Characterization of the Reaction of L-Serine and Indole with *Escherichia coli* Tryptophan Synthase via Rapid-Scanning Ultraviolet–Visible Spectroscopy†

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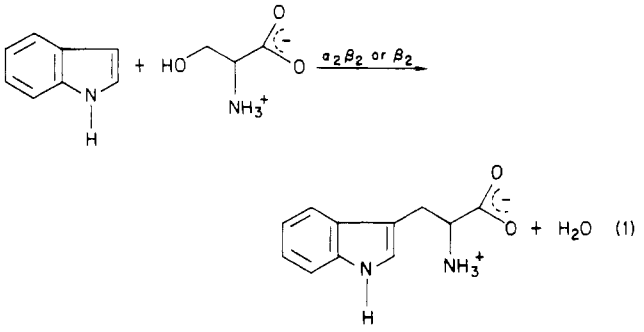
ABSTRACT: The pre-steady-state reaction of indole and L-serine with the $\alpha_2\beta_2$ complex of *Escherichia coli* tryptophan synthase has been investigated under different premixing conditions with rapid-scanning stopped-flow (RSSF) UV–visible spectroscopy for the spectral range 300–550 nm. When $\alpha_2\beta_2$ was mixed with indole and L-serine, the reaction of $\alpha_2\beta_2$ was found to occur in three detectable relaxations ($1/\tau_1 > 1/\tau_2 > 1/\tau_3$) with rate constants identical with the three relaxations seen in the partial reaction with L-serine [Drewe, W. F., Jr., & Dunn, M. F. (1985) *Biochemistry 24*, 3977–3987]. Kinetic isotope effects due to substitution of ^2H for the α-^1H of serine were found to be similar to the effects observed in the reaction with serine only. The observed spectral changes and isotope effects indicate that the aldimine of L-serine and PLP and the first quinoid derived from this external aldimine are transient species that accumulate during τ_1. Conversion of these intermediates to the α-aminoacrylate Schiff base during τ_2 and τ_3 limits the rate of formation of the second quinoidal species (λ_{max} 476 nm) generated via C–C bond formation between indole and the α-aminoacrylate intermediate. The pre-steady-state reaction of the $\alpha_2\beta_2$–serine mixture with indole is comprised of four relaxations ($1/\tau_1^* > 1/\tau_2^* > 1/\tau_3^* > 1/\tau_4^*$). The $1/\tau_1^*$ spectral changes occur within the mixing dead time and result from the perturbation of the spectrum of the $\alpha_2\beta_2$–serine mixture upon interaction with indole, while the appearance of the 476-nm species is characterized by $1/\tau_2^*$, $1/\tau_3^*$, and $1/\tau_4^*$. These experiments demonstrate that the events that occur in τ_1, τ_2, and τ_3 of the L-serine reaction are obligatory for the attainment of the steady state and that the product of the reaction of $\alpha_2\beta_2$ with L-serine is highly reactive toward indole.

The tryptophan synthase from *Escherichia coli* catalyzes the replacement of the β-hydroxyl group of L-serine with indole to yield L-tryptophan and water (eq 1). This pyridoxal



(1)

phosphate (PLP)[1] requiring reaction is catalyzed by the active sites of the β subunits of the native $\alpha_2\beta_2$ enzyme. The α subunits of this bienzyme complex catalyze the cleavage of 3-indole-[D-glycerol 3′-phosphate] to indole and D-glyceraldehyde 3-phosphate (Miles, 1979). The distinctive UV–visible spectral properties of the PLP moiety provide a sensitive probe for the detection and identification of intermediates formed during this β-replacement reaction.

Our investigations of the partial reaction of L-serine with the $\alpha_2\beta_2$ complex (via rapid-scanning, rapid-mixing, stopped-flow spectrophotometry; Drewe, 1984; Drewe & Dunn, 1985) show that this reaction time course is characterized by the rapid formation of the serine external aldimine and the corresponding quinoid. These species then decay to a product mixture that contains a reactive α-aminoacrylate Schiff base species (Miles et al., 1982).

The pre-steady-state phase of the $\alpha_2\beta_2$-catalyzed reaction of indole with L-serine has been studied by single-wavelength stopped-flow methods, and the UV–visible spectra of intermediates that accumulate in the steady state have been determined via conventional UV–visible spectroscopy (Lane & Kirschner, 1983a,b; York, 1970, 1972; Faeder & Hammes, 1970, 1971). The steady-state spectra show the presence of several interesting bands, indicating the accumulation of more than one intermediate. The transient kinetic studies have demonstrated that the pre-steady-state phase is characterized by the formation and decay of intermediates. However, those kinetic studies relied upon a very small sampling of wavelengths for measurement of the pre-steady-state reaction time course. Therefore, the nature of the spectral changes that occur and the chemical identification of the intermediates that form in the pre-steady-state have not been established.

In this paper, we investigate the pre-steady-state phase of the reaction of indole and L-serine with the $\alpha_2\beta_2$ complex via rapid-scanning stopped-flow (RSSF) UV–visible spectroscopy. Rapid-scanning stopped-flow spectroscopy has been shown to

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; $\alpha_2\beta_2$ and β_2, respectively, the native and β_2 forms of *E. coli* tryptophan synthase (EC 4.2.1.20); E and E*, enzyme forms distinguished by differences in reactivity toward substrates; E(Ain), internal ϵ-iminolysyl aldimine form of enzyme-bound PLP; E(S), enzyme–serine Michaelis complex; E(Aex_1) and E(Aex_2), external aldimines formed respectively with L-serine and L-tryptophan; E(T_1) and E(T_2), tetrahedral intermediates formed respectively in the reactions of L-serine and L-tryptophan with the internal aldimine form of enzyme-bound PLP; E(Q_1), E(Q_2), and E(Q_3), quinoidal species formed along the reaction path; E(A-A), enzyme-bound α-aminoacrylate Schiff base with PLP; EDTA, ethylenediaminetetraacetate; DTE, dithioerythritol; RSSF, rapid-scanning stopped flow.

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Table I: Summary of Rate Constants and Amplitudes for the Reactions of *E. coli* Tryptophan Synthase with Serine and Indole under Different Premixing Conditions[a]

syringe 1	syringe 2	wavelength (nm)	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$A_2/(A_2 + A_3)^d$	$1/\tau_3$ (s^{-1})	$A_3/(A_2 + A_3)^d$
$[\alpha_2\beta_2]$ = 13.3 μM	[L-serine] = 40 mM						
	[indole] = 0.1 mM	476		32	+1.0		
		430	>400	83			
	[indole] = 0.2 mM	476		55	+1.0		
		430	>400				
	[indole] = 0.5 mM	476		74	+0.93	7	+0.07
		430	>400	86			
	[indole] = 1.0 mM	476		68	+0.92	12	+0.08
		430	>400	71			
	[indole] = 2.0 mM	476		56	+0.92	6	+0.08
		430	370	57			
	[indole] = 5.0 mM	476		33	+0.93	3	+0.07
		430	139	40			

syringe 1	syringe 2	wavelength (nm)	$1/\tau_{1,Ser}$	A_1^{df}	$1/\tau_{2,Ser}$	A_2^{df}	$1/\tau_{3,Ser}$	A_3^{df}
$[\alpha_2\beta_2]$ = 13.3 μM[b]	[L-serine] = 40 mM	430	*e*	*e*	69	+0.205	13	+0.02
	[L-serine] = 4 mM	430	197	*e*	65	+0.062	13.5	+0.039

syringe 1	syringe 2	wavelength (nm)	$1/\tau_1$*	A_1^{*df}	$1/\tau_2$*	A_2^{*df}	$1/\tau_3$*	A_3^{*df}	$1/\tau_4$*	A_4^{*df}
$[\alpha_2\beta_2]$ = 16.7 μM][c]	[indole] = 5 mM	330	*e*	*e*	220	−0.019	11.5	−0.009		
[L-serine] = 40 mM[c]		430	*e*	*e*	242	+0.024	13.4	+0.021		
		470	*e*	*e*	197	+0.11	47.8	−0.032	2.3	+0.018
		525	>400	+0.010	240	−0.012				

[a] Potassium phosphate buffer (0.1 M), pH 7.80, containing 1 mM EDTA at 25 °C. All concentrations given correspond to conditions *after* mixing the contents of syringes 1 and 2. The errors in reported rate constants and amplitudes are estimated to be ±15%. [b] Values from Drewe and Dunn (1985). [c] Premixed for at least 5 min before initiation of reaction. [d] Signs on the amplitude ratios and amplitudes refer to the direction of the spectral change (+) for increasing absorbance and (−) for decreasing absorbance. [e] These relaxations were found to be too fast to be measured under these conditions with our stopped-flow apparatus (dead time ~3 ms). [f] Absorbance units.

be useful in the study of enzyme catalysis (June et al., 1981a,b; Koerber & Dunn, 1981; Koerber et al., 1983; Drewe & Dunn, 1985). The time-resolved spectra establish that (1) the reaction of $\alpha_2\beta_2$ with (premixed) L-serine and indole involves, as obligatory steps, all of the processes detected in the partial reaction with L-serine, (2) the α-aminoacrylate Schiff base intermediate formed by premixing L-serine with $\alpha_2\beta_2$ is highly reactive toward indole, and (3) the binding of indole alters the spectrum of the α-aminoacrylate prior to covalent bond formation.

Materials and Methods

Chemicals were purchased from Sigma (reagent grade) and used without further purification. Purification of *E. coli* tryptophan synthase, measurement of enzymatic activity, and determination of protein concentrations were as described by Drewe and Dunn (1985). Indole concentrations were determined as described by Lane and Kirschner (1981). All spectroscopic measurements were carried out as described by Drewe and Dunn (1985).

Results

Because most of the mechanistic data that have been reported for *E. coli* tryptophan synthase have been carried out at a pH of either 7.6 or 7.8, a pH of 7.8 was chosen for the herein described work. Detailed steady-state kinetic studies of L-tryptophan synthesis from the reaction of indole with L-serine have been performed by several groups. The steady-state kinetic behavior is complicated by substrate inhibition at high indole concentrations. At pH 7.6 and 25 °C, k_{cat} = 5.3 s^{-1} and K_m^{Ser} = 0.43 mM (Lane & Kirschner, 1983b). Assuming a compulsory sequence of substrate additions (first L-serine, then indole), Faeder and Hammes (1971) determined a value for the L-serine dissociation constant of 27 μM and a Michaelis constant for indole of 13.4 μM at pH 7.8. Assuming a rapid-equilibrium, random sequential mechanism, Heilman (1978) reported values for L-serine binding to $\alpha_2\beta_2$ and to $\alpha_2\beta_2$(indole)$_2$ of 77 μM and 313 μM, respectively, and values for indole binding to $\alpha_2\beta_2$ and $\alpha_2\beta_2$-(L-Ser)$_2$ of 3.7 μM and 14 μM, respectively. Weischet and Kirschner (1976) report two classes of binding sites on the α subunits for indole with K_D = 1.2 mM and K_D = 30 mM. With these constants in mind, we report experiments in this study at indole concentrations ranging from 0.1 to 5 mM and L-serine concentrations of 4 and 40 mM.

Rapid-Scanning Studies of the Reaction of L-*Serine and Indole with Holo-*$\alpha_2\beta_2$ *Tryptophan Synthase.* The time-resolved RSSF spectra for the pre-steady-state reaction of $\alpha_2\beta_2$ with 5 mM indole and 40 mM L-serine at pH 7.80 are shown in Figure 1A. The enzyme (contained in one syringe) is mixed with L-serine and indole (premixed in the second syringe). In Figure 1A, the spectral changes show a rapid burst phase in which the spectrum of the holo-$\alpha_2\beta_2$ (λ_{max} 412 nm, trace 0) shifts to approximately 425 nm (traces 1 and 2) and the absorbance increases. This burst reaction is followed by a decrease in absorbance in the wavelength region 350–450 nm (traces 3–18). In the wavelength regions below 350 and above 450 nm, the absorbance increases. Note that the 18th trace occurs ~2 s after the initiation of reaction and is considered to be the spectrum of the steady state. There are no valid isoabsorptive points (i.e., points of constant absorbance)[2] during the pre-steady-state phases of the reaction. The absence of isoabsorptive points and the red shift during the fast phase are more clearly shown in the family of difference spectra presented in Figure 1B.

Single-Wavelength Stopped-Flow Studies. Single-wavelength rapid-mixing experiments, carried out under experimental conditions identical with those used in the RSSF study, viz., Figure 1, traces a and b, and Figure 2, were undertaken

[2] The term "isoabsorbance point" is used herein to designate those wavelengths where the absorbance remains constant (and nonzero) during one or more phases but not during all phases of the reaction.

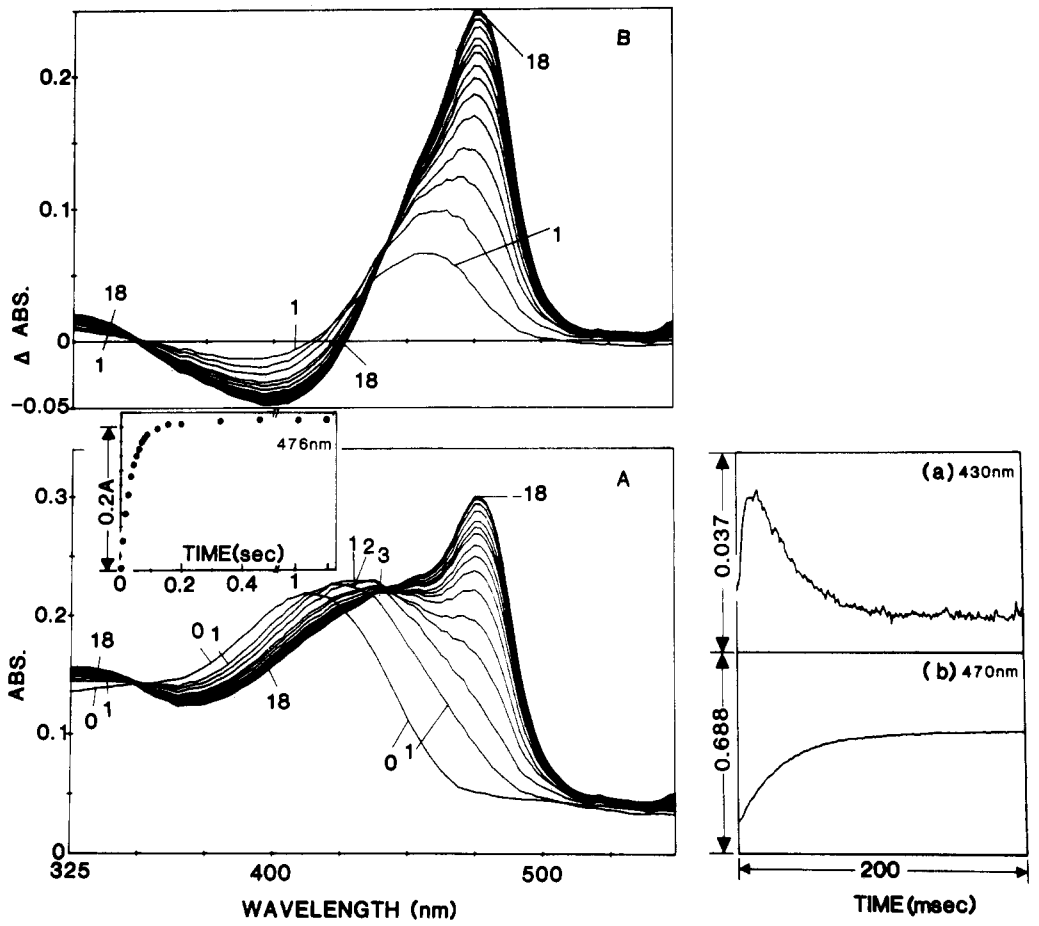


FIGURE 1: Rapid-scanning stopped-flow spectra and difference spectra showing the pre-steady-state spectral changes for the $\alpha_2\beta_2$-catalyzed condensation of indole and L-serine. The spectra (A) and difference spectra (B) were measured at high concentrations of serine and indole. The enzyme contained in one syringe was mixed with indole and L-serine premixed in the second syringe. In (A), the trace designated 0 is the reconstructed spectrum of the reactants. The first scan in (A) was initiated 4 ms after flow stopped. The inset is the 476-nm reaction time course reconstructed from the RSSF data. Difference spectra (B) were computed as $(\text{scan})_t - (\text{scan})_0$ from the data presented in (A). Conditions after mixing: [L-Ser] = 40 mM, [indole] = 5 mM, $[\alpha_2\beta_2]$ = 13.3 μM, 0.1 M potassium phosphate, 1 mM EDTA, pH 7.80, and 25 °C. Timing sequence: (1) 4.0, (2) 12.6, (3) 29.8, (4) 38.4, (5) 47.0, (6) 55.6, (7) 64.2, (8) 72.8, (9) 81.4, (10) 90.1, (11) 98.7, (12) 133, (13) 168, (14) 211, (15) 340, (16) 469, (17) 1114, and (18) 1974 ms after flow stopped. Conventional single-wavelength stopped-flow time courses measured under the same conditions used in the RSSF study are shown in (a) and (b). The best fit assuming the minimum number of consecutive first-order processes: (a) 430 nm, $1/\tau_1 = 138\ s^{-1}$, $1/\tau_2 \geq 41\ s^{-1}$; (b) 470 nm, $1/\tau_2 = 29\ s^{-1}$.

to gather accurate rate parameters (see Table I). At 430 nm, two consecutive first-order processes (designated $1/\tau_1$ and $1/\tau_2$) were found to be necessary to describe the first 200 ms of the time course, while at 470 nm a single first-order process (similar to $1/\tau_2$) was found to be sufficient to describe the time course. Single-wavelength time courses of 500-ms and 2-s duration revealed more complicated time courses. Three ($1/\tau_1$, $1/\tau_2$, and $1/\tau_3$) and two ($1/\tau_2$ and $1/\tau_3$) consecutive first-order processes, respectively, were found necessary to accurately describe the time courses at 430 and 470 nm.

As the concentration of indole is increased, the rates of all three of the relaxations increase to maximum values at about 1 mM indole. At higher concentrations of indole, all three relaxations are slowed. Figure 2 shows the dependencies of $1/\tau_2$ and the amplitude of the 476-nm spectral band on the concentration of indole.[3]

The spectral band formed in τ_1 is very similar to that seen in the reaction of the $\alpha_2\beta_2$ complex with only L-serine [see Figures 1 and 4 in Drewe & Dunn (1985)]. Nevertheless, an apparent discrepancy was encountered when comparing the rate of appearance of the 425-nm species in the two reactions. In the absence of indole, the 425-nm species appears very rapidly ($1/\tau_{1,\text{Ser}} > 400\ s^{-1}$; Drewe & Dunn, 1985), while in the presence of the higher levels of indole (0.5 to 5 mM) the rate of appearance ($1/\tau_1$) decreases from a value > 400 s^{-1} at 1.0 mM to a value of 140 s^{-1} at 5 mM indole (Table I).[3] However, it is known from steady-state kinetic experiments that substrate inhibition with indole is found with concentrations greater than 0.1 mM (Heilmann, 1978). This inhibition appears to account for the decrease in the rate of $1/\tau_1$.

If $1/\tau_{2,\text{Ser}}$ and $1/\tau_{3,\text{Ser}}$ both are obligatory processes for the attainment of the steady state, then as the concentration of indole is increased, $1/\tau_2$ should approach $1/\tau_{2,\text{Ser}}$ and $1/\tau_3$ should approach $1/\tau_{3,\text{Ser}}$. That is, the rate of interconversion of the 425- and 350-nm species ($1/\tau_{2,\text{Ser}}$ and $1/\tau_{3,\text{Ser}}$) in the reaction of L-serine with $\alpha_2\beta_2$ should limit the rate of appearance of the 476-nm species at high indole concentrations where the reaction of indole is no longer rate limiting. This hypothesis was tested by comparing the rates of $1/\tau_2$ and $1/\tau_3$ with the rate of $1/\tau_{2,\text{Ser}}$ and $1/\tau_{3,\text{Ser}}$ (see Figure 2 and Table I). The maximum rates of $1/\tau_2$ (68 s^{-1}) and $1/\tau_3$ (12 s^{-1}) achieved with 40 mM L-Ser and 1 mM indole (Table I) are very close in magnitude to the values of $1/\tau_{2,\text{Ser}}$ and $1/\tau_{3,\text{Ser}}$ observed with 40 mM L-serine (69 and 13 s^{-1}; Drewe & Dunn, 1985). Under these conditions, the relative amplitudes for the

[3] The rate data for the reaction of $\alpha_2\beta_2$ with L-serine and indole presented in Figure 2 and in Table I are from different data sets. Differences in the reported rate constants reflect the level of error associated with the analysis of biphasic and triphasic kinetic time courses.

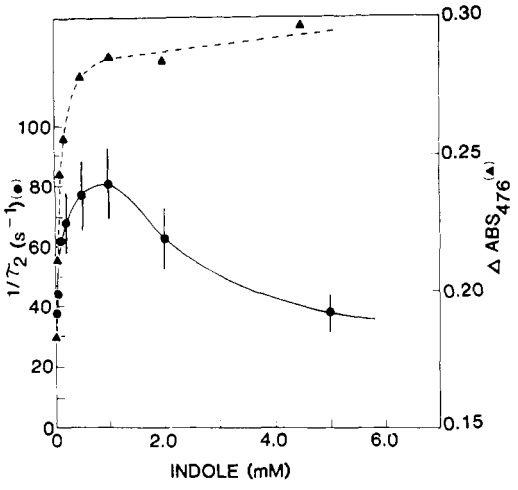


FIGURE 2: Dependence of $1/\tau_2$ and the amplitude of the 476-nm spectral band on the concentration of indole. Before mixing, the enzyme was contained in one syringe while indole and L-serine were premixed in the second syringe. Conditions after mixing: $[\alpha_2\beta_2]$ = 13.3 μM, [L-serine] = 40 mM, 0.1 M potassium phosphate, 1 mM EDTA, pH 7.80, and 25 °C. The rate constants and amplitude changes were evaluated from single-wavelength time courses measured at 476 nm. The amplitude change represents the difference in absorbance between the $\alpha_2\beta_2$ initial spectrum and the maximum absorbance obtained after mixing. The errors in reported rate constants and amplitudes are estimated to be ±15%.[3]

biphasic appearance of the 476-nm species (92% in τ_2) are similar to the relative amplitude changes found during $\tau_{2,Ser}$ and $\tau_{3,Ser}$ (~85% of total amplitude change occurs as $1/\tau_{2,Ser}$, the remainder as $1/\tau_{3,Ser}$).

Indole is a noncompetitive inhibitor of $\alpha_2\beta_2$ both with respect to the binding of L-tryptophan (Lane & Kirschner, 1981) and with respect to the synthesis of L-tryptophan from indole and L-serine (Hielmann, 1978). From stopped-flow studies of L-tryptophan binding, Lane and Kirschner (1981) concluded that the steady-state flux (via the tryptophan quinoid and the tryptophan external aldimine) to free L-tryptophan decreases as the concentration of indole increases. We have found that high indole concentrations (greater than 1 mM) inhibit the reaction of L-serine with the enzyme (i.e., $1/\tau_1$ is slowed, see Table I) and there are similar effects on $1/\tau_2$ and $1/\tau_3$. Weischet and Kirschner (1976) found that in addition to the β subunit catalytic site, indole binds to two different sites on each α subunit of the $\alpha_2\beta_2$ complex. One of these sites appears to be the α subunit catalytic site with K_D = 1.2 mM; the second site with K_D = 30 mM is distinct from the catalytic site. Thus, at indole concentrations that inhibit in $1/\tau_1$, $1/\tau_2$, and $1/\tau_3$ there is considerable binding of indole to the high-affinity site on the α subunit.

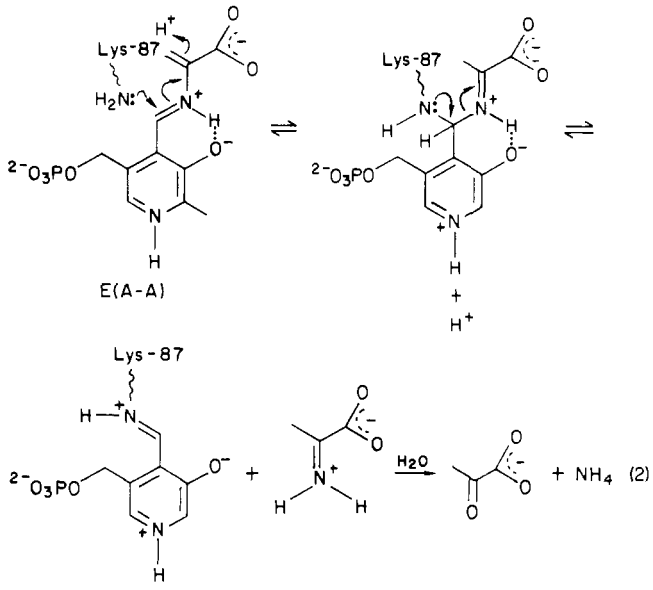
Kinetic Isotope Studies with α-^{1}H- and α-^{2}H-Substituted DL-*Serine.* We also have investigated the transient spectral changes that occur when 80 mM concentrations of DL-serine, with either α-^{1}H or α-^{2}H (Miles & McPhie, 1974), and indole, ranging in concentration from 0.05 to 5 mM, are mixed with the $\alpha_2\beta_2$ complex. These experiments (data not shown) establish that substitution of deuterium gives a primary kinetic isotope effect $(1/\tau_2)_H/(1/\tau_2)_D \sim 2$, on the appearance of the 476-nm band. The RSSF spectra show that deuterium substitution results in a substantial increase in the amplitude of the 425-nm band, a substantial decrease in the width of the 425-nm band, and a decrease in the amplitude of the 476-nm band during the τ_2 process. This result is analogous to the effects of deuterium substitution observed in the partial reaction of [α-^{1}H]- and [α-^{2}H]-DL-serine with the $\alpha_2\beta_2$ enzyme (Drewe, 1984; Drewe & Dunn, 1985).

Rapid-Scanning Studies of the Reaction of Premixed Holo-$\alpha_2\beta_2$ and L-*Serine with Indole.* The time-resolved spectra obtained when enzyme preincubated with 40 mM L-serine is then mixed with 5 mM indole in the RSSF apparatus are shown in Figure 3A. The insets compare the single-wavelength time courses (see below) measured in the presence of 40 mM L-serine, panels a–d, and 4 mM L-serine, panels e–h. In the RSSF experiment, the spectrum of the enzyme–serine reaction product (trace 0) is shown. Under the premixing conditions used, $\alpha_2\beta_2$ reacts with L-serine to give spectrum 0 (within 2–3 s), which is stable for at least 30 min. Upon mixing with indole, the reaction is characterized by two fast phases (τ_1* and τ_2*) with absorbance changes between 325 and 550 nm. At both 40 and 4 mM L-serine, the absorbance in the wavelength region above 500 nm undergoes a very rapid increase (τ_1*), which is complete within the time required to measure the first spectrum. The absorbance then decreases during the fast phase (τ_2*) in an apparently monophasic manner. These faster phases are followed by two slower changes. In the wavelength region of 325–380 nm, the absorbance decreases; while between 380 and 500 nm the absorbance increases. In the presence of 40 mM serine (Figure 3A), the observed time course is triphasic ($1/\tau_2$* > $1/\tau_3$* > $1/\tau_4$*). This triphasicity is most noticeable in the 476-nm region (see the inset to Figure 3A). At this wavelength, the initial increase in absorbance (τ_2*) is followed first by a smaller absorbance decrease and then an increase in absorbance. At 4 mM L-serine, the time course in the 476-nm region (see inset g) is biphasic (τ_2* and τ_3*; RSSF data not shown). No true isosbestic points were found; however, at 415 nm there is an isoabsorptive point where only the slower phase is observed.

Single-Wavelength Stopped-Flow Studies. In order both to accurately determine rate constants and to verify the unusual absorbance changes detected at 525 nm in the RSSF studies, single-wavelength time courses for 40 mM L-serine (a–d) and for 4 mM L-serine (e–h) were measured at 330, 430, 470, and 525 nm under experimental conditions identical with those used in the RSSF studies (see Table I and the two sets of traces a–d and e–h attached to Figure 3). The time courses measured at 330 and 430 nm were found to be adequately fit with two consecutive first-order processes, while the time courses observed at 525 nm were apparently monophasic. At 40 mM L-serine (traces a–d), three consecutive first-order processes were found to be necessary to accurately describe the time course at 470 nm (viz., trace c of Figure 3), while at 4 mM L-serine only two processes were needed to describe the 470-nm time course (viz., trace g of Figure 3). Note that the absorbance at 525 nm increases within the instrument dead time (in agreement with the results of the RSSF studies, Figure 3A). We find that the magnitudes of the apparent rate constants for the individual relaxations, measured at the different wavelengths, are very similar (±20%) for each of the two L-serine concentrations used.

DISCUSSION

According to the chemical steps outlined in Scheme I, the imperative for efficient catalysis dictates the formation of an activated α-aminoacrylate Schiff base intermediate poised for reaction with bound indole. However, prior to indole binding, the α-aminoacrylate Schiff base must be shielded at the C-4′ position of the PLP moiety from reaction with interfering nucleophiles such as water or nucleophilic residues at the site. For example, in the absence of indole, transimination involving the active site ϵ-amino group of Lys-87 and the α-aminoacrylate intermediate (eq 2) is a very slow side reaction (Miles, 1979; Lane & Kirschner, 1983b; M. Roy and M. F. Dunn,



unpublished results). Nevertheless, a few steps further along the pathway (viz., Scheme IB), transimination is an obligatory, rapid step for the release of L-tryptophan.

Since the reacting substrates undergo a series of geometrical changes involving $sp^3 \rightleftharpoons sp^2$ hybridization changes (both at the α- and β-carbons of serine and at the 3-carbon of indole), it is logical to expect a complementary set of changes to occur in the three-dimensional structure of the site. Changes in charge distribution from one transition state to the next undoubtedly are matched by obligatory rearrangements of charges and dipoles contributed by the site so as to lower the energy barriers of steps along the catalytic path. Consequently, we view these protein transconformation/electronic changes to be obligatory for the catalysis of chemical changes in the reacting substrates, and we propose that weak binding interactions between the substrates and the site are used to help drive these conformation changes.

Heretofore, the investigation of the events that occur during tryptophan synthase catalysis has been restricted to the determination of UV–visible spectra in the steady state (Goldberg et al., 1968; York, 1970; Miles, 1980) and to the measurement of the pre-steady-state absorbance and fluorescence changes at a few wavelengths (Faeder & Hammes, 1970, 1971; Lane & Kirschner, 1983b; York, 1970, 1972). The time-resolved spectra and the single-wavelength time courses presented in Figures 1 and 3 define the changes in the electronic spectrum of the PLP chromophore as the steady state is approached, both in the reaction of $\alpha_2\beta_2$ with L-serine and indole (Figure 1) and in the reaction of the α-aminoacrylate Schiff base intermediate with indole (Figure 3). We detect at least three relaxations ($1/\tau_1$, $1/\tau_2$, and $1/\tau_3$) in the reaction of $\alpha_2\beta_2$ with L-serine and indole. As will be shown, the reaction of $\alpha_2\beta_2$ with L-serine and indole includes as obligatory steps all of the processes and the same transient intermediates seen in the partial reaction with L-serine (Drewe, 1984; Drewe & Dunn, 1985). The chemical events proposed to occur during this partial reaction are shown in Scheme IA.

The reaction of the α-aminoacrylate intermediate, E(A-A), with indole yields a time course (viz., Figure 3) consisting of four relaxations ($1/\tau_1$*, $1/\tau_2$*, $1/\tau_3$*, and $1/\tau_4$*). The events that occur in τ_1* appear to result from a perturbation of the spectrum of the E(A-A) (spectrum 0). The spectral changes that accompany nucleophilic attack by C-3 of indole on C-β of the E(A-A) occur during τ_2*. The processes that take place during τ_3* and τ_4* reflect the final approach to the steady state. The steady-state mixture contains detectable amounts of a quinoidal species, presumed to be $E(Q_3)$, with λ_{max} 476 nm and the L-tryptophan external aldimine, $E(Aex_2)$, with λ_{max} 420 nm, derived from the protonation of the α-carbon proton of this quinoid. This sequence of chemical transformations is shown in Scheme IB.

Intermediates Formed during τ_1 and τ_2 in the $\alpha_2\beta_2$ Reaction with L-Serine and Indole. The experiments presented in Figures 1 and 2 and Table I establish two points: (1) the envelope of spectral bands (with λ_{max} 425 nm) formed in the fast phase ($\tau_{1,Ser}$) of the partial reaction of $\alpha_2\beta_2$ with serine (Drewe & Dunn, 1985) is the same as that formed in the fast phase (τ_1) of the complete reaction when L-serine and indole are reacted with $\alpha_2\beta_2$ (Figure 1), and (2) the 425-nm spectral envelope consists both of the band for the external aldimine (with $\lambda_{max} \simeq 420$ nm) and of the band for the first quinodial species (with $\lambda_{max} \simeq 460$ nm). We have previously shown that the 425-nm spectral band consists of two species, the highly fluorescent serine external aldimine, $E(Aex_1)$ with $\lambda_{max} \sim 420$ nm, and the delocalized carbanion (quinoid), $E(Q_1)$ with $\lambda_{max} \simeq 460$ nm, derived from abstraction of the α-proton from the external aldimine [see Drewe & Dunn (1985)]. Furthermore, the primary isotope effect $(1/\tau_2)_H/(1/\tau_2)_D \sim 2$ resulting from ^2H substitution for the α-^1H of serine is constant with a mechanism in which the rate of conversion of $E(Aex_1)$ to $E(Q_1)$ (Scheme IAB) is partially rate determining for the appearance of $E(Q_3)$.

The rate and amplitude data presented in Figure 2 and Table I show biphasic dependencies on the concentration of indole. Both $1/\tau_2$ and A_2 exhibit dependencies, at low indole concentrations, which reflect a relatively high-affinity interaction between indole and tryptophan synthase. The increases in the initial phases of each curve reach a half-maximal value of ≤ 0.1 mM. This dependence is consistent with the K_m values derived from steady-state studies for the interaction of indole with E(A-A) (Faeder & Hammes, 1971; Heilman, 1978). Although the studies of Lane and Kirschner (1983b) were not extended to high indole concentrations, they inferred similar conclusions about the origins of the dependencies of $1/\tau_2$ and A_2. At the higher indole concentrations presented in Figure 2 and Table I, both $1/\tau_2$ and A_2 show dependencies that correlate with a weaker interaction (viz., the second phase present in each curve of Figure 2). We propose that the decreases in $1/\tau_1$ (Table I) and $1/\tau_2$ (Table I and Figure 2) are the consequence of an allosteric effect involving the interaction of indole at the active sites of the α subunits with an apparent K_D in the 1 mM range. Weischet and Kirschner (1976) report that the α subunits are the loci of two classes of indole binding sites, one with $K_D = 1.2$ mM (presumed to be part of the catalytic site on α) and the other with $K_D = 30$ mM. Clearly, the dependencies seen in Figure 2 and Table I cannot be due to the binding of indole to a site with $K_D = 30$ mM.

Analysis of Spectral Changes during τ_1 of Reaction of E(A-A) with Indole.* The τ_1* spectral changes (Figure 3) are most likely the result of a perturbation of the concentrations of species present in the initial $\alpha_2\beta_2$–serine mixture. This equilibrium mixture previously has been shown to include the α-aminoacrylate Schiff base (Miles et al., 1982). The perturbation of this equilibrating system by the binding of indole could increase the concentration of an α-aminoacrylate species absorbing at longer wavelengths ($\lambda_{max} \simeq 480$ nm). As evidenced by the absorbance increase in the 500–540-nm region, there are spectral changes in τ_1* that occur within the mixing dead time (4 ms), whereas the spectral band centered at 476

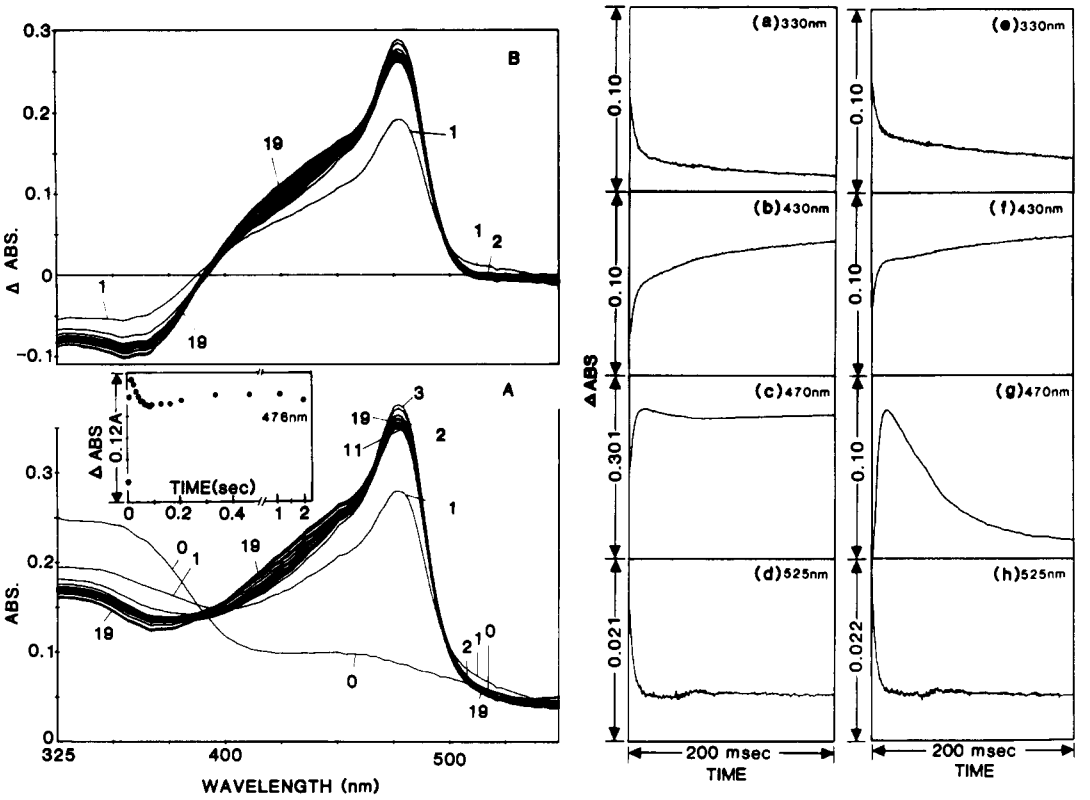


FIGURE 3: Rapid-scanning stopped-flow spectra and difference spectra showing the pre-steady-state spectral changes for the reaction of the α-aminoacrylate Schiff-base intermediate (scan 0) with indole measured at high substrate concentrations. The single-wavelength time courses were measured in the presence of 40 mM L-serine (a–d) and 4 mM L-serine (e–h). Both the enzyme and L-serine were premixed in one syringe while indole was present in the second syringe. The initiation of scanning occurred 1 ms after flow stopped. Difference spectra (B) were computed as $(\text{scan})_t - (\text{scan})_0$ from the data presented in (A). The 476-nm time course reconstructed from the RSSF data is shown in the inset to (A). Conditions after mixing: [L-Ser] = 40 mM, [indole] = 5 mM, $[\alpha_2\beta_2]$ = 13.3 μM, 0.1 M potassium phosphate buffer, 1 mM EDTA, pH 7.80, and 25 °C. Timing sequence: (1) 1.0, (2) 9.6, (3) 18.2, (4) 26.8, (5) 35.4, (6) 44.0, (7) 52.6, (8) 61.2, (9) 69.8, (10) 78.4, (11) 87.1, (12) 95.7, (13) 130, (14) 165, (15) 208, (16) 337, (17) 466, (18) 1111, and (19) 1971 ms after mixing. Conventional single-wavelength stopped-flow time courses (a–d) were measured under the same conditions used in (A). Assuming the minimum number of consecutive first-order processes, the best fit rate constants are as follows: (a) $1/\tau_2^* = 220\ s^{-1}$, $1/\tau_3^* = 11.5\ s^{-1}$; (b) $1/\tau_2^* = 242\ s^{-1}$, $1/\tau_3^* = 13.4\ s^{-1}$; (c) $1/\tau_2^* = 197\ s^{-1}$, $1/\tau_3^* = 47.8\ s^{-1}$, $1/\tau_4^* = 2.3\ s^{-1}$; (d) $1/\tau_2^* = 240\ s^{-1}$. Time courses e–h were measured with $\alpha_2\beta_2$ preincubated with 4 mM L-serine. Best fit rate constants: (e) $1/\tau_2^* = 215\ s^{-1}$, $1/\tau_3^* = 12.5\ s^{-1}$; (f) $1/\tau_2^* = 225\ s^{-1}$, $1/\tau_3^* = 8.5\ s^{-1}$; (g) $1/\tau_2^* = 210\ s^{-1}$, $1/\tau_3^* = 20\ s^{-1}$; (h) $1/\tau_2^* = 238\ s^{-1}$.

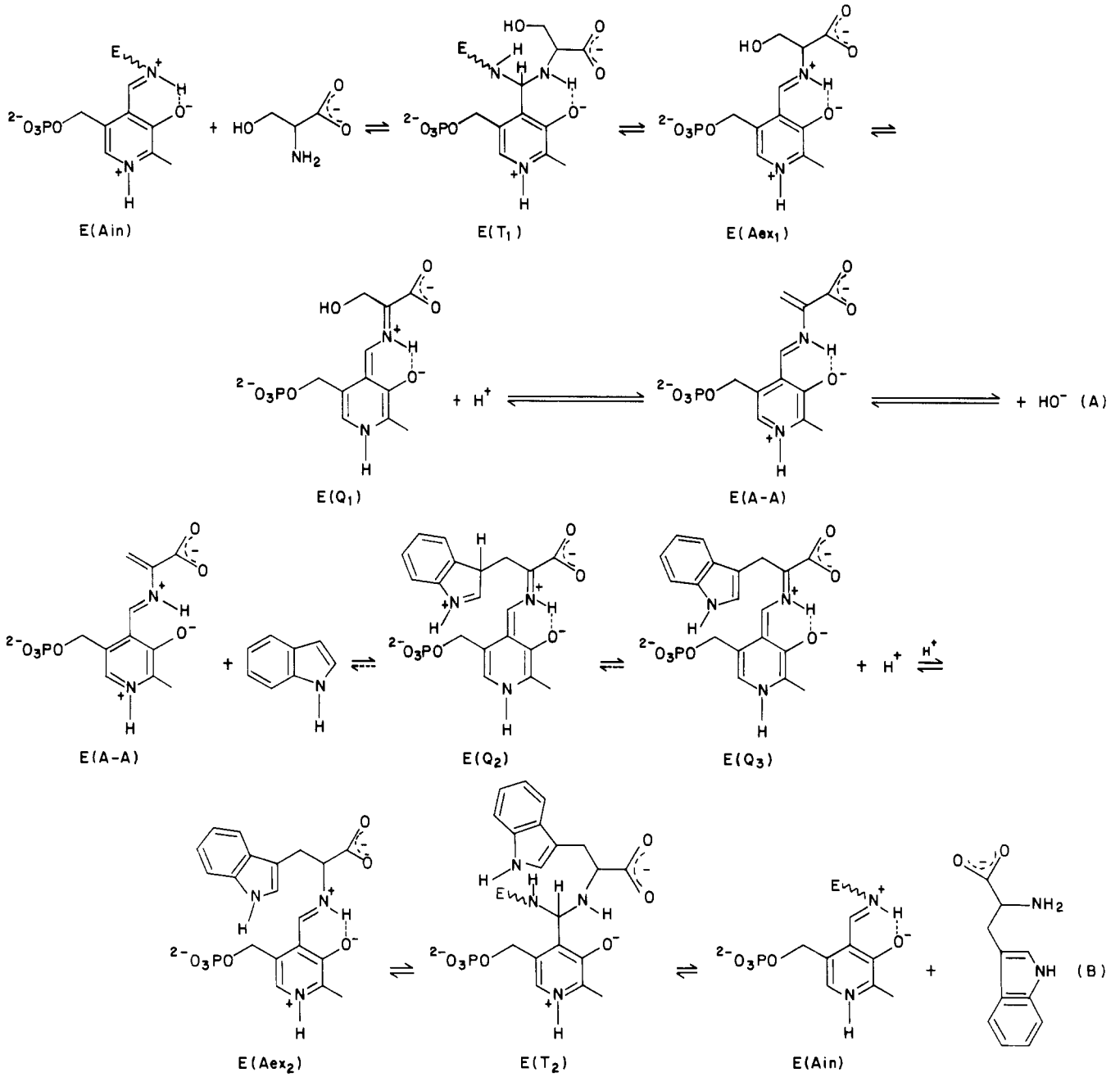
nm forms more slowly during τ_2^*. The detection of these (heretofore unexpected) spectral changes via RSSF is verified by the single-wavelength stopped-flow data (see the single-wavelength 525-nm traces in Figure 3, insets d and h). Although the τ_1^* process can best be directly observed above 500 nm, there are rapid changes in both the 325–400- and 500–525-nm regions (compare traces 0 and 1 in Figure 3). The changes in τ_1^* do not appear to arise from the formation of a quinoidal species; the 476-nm quinoid that forms during τ_2^* is characterized by a relatively narrow band with almost no absorbance above 500 nm, while the changes in τ_1^* are rather different. The spectral changes in τ_1^* appear similar in overall shape to the 325–400- and 500–525-nm regions of the spectrum of the α-aminoacrylate Schiff base (viz., spectrum 0 of Figure 3), whereas all previously reported quinoidal species detected in the reactions of substrates or substrate analogues with tryptophan synthase are characterized by narrow, intense, low-energy spectral bands with higher energy shoulders (Miles, 1979; Phillips et al., 1984; Tschopp & Kirschner, 1980a,b; Lane & Kirschner, 1981; Drewe & Dunn, 1985).

Appearance of the 476-nm Spectral Band. When $\alpha_2\beta_2$ is premixed with L-serine and then reacted with indole, the appearance of the 476-nm spectral band is triphasic ($1/\tau_2^*$, $1/\tau_3^*$, and $1/\tau_4^*$). This finding is in agreement with the single-wavelength studies of Lane and Kirschner (1983b). With both 40 and 4 mM L-Ser, the same maximum amount of the 476-nm transient is formed in $1/\tau_2^*$ (compare insets c and g of Figure 3), and the same amplitude at 525 nm is also produced. Under these conditions, it appears that almost all of the enzyme sites (80–90%) are titrated as a highly reactive species in τ_2^*. Using sodium borohydride, Miles et al. (1982) have trapped an α-aminoacrylate Schiff base by reduction to (phosphopyridoxyl)alanine. Therefore, the highly reactive species that reacts in τ_2^* must be an α-aminoacrylate Schiff base that (within the complex with bound indole) is activated at the β-carbon for reaction with indole.

In agreement with many other investigators (York, 1972; Miles, 1980; Lane & Kirschner, 1981, 1983b), we conclude that the 476-nm spectral band must be a quinoidal species derived from the nucleophilic attack of indole on the α-aminoacrylate, but whether or not C-3 on the indole moiety is sp^3 or sp^2 has not been ascertained. The two forms of the quinoid, $E(Q_2)$ and $E(Q_3)$ (Scheme IB), should have similar, if not identical, long-wavelength spectral bands.

Spectral Changes during $1/\tau_3^*$ and $1/\tau_4^*$. The spectral changes during $1/\tau_3^*$ and $1/\tau_4^*$ and the spectrum at 2 s (the "steady-state" spectrum, Figure 3) depend drastically on the concentration of L-serine (compare the single-wavelength time courses, insets c and g measured at 470 nm). This dependence cannot be due to depletion of the L-serine initially present. The maximum amount of L-serine converted during the pre-steady-state can be calculated to be $\leq$300 μM. This calculation is based on the assumption of an "instantaneous" conversion of L-serine to L-tryptophan by the 26.7 μM β subunits,

Scheme I



which then is followed by 270 turnover cycles of the active sites (based on a k_{cat} = 5 s^{-1} at 25 °C, 26.7 μM β sites, pH 7.8, and 2 s) (Lane & Kirschner, 1983b). Consequently, on the basis of these findings and in agreement with the work of Heilmann and Bürger (1981), we conclude that L-serine acts both as substrate and as an allosteric effector that influences the distribution of bound species in the steady state.

The appearance of the L-tryptophan quinoid (λ_{max} 476 nm) occurs before the appearance of the species that contributes the higher energy (400–460 nm) shoulder is complete. The presence of the isoabsorptive point at 435 nm supports our contention that the formation of the L-tryptophan external aldimine, E(Aex_2) with $\lambda_{max} \simeq 420$ nm, is concomitant with the decay of the quinoid.

The rapid rate of C–C bond synthesis in τ_2* to form the 476-nm quinoidal intermediate (Figure 1 and Scheme I) is indicative either of the preexistence of an activated α-aminoacrylate species or of the preexistence of a species that is rapidly converted to the chemically activated species as a consequence of indole binding. Since it is unlikely that the observed spectral changes in τ_1* arise from covalent bond formation to indole, we speculate that the very rapid spectral change that occurs in τ_1* is the result of a rapid redistribution of species to form the reactive α-aminoacrylate and that this redistribution is brought about by the binding of indole.

These experiments provide the basis for several interesting observations about the tryptophan synthase catalytic mechanism. Herein, we conclude the following: (1) The enzyme-bound covalent intermediates detected in the partial reaction between $\alpha_2\beta_2$ and L-serine (Drewe & Dunn, 1985) are obligatory species necessary for the complete reaction between L-serine and indole. The accumulated chemical, kinetic, and spectroscopic evidence (Miles et al., 1982; Miles, 1979; Drewe & Dunn, 1985) identifies these intermediates as E(Aex_1), E(Q_1), and E(A-A). (2) The β-carbon of the α-aminoacrylate Schiff base, E(A-A), is chemically activated for reaction with indole, while the C-4′ of the E(A-A) PLP moiety is protected from attack by nucleophiles (e.g., the ϵ-NH_2 group of Lys-87).

(3) The binding of indole induces a very rapid change in the spectrum of the E(A-A) in τ_1* prior to covalent bond formation between the C-3 of indole and C-β of the α-aminoacrylate moiety.

Together, these findings indicate that the catalytic events that take place at the β-subunit active site involve a subtle interplay between protein conformational changes and covalent bond-scission/bond-formation events. Transconformation changes in the protein must take place such that catalysis occurs where catalysis is most needed while constraining the interconversions of intermediates to those that lie on the pathway for the synthesis of L-tryptophan. This stereoelectronic control exerted by the protein must arise from conformational changes that assist the interconversion of intermediates on the pathway by lowering activation energy barriers but at the same time impede side reactions such as the transimination of the α-aminoacrylate to yield pyruvate and NH_4^+ (as depicted in eq 2). The driving forces for these conformation changes must derive from the interaction of substrates and effectors with the enzyme. The available evidence (viz., Figures 2 and 3; Table I) indicates that these ligand-mediated effects involve sites on both the α and β subunits; α–β subunit interactions have been shown to exert a critical influence on the kinetics and thermodynamics of the conversion of $E(Aex_1)$ to $E(Q_1)$ and to E(A-A) (Miles, 1979; Drewe, 1984; Drewe & Dunn, 1985; these studies). For example, although E(A-A) is the predominating species that forms in the reaction of $\alpha_2\beta_2$ with L-serine, the reaction of β_2 with L-serine in the absence of indole yields $E(Aex_1)$ as a quasi-stable species (Goldberg & Baldwin, 1967; Goldberg et al., 1968; Miles, 1979; Drewe & Dunn, 1985;) furthermore, the steady-state rate of L-tryptophan synthesis from indole and L-serine by β_2 is 50-fold slower than that of $\alpha_2\beta_2$. Lane and Kirschner (1981) have shown that the binding of either 3′-(indol-3-yl)propyl phosphate or benzimidazole to the α subunits of $\alpha_2\beta_2$ increases the affinity of the β sites for ligands. The inhibitory and activating effects respectively of high concentrations of indole and L-serine (Lane & Kirschner, 1983b; Kirschner et al., 1975; Heilmann, 1978; Heilmann & Bürger, 1981) provide further indications that ligand-mediated site–site interactions are transmitted across the α–β subunit interface. It therefore is likely that such allosteric effects are responsible both for the inhibition of the reaction of $\alpha_2\beta_2$ with L-serine by high indole concentrations (viz., Figure 2) and for the spectral changes detected in τ_1* (viz., Figures 4 and 5).

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Registry No. Tryptophan synthase, 9014-52-2; indole, 120-72-9; L-serine, 56-45-1.

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