 1985; Lane & Kirschner, 1983a), and Gly, there is no detectable exchange of α-^1H for solvent ^2H when D-His is incubated with $\alpha_2\beta_2$. Hence, the spectrum of the products of the D-His reaction with $\alpha_2\beta_2$ is dominated by absorption bands corresponding to E(Aex-K) and E(Aex-E) in Scheme I. The 330-nm region of the spectrum likely contains contributions from either E(Aex-E) or E(GD), or both, and other tetrahedral C-4′ adducts are possibilities.

Analogue Reactions with β_2. The absorption spectra of the β_2 complexes of L-His, Gly, and L-Ala lack any absorption in the 440–480-nm region that can be attributed to quinonoid structures (Figure 2, spectra b–d). Consequently, it appears that the amounts of quinonoid species formed with β_2 are not detectable by UV–visible spectroscopy. Nevertheless, the ^1H NMR studies demonstrate that β_2 catalyzes the exchange of the α protons of both L-His and Gly, albeit at rates that are

slower than those observed for $\alpha_2\beta_2$ under similar conditions. In the β_2 reactions, the spectral band of E(Ain-K) is replaced by absorption bands that are shifted to longer wavelength but still retain a band shape characteristic of a ketoenamine tautomer. We assign these bands to the E(Aex-K) tautomer with the respective amino acid. Previous static and RSSF studies of the reaction between L-Ser and β_2 also failed to detect any spectral bands corresponding to intermediates beyond E(Aex-K) and E(Aex-E) (Miles, 1979; Drewe & Dunn, 1985). Taken together, these UV–visible and NMR spectroscopic studies indicate that the differences in ground-state energies between the external aldimine and the quinonoid species formed with L-His and with Gly are larger for β_2 than for $\alpha_2\beta_2$. This stabilization of the quinonoid species by $\alpha_2\beta_2$ is sufficient to make possible their detection by UV–visible spectroscopy, while this is not the case in the β_2 system. Without a more detailed study, we are unable to give a detailed explanation for the different ratios of $k(\alpha_2\beta_2)/k(\beta_2)$ measured for L-His and Gly; the difference in ratios ($\sim$11 for L-His vs 1.9 for Gly) indicates these two systems may have different rate-limiting steps for the exchange.

Role of Subunit Interactions in Tryptophan Synthase. The previously described reaction of β_2 with L-Ser (Miles, 1979; Drewe & Dunn, 1985) is analogous to the herein-described reactions of β_2 with the analogues; the spectrum formed with L-Ser and with each analogue reveals no detectable amount of intermediate along the tryptophan synthase catalytic pathway beyond the external aldimine [i.e., $\beta_2(\text{Aex})_2$ of eq 5 (where L-aa is L-His or Gly)]. Whereas the reaction of L-Ser

$$\beta_2 + \text{L-aa} \rightleftharpoons \beta_2(\text{L-aa})_2 \rightleftharpoons \beta_2(\text{Aex})_2 \rightleftharpoons \beta_2(\text{Q})_2 \quad (5)$$

with $\alpha_2\beta_2$ yields the α-aminoacrylate [i.e., $\alpha_2\beta_2(\text{A-A})_2$ of eq 6], the reactions of Gly, L-His, and L-Ala with $\alpha_2\beta_2$ yield new absorption bands with λ_{max} in the 400–420- and 440–480-nm regions corresponding to the external aldimines and the quinonoids [i.e., $\alpha_2\beta_2(\text{Aex})_2$ and $\alpha_2\beta_2(\text{Q})_2$ of eq 6]. Therefore,

$$\alpha_2\beta_2(\text{L-Ser})_2 \rightleftharpoons \alpha_2\beta_2(\text{Aex})_2 \rightleftharpoons \alpha_2\beta_2(\text{Q})_2 \rightleftharpoons \alpha_2\beta_2(\text{A-A})_2 \quad (6)$$

we conclude that all the intermediates along the pathway up to and including the quinonoid (Scheme I) either are present in the reaction mixtures of the analogues (and contribute to the static spectra, viz., Figure 1b–d) or were traversed in going from the initial internal aldimine to the external aldimine and then to the quinonoid structure. Our kinetic measurements indicate these intermediates all interconvert moderately rapidly. From the spectra measured at equilibrium, it is apparent that the distribution of these intermediates is different for each analogue.

The importance of protein–protein interactions between enzyme pairs that catalyze sequential steps has been documented. Interactions between enzyme pairs can contribute to a significant increase in metabolic rate, largely through direct transfer of metabolite between sequential enzyme pairs (Srivastava & Bernhard, 1986a,b). In the tryptophan synthase bienzyme complex, the transfer of common metabolite (indole) occurs via a 25-Å-long tunnel that links the α- and β-sites (Yanofsky & Rachmeler, 1958; De Moss, 1962; Creighton, 1970; Matchett, 1974; Dunn et al., 1987b; Hyde et al., 1988). This direct transfer and α–β subunit interactions are important for the efficient conversion of substrate to product in the physiological reaction (eq 1) (Yanofsky & Crawford, 1972; Miles, 1979). The β_2 species exists in an equilibrium between at least two conformations, one of which is stabilized by formation of the $\alpha_2\beta_2$ complex (Faeder & Hammes, 1971; Tschopp & Kirschner, 1980b; Lane & Kirschner, 1983a,b). In the absence of α subunit, the specificity of the reaction of β_2 with L-Ser is greatly reduced; the L-Ser reaction with β_2 produces pyruvate and ammonia, while addition of α subunit significantly inhibits formation of these products (Kumagai & Miles, 1971). The reaction described by eq 3 is 50-fold more efficient with $\alpha_2\beta_2$ than with β_2 (Yanofsky & Crawford, 1972). This rate enhancement is primarily due to a lowered activation energy for the removal of the C-α proton from E(Aex-K) (Miles, 1979). Since the rate enhancements for the exchange of the α-^{1}H protons of L-His and Gly are somewhat smaller (11- and 1.9-fold, respectively), and since we find no kinetic isotope effect in the reaction of Gly with $\alpha_2\beta_2$, we conclude that removal of the C-α proton is not rate limiting in the exchange reaction.

The enhancement of β-catalysis exerted by the α subunits is due in part to the lowering of the activation energy for removal of the C-α proton from E(Aex-K) (Miles & McPhie, 1974; Lane & Kirschner, 1983a). Since $\beta_2(\text{Aex})_2$ accumulates in the β_2 reaction with L-Ser (eq 5), while it is $\alpha_2\beta_2$(A-A) that accumulates with $\alpha_2\beta_2$ (eq 6), the relative ground-state energies of covalent intermediates along the reaction pathway are altered by formation of the α–β subunit interfaces (Faeder & Hammes, 1970, 1971; Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985; Dunn et al., 1987a,b). The E(Q)_2 and E(A-A)_2 intermediates (compare eq 5 and 6) are stabilized to a greater degree by $\alpha_2\beta_2$ than by β_2 alone. Allosteric interactions between α and β subunits cause these differences in catalytic activity and intermediate stability. We speculate that there are protein conformational changes that occur in concert with, and that are obligatory for, the interconversion of covalent intermediates in the tryptophan synthase system, and the energy barriers to some of these conformational changes are lower in $\alpha_2\beta_2$ than in β_2.

In subsequent studies (K. F. Houben and M. F. Dunn, unpublished results), we will show that allosteric interactions across the α–β subunit interface alter the quilibrium distribution of intermediates formed in the reactions of L-His and Gly with the β subunits. These allosteric effects are modulated by structural analogues of 3-indole-D-glycerol 3′-phosphate and D-glyceraldehyde 3-phosphate (the substrates/products for the α-site, viz., eq 2).

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