Allosteric Linkages between β-Site Covalent Transformations and α-Site Activation and Deactivation in the Tryptophan Synthase Bienzyme Complex†

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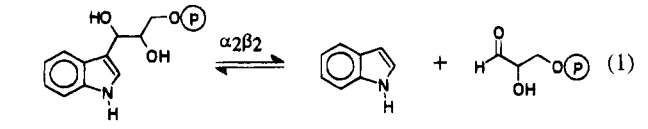
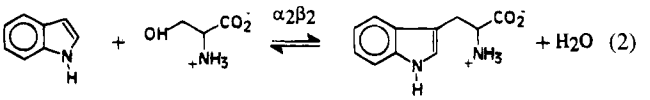
ABSTRACT: This work examines two aspects of the catalytic mechanism and allosteric regulation of the tryptophan synthase bienzyme complex from *Salmonella typhimurium*: (a) the chemical mechanism by which indole and other nucleophiles react with the enzyme-bound α-aminoacrylate Schiff base intermediate, E(A-A), to form quinonoidal intermediates, E(Q), and (b) the effects of covalent transformations at the β-site on the catalytic activity of the α-site. Transient kinetic studies in combination with α-secondary deuterium isotope effects are undertaken to determine the mechanism of nucleophile addition to E(A-A). These studies establish that nucleophilic attack is best described by a two-step reaction sequence consisting of a binding step that is followed by Michael addition to the conjugated double bond of E(A-A). Analysis of isotope effects suggests that the transition state for indole addition gives an E(A-A) β-carbon that resembles an sp^3 center, while the stronger nucleophiles, indoline and β-mercaptoethanol, have transition states that appear to more closely resemble an sp^2 β-carbon. The effects of β-site covalent transformations on α-site catalysis were studied using quasi-stable β-site intermediates and the α-site substrate analogue 3-[6-nitroindole]-D-glycerol 3′-phosphate (6-nitro-IGP). It was found that the cleavage of 6-nitro-IGP is strongly activated by the formation of E(A-A) and various E(Q) species at the β-site but not by external aldimine species. Therefore, we conclude that the conversion of the L-Ser external aldimine to E(A-A) is the β-site process which activates the α-site, while conversion of E(Q) to the L-Trp external aldimine triggers deactivation of the α-site. These findings are discussed within the context of allosteric regulation of substrate channeling in tryptophan synthase catalysis.

Since publication of the *Salmonella typhimurium* tryptophan synthase ($\alpha_2\beta_2$)[1] crystal structure in 1988 (Hyde et al., 1988), this bienzyme complex has become an important and unusual example of the relationship between protein structure and biological function. The complex catalyzes the last two steps in the biosynthesis of L-Trp, herein designated as the αβ-reaction (see Miles, 1986). The first of these steps (designated as the α-reaction, eq 1) is a simple, "aldolytic-like", cleavage of indole glycerol phosphate (IGP) to indole and D-glyceraldehyde 3-phosphate (G3P) that likely involves a single chemical intermediate (Scheme 1). The second step (designated as the β-reaction, eq 2) is a PLP-requiring β-replacement wherein the β-hydroxyl of L-Ser is exchanged for indole.



(1)



(2)

This transformation involves two stages and at least eight covalent intermediates (Scheme 1) [see Brzovic' & Dunn (1992)]. In the first stage, L-Ser reacts to form the Schiff base of α-aminoacrylate, E(A-A); in the second stage, indole makes a nucleophilic attack on E(A-A) to form a quinonoid species that then is converted to L-Trp. While the overall reaction (designated the αβ-reaction) is the physiologically important pathway for the *in vivo* synthesis of L-Trp, the synthesis of L-Trp can be carried out both *in vitro* and *in vivo* by the reaction of indole with L-Ser at the β-site, herein designated the β-reaction (Miles, 1986).

Each αβ subunit pair of the $\alpha_2\beta_2$ tetramer acts as a functional allosteric unit in carrying out these two reactions in a synchronous fashion so that the common metabolite, indole, is efficiently "channeled" between the α- and β-sites (Yanofsky & Rachmeler, 1958; DeMoss, 1962; Creighton, 1970; Kirschner et al., 1975). The structural explanation for

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[1] Abbreviations: $\alpha_2\beta_2$, tryptophan synthase bienzyme complex; IGP, indole glycerol phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerol phosphate; α-reaction, cleavage of IGP to G3P and indole; β-reaction, conversion of L-serine (L-Ser) and indole to tryptophan (L-Trp) and H_2O; αβ-reaction, conversion of IGP and L-Ser to L-Trp, G3P, and H_2O; PLP, pyridoxal phosphate; E(Ain), internal aldimine; $E(GD_1)$ and $E(GD_2)$, *gem*-diamine intermediates formed, respectively, from the reactions of L-Ser and L-Trp with enzyme-bound PLP; $E(Aex_1)$ and $E(Aex_2)$, L-Ser and L-Trp external aldimines, respectively; $E(Q_1)$, $E(Q_2)$, and $E(Q_3)$, the quinonoidal intermediates formed, respectively, from L-Ser and L-Trp; E(A-A), the α-aminoacrylate Schiff base of PLP (see Scheme 1); Nu, nucleophile; $E(Q)_{Nu}$, quinonoids formed from the reactions of nucleophiles with E(A-A); $E(Q)_{aniline}$, the quinonoid formed from reaction of aniline with E(A-A); SWSF, single-wavelength stopped-flow; RSSF, rapid-scanning stopped-flow; τ_1, first observed relaxation in the reactions of nucleophiles with E(A-A); τ_{ex}, relaxation measured for the interconversion of substituents in $E(Q)_{Nu}$; KIE, kinetic isotope effect.

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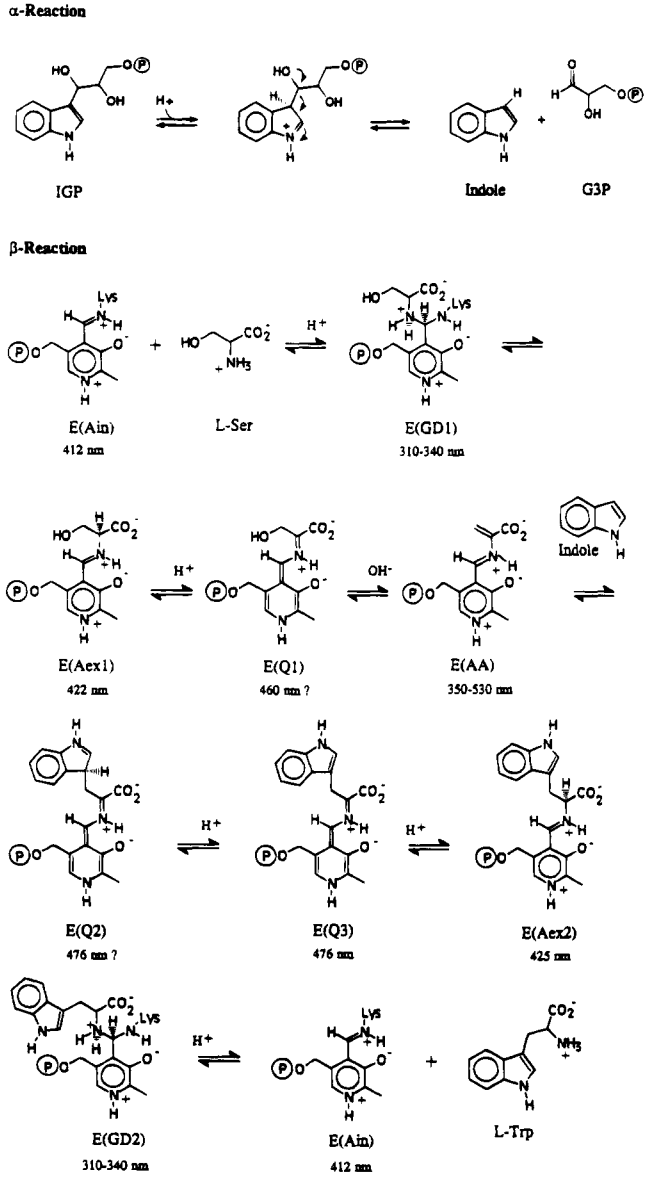
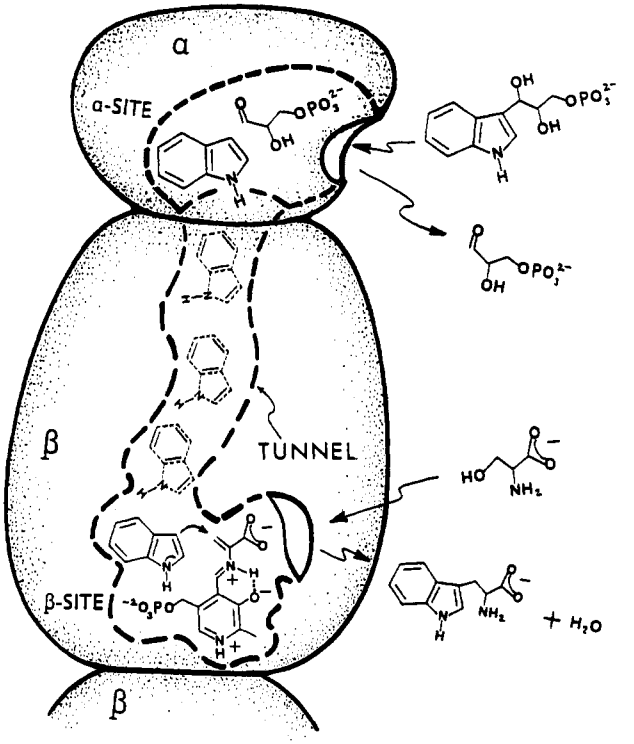
Scheme 1: Structures of Species Believed To Lie along the Reaction Pathways Catalyzed by the Tryptophan Synthase $\alpha_2\beta_2$ Bienzyme Complex[a]



[a] α-Reaction, the reaction catalyzed by the α-subunit. β-Reaction, the reaction catalyzed by the β-subunit. Absorption maxima for species which have been characterized are given. The β-reaction occurs in two stages, the conversion of L-Ser to E(A-A) (stage I) and the conversion of E(A-A) and indole to L-Trp and E(Ain)[1] (stage II).

this "channeling" became obvious when the X-ray structure of *S. typhimurium* enzyme was solved (Hyde et al., 1988). The active sites of each $\alpha\beta$ promoter are linked by an interconnecting 25-Å-long tunnel (Scheme 2) with an architecture that can accommodate the passage of indole between the α and β catalytic sites. To investigate the functional role of the tunnel, Dunn et al. (1990) carried out transient kinetic studies of the reaction of indole and indole analogues with E(A-A) (eq 3). These studies established two important findings: (a) indole and indole structural analogues (e.g., indoline, aniline) enter the β-catalytic site via the α-site and tunnel, and (b) the binding of a structural analogue of G3P, α-glycerol phosphate (GP), to the α-site strongly inhibits the access of indole (and indole analogues) to the β-site by blocking the entrance to the tunnel. These

Scheme 2: Cartoon Depicting the Active Sites of the α- and β-Subunits, the Interconnecting Tunnel, the Sequence of Reactions, and the Routes for Entry and Exit of Substrates and Products for the αβ-Reaction[a]



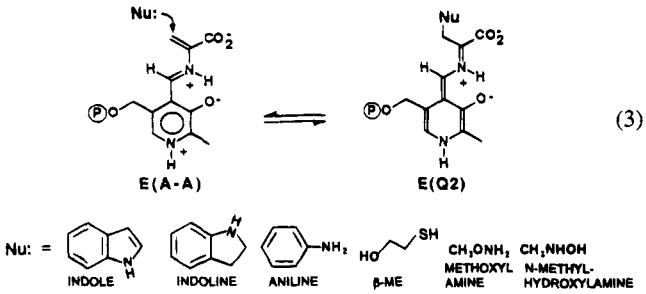
[a] The immediate products of the α-reaction (indole and G3P) are shown bound to the α-site. The pathway for diffusion of the common metabolite, indole, along the tunnel from the α-site to the β-site is shown by the three dashed structures. Upon arrival at the β-site, indole is shown making a nucleophilic attack on the α-aminoacrylate Schiff base intermediate. Adapted from Dunn et al. (1990) with permission.

findings established that the tunnel plays a functional role in the transfer of indole between the α- and β-sites. The kinetic studies of Lane and Kirschner (1991), Kirschner et al. (1991) and Anderson et al. (1991) provided further evidence supporting a functional role for the tunnel in the channeling of indole. Lane and Kirschner (1991) showed that the reaction of L-Ser with the β-site activates catalysis at the α-site. Brzovic' et al. (1992a) demonstrated that this activation is the consequence of an allosteric transition triggered by the conversion of $E(Aex_1)$ to E(A-A) at the β-site,[2] whereas the external aldimine intermediates on the β-catalytic pathway do not activate the α-subunit. Brzovic' et al. (1992a) proposed that the catalytic cycle must involve the coordinated activation and deactivation of the α- and β-sites; formation of E(A-A) activates, while a subsequent step deactivates. Dunn et al. (1990) and Brzovic' et al. (1992b, 1993) have shown that the binding of G3P (or GP, a G3P analogue) to the α-subunit drives a change in conformation to a "closed" structure that "caps" the openings

[2] The experiments to identify the β-site chemical processes which trigger activation and deactivation of the α-site show that α-site activation accompanies E(A-A) formation and that artificial quinonoids derived from the reactions of β-mercaptoethanol, methoxylamine, or indoline also activate the α-site, whereas $E(Aex_2)$ does not. However, one ambiguity remains: in stage I of the β-reaction, $E(Q_1)$ is a transient intermediate with a rate of decay that exceeds its rate of formation (Drewe & Dunn, 1985). Consequently, the possibility that $E(Q_1)$ formation rather than E(A-A) formation triggers activation of the α-site cannot be ruled out by the available data.

to the tunnel at both ends. Brzovic' and Dunn (1992) and Brzovic' et al. (1992b, 1993) hypothesize that the interconversion between open and closed conformations is an essential component both for the coupling of the activities of the α- and β-sites and for the efficient channeling of indole.

The nucleophilic attack of indole on E(A-A) (eq 3) gives $E(Q_2)$, $E(Q_3)$, and then $E(Aex_2)$; these are key steps in the chemical transformation, and one of these steps must provide the allosteric signal to deactivate catalysis at the α-site. To further characterize these critically important components of the β-subunit catalytic cycle, herein we undertake transient kinetic studies in combination with α-secondary deuterium kinetic isotope effects (KIEs) and steady-state kinetic measurements to determine both the mechanism of the reaction of indole and nucleophilic indole analogues with E(A-A) and the effects of E(Q) and $E(Aex_2)$ formation at the β-site on the catalytic activity of the α-site.



(3)

MATERIALS AND METHODS

Materials. Indoline, β-mercaptoethanol (β-ME), and indole were purchased from Aldrich Chemical Co. Indoline was further purified by vacuum distillation; β-ME and indole were used without further purification. [β,β-$^2H_2$]-D,L-Ser containing 98.0 atom % ^2H incorporation was purchased from Cambridge Isotopes, Inc. D,L-Ser, GP, and Bicine were purchased from Sigma. Purification of *S. typhimurium* tryptophan synthase, determination of protein concentrations, and measurement of enzyme activity have been previously described (Kawasaki et al., 1987; Miles et al., 1987, 1989; Brzovic' et al., 1992a). 6-Nitro-IGP was synthesized enzymatically from 6-nitroindole and fructose 1,6-bisphosphate using a mixture of three enzymes, aldolase, triosephosphate isomerase, and tryptophan synthase. The 6-nitro-IGP was purified as described by Kirschner et al. (1991).

UV−Visible Spectroscopy. All static spectral measurements were performed with a Hewlett-Packard 8450A diode-array spectrometer. All reactions and static spectra were measured in 0.05 M Bicine buffer, pH 7.8, containing 1 mM EDTA and 25 mM Na^+ at 25 °C.

Stopped-Flow Kinetic and Spectral Measurements. Single-wavelength stopped-flow (SWSF) kinetic studies were carried out as previously described (Dunn et al., 1979). All SWSF time courses were fitted to eq 4 by nonlinear least-squares regression analysis.

$$A_t = A_\infty \pm \sum_{i=1}^{N} A_i \exp\left(\frac{-t}{\tau_i}\right) \quad (4)$$

In eq 4, A_t and A_∞ are the absorbance values at time t and the time infinity, respectively; A_i and t_i are the ith amplitude and the ith relaxation time, respectively. All time courses were collected under pseudo-first-order conditions (i.e., [Nu], [D,L-Ser] ≫ [E(A-A)]), and the reported rate constants represent an average of at least three separate determinations. The upper and lower limits for the values of the slopes and y-intercepts were calculated by performing linear regressions with the maximum and minimum limits of the relaxation rates. The maximum and minimum values of the relaxation rates were determined from the standard deviation of the mean values. These data were compared with the computer-generated standard deviation of each fit and the larger error is reported.

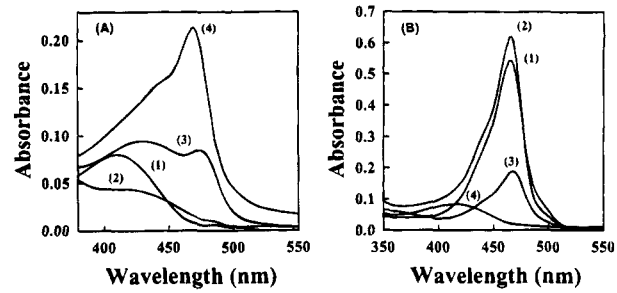


FIGURE 1: Steady-state and quasi-equilibrium spectra of intermediates formed in the reaction of E(A-A) with nucleophiles. (A) Comparison of the spectra of the E(Ain) form of $\alpha_2\beta_2$ (1) with E(A-A) (2), with the species formed upon reaction of indole with E(A-A) (3), and with the quinonoid formed upon reaction of β-mercaptoethanol with E(A-A) (4). (B) Spectra of the quinonoids formed in the reactions of E(A-A) with methoxylamine (1), indoline (2), and aniline (3). Spectrum 4 is the mixture of species formed upon reaction of E(Ain) with L-Trp [$E(Aex_2)$ is the predominant species]. The spectra represent the steady state obtained after $\alpha_2\beta_2$ and L-Ser were mixed in 50 mM TEA buffer, pH 7.8, containing 100 mM NaCl at 25 °C with the nucleophile indicated. Conditions after mixing: $[\alpha_2\beta_2]$ = 5 μM; [L-Ser] = 40 mM. Nucleophile concentrations were [β-mercaptoethanol] = 100 mM; [methoxylamine] = 20 mM; [indoline] = 30 mM; [aniline] = 30 mM. In spectrum 4, the L-Trp concentration was 15 mM.

Turnover Measurements. The α-reaction activity in the presence of different β-subunit-bound intermediates was measured using 6-nitro-IGP as the substrate. 6-Nitro-IGP is cleaved at the α-active site to G3P and 6-nitroindole, which is, however, not a substrate for the β-reaction (Kirschner et al., 1991). A coupled enzyme assay with glyceraldehyde-3-phosphate dehydrogenase (GPDH) was used in all turnover measurements. The appearance of NADH was followed spectrophotometrically at 340 nm. All turnover measurements were measured in 50 mM triethanolamine (pH 7.8) in the presence of 100 mM NaCl.

RESULTS

The purpose of the work presented herein is to determine the nature of the rate-limiting step for the reaction of nucleophiles (Nu) with E(A-A) to form $E(Q)_{Nu}$ (eq 3) and the influence of $E(Q)_{Nu}$ formation at the β-site on the catalytic activity of the α-site. The pre-steady-state period of the reaction of indole with E(A-A) is a multiphasic process that involves the rapid appearance of a quinonoid which undergoes conversion to a steady-state mixture of species dominated by the spectra of a quinonoid and the L-Ser and L-Trp external aldimines, $E(A_{ex1})$ and $E(A_{ex2})$ (Drewe & Dunn, 1986; Woehl and Dunn, in preparation). The first observable relaxation (τ_1) involves the appearance of a quinonoid species with λ_{max} 476 nm (Figure 1A, spectrum 3). The dependence of the rate of this relaxation ($1/\tau_1$) on the concentration of

indole is hyperbolic, with a near-zero y-intercept (Lane & Kirschner, 1983a; Drewe & Dunn, 1986; Dunn et al., 1990). However, other nucleophiles do not display this behavior (Dunn et al., 1990). While the reactions of aniline and *N*- and *O*-methylhydroxylamine with E(A-A) give quinonoidal species (Figure 1B) that are quasi-stable, we have not been able to detect the formation of new amino acids with these nucleophiles (Roy et al., 1988; Dunn et al., 1987, 1990). Both the indoline and the β-ME quinonoids (Figure 1) yield amino acid products. In these systems, the dependence of the first relaxation of the quinonoid-forming step on the Nu concentration is characterized by rates that display a linear dependence on [Nu] and nonzero y-intercepts (Dunn et al., 1990). To examine the mechanisms of these reactions, α-secondary KIEs were measured by comparing the rates of the reactions of nucleophiles with the E(A-A) species derived from [β,β-$^2H_2$]- and isotopically normal D,L-Ser.

Kinetic Isotope Effects. SWSF kinetic studies of the reactions of indole, indoline, and β-ME with E(A-A) using [β,β-$^2H_2$]- and isotopically normal D,L-Ser were carried out to determine if the transition state of the rate-determining step involves bond formation between Nu and the β-carbon of E(A-A). The studies of Lane and Kirshner (1983a) and Drewe and Dunn (1985) have established that only L-Ser reacts with tryptophan synthase; D-Ser has no effect upon the kinetics of the reaction of L-Ser, and D-Ser does not alter the spectrum of PLP bound to the native enzyme. Therefore, the commercially available [β,β-$^2H_2$]-D,L-Ser mixture was used in these studies. Because the deuterium atoms at the β-carbon of the E(A-A) are not directly involved in bond formation/scission in these reactions, any KIE on the rate of Nu reaction at the β-carbon must be considered α-secondary and therefore is expected to be relatively small (k_H/k_D = 0.98−0.71; Halevi, 1963; Saunders, 1986; Cook, 1991).

Reaction of Indole with E(A-A). The reactions of nucleophiles with E(A-A) bring about large changes in the spectrum of the PLP chromophore (Figure 1). The spectrum of E(A-A) is characterized by a $\Pi-\Pi^*$ transition with λ_{max} 350 nm (ϵ_{max} = 6000 M^{-1} cm^{-1}), and a broad shoulder made up of one or more bands extending to >500 nm (spectrum 2 of Figure 1A). The spectra of the quinonoids formed from the reactions of indole, indoline, methoxylamine, and β-ME all give intense $\Pi-\Pi^*$ transitions with λ_{max} between 462 and 476 nm (Figure 1). The time courses for the appearance of these quinonoidal species can be observed via UV−visible absorbance RSSF measurements. The appearance of the quinonoidal spectral band provides a direct measure of the processes that limit bond formation between the nucleophile and the β-carbon of the α-aminoacrylate species as the Michael addition occurs.

Figure 2A compares the effect of β-deuteration on the dependence of $1/\tau_1$ on [indole] for the reaction of indole with E(A-A). From inspection of these data, it is evident that the substitution of deuterium for both hydrogens at the β-carbon alters this dependence; the y-intercept is increased from 25 ± 3.9 to 31 ± 4.5 s^{-1}, the limiting value reached at high indole concentration is increased from 390 ± 15 to 500 ± 15 s^{-1}, and the value of the initial slope is changed from 6.06 × 10^5 to 7.00 × 10^5 M^{-1} s^{-1} (Table 1). In Figure 2B, the same comparison is made for the reaction run in the presence of 50 mM GP. These data show that the presence of GP greatly reduces the apparent rate of quinonoid formation, and there is no detectable KIE.

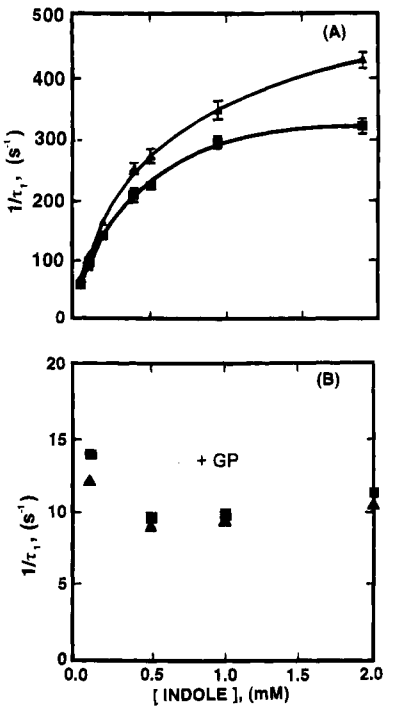


FIGURE 2: Effects of deuterium substitution at the β-carbon of D,L-Ser on the dependence of $1/\tau_1$ on the rate of $E(Q)_{indole}$ formation from reaction of E(A-A) with indole in the absence (A) and in the presence (B) of GP. Data are derived from SWSF reaction time courses for the appearance of $E(Q)_{indole}$ measured at 476 nm in 50 mM Bicine buffer, pH 7.8, at 25 °C. Conditions after mixing: 20 mM D,L-Ser, 5.0 μM $\alpha_2\beta_2$, and variable concentrations of indole. When present, the GP concentration was 50 mM. Prior to mixing, one syringe contained $\alpha_2\beta_2$ preincubated with either [β-$^2H_2$]-D,L-Ser (▲), or isotopically normal D,L-Ser (■).

Reactions of Nucleophilic Indole Analogues with E(A-A). Figure 3 summarizes the results obtained in the comparisons of the reactions of indoline (Figure 3A) and β-ME (Figure 3B) with the E(A-A) species derived from isotopically normal and [β,β-$^2H_2$]-D,L-Ser. Inspection of these figures reveals that both nucleophiles show a linear dependence of relaxation rate on [Nu], and the slopes of these plots show little or no KIE, while the y-intercept differences indicate a significant KIE. The reaction with isotopically normal E(A-A) gives a larger y-intercept than does the β,β-dideuterated species. The y-intercept and slope values from each curve in Figures 2 and 3 are summarized in Table 1.

Kinetics of Nucleophile Exchange. Figure 4 shows the effects of GP on the SWSF time courses for the exchange of indole for aniline in the following reaction sequence:

$$E(Q)_{aniline} \underset{k_{-A}}{\overset{k_A}{\rightleftharpoons}} E(A\text{-}A) + \text{aniline} \quad (5)$$

$$E(A\text{-}A) + \text{indole} \underset{k_{-Q}}{\overset{k_Q}{\rightleftharpoons}} E(Q)_{indole} \quad (6)$$

In the absence of GP, the exchange rate ($1/\tau_{ex}$) is very rapid (viz. trace a of Figure 4); in the presence of GP, $1/\tau_{ex}$ is slowed by 1−2 orders of magnitude (viz. traces b and c of Figure 4). Because the allosteric effects of GP binding shift the position of equilibrium for eq 5 in favor of $E(Q)_{aniline}$ (Dunn et al., 1987, 1990), the amplitude of $1/\tau_{ex}$ is larger in the presence of GP than in its absence. The effects of indole concentration on $1/\tau_{ex}$ are shown in Figure 5A. In the absence of GP (curve a), the rate of exchange increases

Table 1: Summary of Apparent α-Secondary Kinetic Isotope Effects (KIEs) on the Reactions of Nucleophiles

nucleophile	y-intercept $(1/\tau_1 = k_{-2})^b$ (s^{-1})		initial slope $= k_2K_1{}^b$ (M^{-1} s^{-1})		$1/\tau_{max}$ $(k_2 + k_{-2})^b$ (s^{-1})		$K_1{}^c$	$k_{-1}{}^c$ (s^{-1})	$(k^H{}_{-2})/(k^D{}_{-2})^b$	$k_2{}^b$ (s^{-1})		$k^H{}_2/k^D{}_2{}^b$
	H	D	H^b	D	H	D				H	D	
indole	25 ± 3.9	31 ± 4.5	(6.06 ± 0.15) × 10^{-5}	(7.00 ± 0.15) × 10^{-5}	390 ± 15	500 ± 25	(6.4 ± 0.3) × 10^{-4} M	350	0.84 ± 0.21	365 ± 15	470 ± 26	0.78 ± 0.10
indoline	105 ± 2.6	75 ± 2.3	160 ± 4.6	142 ± 4.7					1.41 ± 0.04			
β-ME	132 ± 3.6	107 ± 1.2	10.8 ± 0.4	10.9 ± 0.4					1.23 ± 0.03			

[a] Data are derived from the reactions of nucleophiles with the E(A-A) intermediate prepared from isotopically normal and [β,β-$^2H_2$]-D,L-serine (data taken from Figures 2–4). [b] Data were analyzed according to the mechanistic assumptions of eqs 7 and 8 (see text). [c] Data were analyzed according to the mechanistic assumptions of eqs 5, 6, 8, and 10 (see text).

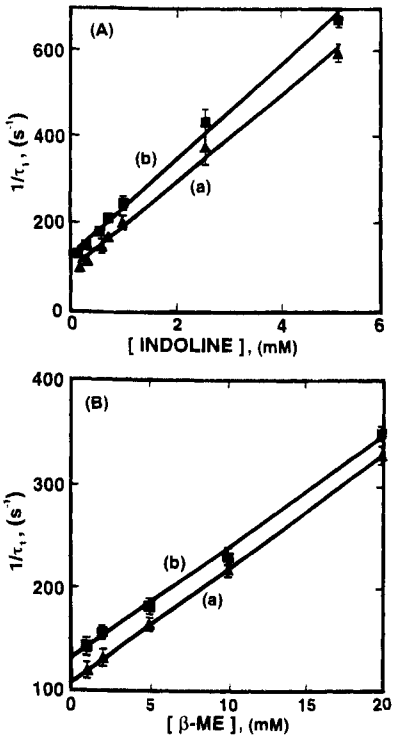


FIGURE 3: Deuterium isotopic effects on the reactions of indoline (A) and β-ME (B) with isotopically normal E(A-A) (a) (■) and [β-$^2H_2$]E(A-A) (b) (▲). Values of $1/\tau_1$ were measured at 464 nm where the indoline and β-ME quinonoids strongly absorb (viz. Figure 1). Conditions were the same as in Figure 2A except that indoline (A) or β-ME (B) was used in place of indole.

linearly as the indole concentration is increased; the slope of this curve is 5.5×10^5 M^{-1} s^{-1} and the y-intercept (where [indole] = 0) is ~30 s^{-1}. In the presence of 50 mM GP (curve b), $1/\tau_{ex}$ is independent of [indole] with an average value of ~5 s^{-1}. The data in Figure 5B show that $1/\tau_{ex}$ is strongly dependent on [GP] in the concentration range 0–5 mM and decreases with increasing concentration. Between 5 and 50 mM GP, there is a gradual further decrease in $1/\tau_{ex}$, and above 50 mM GP, $1/\tau_{ex}$ is constant at about 5 s^{-1}.

Influence of Covalent Intermediates at the β-Active Site on α-Subunit Activity. Activity measurements using the substrate analogue 6-nitro-IGP were performed to investigate the dependence of the α-reaction activity on certain β-subunit-bound intermediates. The activity of the α-reaction in the absence of any covalent intermediates at the β-active site was used as the reference for activities measured in presence of E(A-A), $(E(Q)_{Nu}$, or $E(Aex_2)$ (Table 2). As reported previously, the E(A-A) species at the β-active site activates the α-reaction (Kirschner et al., 1991; Brzovic' et al., 1992a). For the 6-nitro-IGP system, the activity was measured

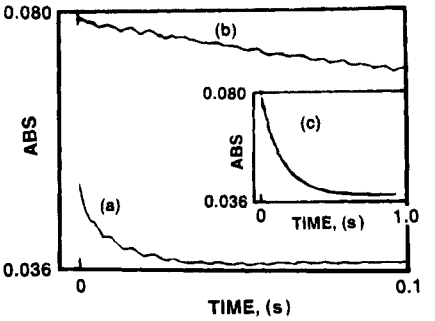


FIGURE 4: Comparison of the SWSF time courses for the exchange of indole for aniline during the interconversion of the aniline and indole quinonoids (eqs 5 and 6) measured in the absence (a) and presence (b, c) of 50 mM GP. The time courses were measured at 466 nm (viz. Figure 1). Note the different time scale of the inset (c). Conditions after mixing: 4 mM $\alpha_2\beta_2$; 40 mM L-Ser; 10 mM aniline; 1.0 mM indole; 50 mM Bicine buffer, pH 7.8; 25 °C. Both syringes contained identical concentrations of aniline and L-Ser. One syringe contained $\alpha_2\beta_2$, the other indole. In traces b and c, GP was present in both syringes.

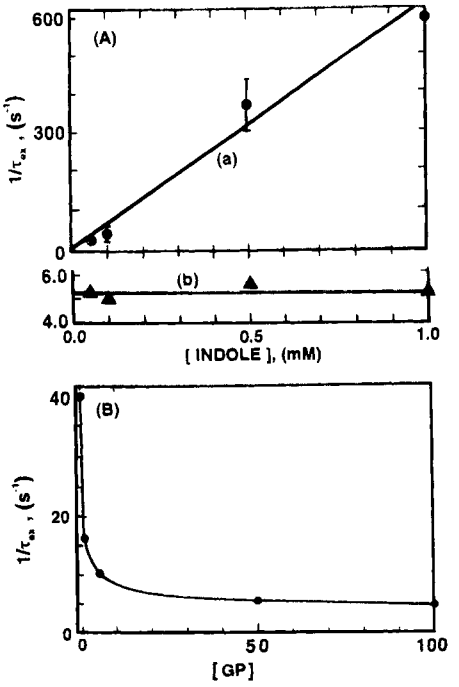


FIGURE 5: Dependencies of the relaxation rate shown in Figure 4 on the concentrations of (A) indole in the absence (a) and presence (b) of 50 mM GP and on the concentration of GP (B). Conditions are the same as in Figure 4 except that variable concentrations of indole (A) or GP (B) are used.

multiple times and an average activation of 4-fold was found when the β-site was in the E(A-A) form. The extent of activation is somewhat lower than that reported for IGP (viz.

Table 2: Effects of β-Site Ligation on the α-Site-Catalyzed Cleavage of 6-Nitro-IGP[a] and IGP

covalent species at β-site	β-site ligands/substrates	relative activity[b] 6-nitro-IGP	IGP[c]
E(Ain)		1	1
E(A-A)	L-Ser	4	27.7
E(Q)	L-Ser, methoxylamine	5.5	
	L-Ser, indoline	4	
	L-Ser, aniline	4	
E(Aex2)	L-Trp	1	0.85

[a] The activity of the α-subunit-catalyzed cleavage of 6-nitro-IGP was determined in a coupled enzyme assay with glyceraldehyde-3-phosphate dehydrogenase (GPDH) by monitoring the appearance of NADH at 340 nm (Kirschner et al., 1991). [b] The activity of the α-reaction in the absence of β-subunit bound intermediates was used as a reference; relative activity = activity measured in the presence of β-site ligand and/or substrate divided by the activity measured in their absence [i.e., when the β-site = E(Ain), see Materials and Methods and Results]. Each assay was repeated multiple times. The concentration of L-Ser, when present, was always 40 mM. Concentrations: $[\alpha_2\beta_2]$ = 1.36 μM, [L-Ser] = 40 mM, [6-nitro-IGP] = 0.4 mM, [methoxyamine] = 10 mM, [indoline] = 5 mM, [aniline] = 10 mM, [L-Trp] = 15 mM. All measurements were performed at 100 mM NaCl. [c] Data calculated from Brzovic et al. (1992b).

Table 2) (Brzovic' et al., 1992a). The presence of quinonoidal species at the β-active site also resulted in stimulation of the α-site-catalyzed cleavage of 6-nitro-IGP. The extent of the stimulation, however, was slightly dependent on the nucleophile used. The quinonoid derived from indoline gave an average activation of 4-fold. The methoxylamine quinonoid gave a 5.5-fold stimulation, and the aniline quinonoid gave a 4-fold stimulation (Table 2). These data and Figure 1 show that the extent of activation appears to depend on the ability of the nucleophile to form quinonoidal species. All three nucleophiles react with E(A-A) to give quinonoidal species with intense Π−Π* transitions with λ_{max} in the 462−468-nm region (Figure 1). However, the amplitudes of these bands are different, indicating either the yield of $E(Q)_{Nu}$ or its extinction coefficient is dependent on Nu structure. With L-Trp bound at the β-active site, which forms mainly $E(Aex_2)$, the α-site catalyzed cleavage of 6-nitro-IGP is not activated relative to its activity in absence of PLP-bound intermediates (Table 2), whereas the cleavage of IGP is slightly inhibited (Brzovic' et al., 1992a).

DISCUSSION

It is now apparent that certain covalent transformations which occur at the β-site are tightly linked to allosteric subunit interactions in the bienzyme complex; these interactions regulate the catalytic activity of the α-site, the transition between open and closed subunit conformations, and therefore, the functioning of the tunnel as a conduit for the transfer of indole between the α- and β-sites in the physiological reaction (Houben & Dunn, 1990; Dunn et al., 1990, 1994; Lane & Kirschner, 1991; Brzovic' et al., 1992a,b, 1993; Brzovic' & Dunn, 1992). The conversion of $E(Aex_1)$ to E(A-A) activates the α-site, while a subsequent step deactivates the α-site, and this cycle of activation and deactivation is accompanied by conformational transitions that shift the subunits between open and closed conformations. One function of the shift to a closed conformation is to prevent the escape of indole from the confines of the α- and β-sites and the tunnel, thus constraining the indole produced in the cleavage of IGP to reaction with E(A-A) (Brzovic' et al., 1992a,b, 1993; Brzovic' & Dunn, 1992). The strongly inhibitory effect of GP on the reactions of indole, indoline, and aniline presented in this work (viz. Figures 2, 4, and 5) is consistent with the hypothesis that, in the physiological αβ-reaction, the binding of G3P at the α-site is the interaction that constrains both the α- and β-subunits of E(A-A) to closed conformations.

Lane and Kirschner (1983a,b) and Drewe and Dunn (1985, 1986) undertook single-wavelength, fluorescence, and rapid-scanning stopped-flow kinetic studies to characterize both the processes of E(A-A) formation and E(A-A) reaction with indole. Lane and Kirschner (1983a) found that the decay of the highly fluorescent $E(Aex_1)$ is subject to a substantial primary kinetic isotope effect when [α-^{2}H]-D,L-Ser is substituted for isotopically normal D,L-Ser, and this observation was confirmed and extended by Drewe and Dunn (1985). It was further reported by Lane and Kirschner (1983b) that no isotope effect was observed when the reaction of isotopically normal and 3-^{2}H-substituted indole were compared, indicating that deprotonation of the indole ring C-3 carbon of $E(Q)_2$ is not part of the transition state for the appearance of the 476-nm-absorbing quinonoid. This work extends those mechanistic studies by probing the nature of the transition state for the reactions of nucleophiles with E(A-A) via α-secondary kinetic isotope effects in the absence and the presence of the G3P analogue, GP. While it has been established that the formation of E(A-A) provides the trigger for the allosteric transition that activates the α-site (Brzovic' et al., 1992a), prior to these studies, the chemical transformation which deactivates the α-site had not been determined. The effects of $E(Q)_{Nu}$ and $E(Aex_2)$ formation at the β-site on catalysis at the α-site provide new insight into the events that modulate reactions at the α- and β-sites.

Secondary Kinetic Isotope Effects. Secondary KIEs arise when reaction rates are altered as a consequence of isotopic substitution of atoms to which no bonds are broken or formed during the course of reaction. Provided there is no coupled motion with a C−H bond undergoing transformation in the transition state, deuterium α-secondary effects are believed due solely to changes in the zero-point energies of ordinary vibrations that result from changes in bond hybridization at the atom to which the isotope is bonded (Halevi, 1963). These effects have been extensively studied for a wide variety of reactions involving the transformation between sp^2 and sp^3 hybridization (Saunders, 1986; Cook, 1991). For the sp^2-sp^3 case, the out-of-plane bending mode of a C−^{1}H or C−^{2}H bond is believed to make the dominant contribution to the secondary isotope effect (Buddenbaum & Shiner, 1976; Herzberg, 1950; Streitwieser et al., 1958). According to this analysis, the conversion from sp^2 to sp^3 hybridization results in an increase in the force constant of this bond, and the curvature of the potential energy well increases, thus widening the gap between the H and D zero-point energies in the transition state. The result is a ratio of $k_H/k_D < 1.0$. Analysis of the out-of-plane bending frequencies for a C−H bond undergoing the sp^2 to sp^3 transformation led to the prediction of a lower limit of 0.71 for k_H/k_D (Buddenbaum & Shiner, 1976; Streitwieser et al., 1958). Estimates by others and empirical observations suggest values of 0.74−0.88 may be more reasonable (Herzberg, 1950; Streitwieser et al., 1958).

α-Secondary Kinetic Isotope Effects on the Reactions of Nucleophiles with E(A-A). Figures 2 and 3 and Table 1 demonstrate that the reactions of indole, indoline, and β-ME with isotopically normal and [β,β-$^2H_2$]E(A-A) all exhibit significant secondary KIEs in the absence of GP. In the presence of GP, the KIEs on the indole and β-ME reactions are abolished (the effect of GP on the indoline reaction was not tested), indicating a change in rate-determining step.

The reactions of these nucleophiles with E(A-A) are Michael additions that change hybridization of the α-aminoacrylate β-carbon from sp^2 to sp^3 as the corresponding quinonoidal species is formed. Under circumstances where this process is part of the transition state of the rate-limiting step, the reaction should be subject to a secondary KIE when the β-protons are replaced by deuterium. The observed KIEs are consistent with this prediction. The analysis of these effects within the framework of relaxation kinetic theory is presented below.

Analysis of the Indole Reaction. The dependence of $1/\tau_1$ for $E(Q_2)$ formation on the concentration of indole shown in Figure 2A is consistent with a two-step reaction mechanism wherein indole forms a Michaelis complex, E(A-A)(indole), in a rapid, preequilibrium step that is followed by nucleophilic attack on the β-carbon of the α-aminoacrylate to yield $E(Q_2)$:

$$E(A\text{-}A) + Nu \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(A\text{-}A)(Nu) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E(Q_2) \qquad (7)$$

According to relaxation kinetic theory (Bernasconi, 1976), the expression for the appearance of the quinonoidal species, $1/\tau_1$, is given by

$$1/\tau_1 = \frac{k_2 K_1 [Nu]_0}{1 + K_1 [Nu]_0} + k_{-2} \qquad (8)$$

where $K_1 = k_1/k_{-1}$, provided the reaction is run under pseudo-first-order kinetic conditions (i.e., [indole] ≫ [E(A-A)]). According to eq 8, the curves in Figure 2A give *y*-intercept values = k_{-2}, initial slopes = k_2K_1, and rates at substrate saturation = $k_2 + k_{-2}$.

Comparison of the effects of deuterium substitution on the rate ($1/\tau_1$) for $E(Q)_{indole}$ formation in the reaction of indole with the E(A-A) (Figure 2A) shows an obvious influence of isotopic substitution on the observed relaxation rates. According to eq 8, the *y*-intercepts for these plots give values for k_{-2} of 25 ± 3.9 and 31 ± 4.5 s^{-1} for the ^1H and β,β-$^2H_2$ samples, respectively. These two values are well within 2 standard deviations of one another, making it impossible to distinguish one from the other. However, the maximum rates achieved at substrate saturation ($1/\tau_{max}$) are easily distinguished. When analyzed according to eq 8, $1/\tau_{max} = k_2 + k_{-2}$, thus giving k_2 values of 365 ± 15 and 470 ± 26 s^{-1} for the ^1H and ^2H samples, respectively. These values give a ratio of k^H_2/k^D_2 of 0.78 ± 0.10, a value consistent with a transition state that includes the conversion from sp^2 to sp^3 hybridization. For the reverse reaction (the KIE given by the *y*-intercepts in Figure 2A), the ratio k^H_{-2}/k^D_{-2} is 0.84 ± 0.21, a value indistinguishable from 1.0.

Binding of Indole to E(A-A). According to eq 8, the initial slopes of the curves presented in Figure 2A provide measures of k_2K_1. Using the values of k^H_2 (365 ± 15 s^{-1}) and k^D_2 (470 ± 26 s^{-1}) extracted from the data, these slopes yield dissociation constants for indole binding to E(A-A) of 6.02×10^{-4} M (isotopically normal sample) and 6.72×10^{-4} M (dideuterated sample). Since dideuteration of the α-aminoacrylate β-C should have a very small effect on the affinity for indole, a value of (6.4 ± 0.3) × 10^{-4} M seems a reasonable estimate for the indole dissociation constant.

The dependence of the relaxation rate ($1/\tau_{ex}$) for the reaction of indole with the aniline quinonoid (Figures 4 and 5) provides additional information about the kinetics of indole binding to E(A-A). This exchange reaction can be treated as a two-step process involving a rapid dissociation of $E(Q)_{aniline}$ to give E(A-A) and aniline (eq 5), followed by a slower process in which indole and E(A-A) give $E(Q)_{indole}$ (eq 6). The relaxation equation appropriate to this system (Bernasconi, 1976) is given by

$$1/\tau_{ex} = k_Q[E(A\text{-}A)] + k_Q[\text{indole}]\frac{(K_{aniline} + [E(A\text{-}A)])}{K_{aniline} + [E(A\text{-}A)] + [\text{aniline}]} + k_{-Q} \qquad (9)$$

where $K_{aniline}$ is the equilibrium constant for the reaction of aniline with E(A-A). Under conditions where the concentration of E(A-A) can be neglected, this equation simplifies to

$$1/\tau_{ex} = \frac{k_2[\text{indole}]K_{aniline}}{K_{aniline} + [\text{aniline}]} + k_{-Q} \qquad (10)$$

and if $K_{aniline} \geq$ [aniline], then plots of $1/\tau_{ex}$ vs [indole] should be linear with slope $\cong k_Q$, and *y*-intercept = k_{-Q}; the dependence of $1/\tau_{ex}$ on [indole] in the absence of GP (Figure 5A) appears to conform to this prediction. This treatment yields a lower limit estimate for k_Q of ~5.5×10^5 M^{-1} s^{-1}. Since the reaction of indole with E(A-A) actually occurs by the two-step process depicted in eq 7, the *y*-intercept in Figure 2A should be the rate constant for conversion of $E(Q)_{indole}$ to E(A-A)(indole), not the rate constant for the dissociation of indole from E(A-A)(indole). The value observed (30 s^{-1}) is in good agreement with the intercept obtained in Figure 2A for the isotopically normal sample (25 ± 3.9 s^{-1}). Therefore, a value of 350 s^{-1} for the rate constant of indole dissociation (k_{-1} of eq 7) can be estimated from K_1 and k_Q. These values are included in Table 1.

Analysis of the Reactions of Indole Analogues. The data presented in Figure 3 and Table 1 summarize the secondary KIEs on the reactions of indoline and β-ME with E(A-A). Indoline and β-ME give qualitatively very similar behavior. From inspection of Figure 3, we conclude that there are observable KIEs on the *y*-intercept values, while the slopes appear very similar. For indoline, the intercepts gave values of 105 ± 2.6 and 75 ± 2.3 s^{-1} and slopes of 160 ± 4.6 and 142 ± 4.7 M^{-1} s^{-1} for the ^1H and ^2H samples, respectively; the β-ME data gave *y*-intercept values of 132 ± 3.6 and 107 ± 1.2 s^{-1} and slopes of 10.8 ± 0.4 and 10.9 ± 0.4 M^{-1} s^{-1} for the ^1H and ^2H samples, respectively (Table 1). The linear dependences of $1/\tau_1$ with respect to [Nu] exhibited by these two nucleophiles could reflect a reaction mechanism wherein nucleophilic attack is a bimolecular process:

$$E(A\text{-}A) + Nu \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E(Q) \qquad (11)$$

In this case, the slopes and intercepts would correspond

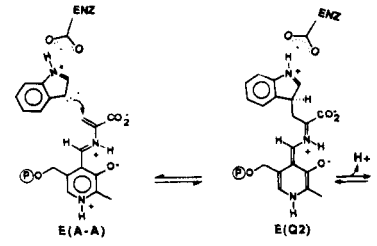
respectively to the forward (k_f) and reverse (k_r) rate constants of eq 11. Alternatively, provided that the relationship $K_1 \gg$ [Nu] (eq 8) holds over the concentration ranges investigated, these reactions could occur via the same mechanism proposed for the indole reaction (i.e., eqs 7 and 8). In this instance, the slopes of these plots provide measures of k_2K_1, while the y-intercepts measure k_{-2}. Irrespective of which mechanism is assumed to better describe these reactions, the y-intercept values provide measures of the KIE for the reverse reaction, a process involving an sp^3 to sp^2 change in bond hybridization at the β-carbon of the α-aminoacrylate. The KIEs calculated from the relaxation rates defined by the y-intercepts, $(1/\tau^H_1)/(1/\tau^D_1)$, are 1.41 ± 0.04 for indoline and 1.23 ± 0.03 for β-ME, values fully consistent with the occurrence of an sp^3 to sp^2 transformation in the transition state of the reaction. Because the slopes measured for the isotopically normal and deuterated samples are so similar, we conclude that the isotope effects on the forward direction are very small (i.e., $k^H_2/k^D_2 \simeq 1.0$).

Equilibrium Isotope Effects with β-ME. Because β-ME yields an E(Q) species that is relatively slowly converted to products, the quasi-equilibrium α-secondary isotope effect on this reaction could be estimated from the amplitudes of the relaxations for $E(Q)_{\beta\text{-ME}}$ formation. The value of the amplitude ratio, $A_H/A_D = 0.83 \pm 0.05$, is consistent in magnitude with values expected for an sp^2 to sp^3 interconversion.

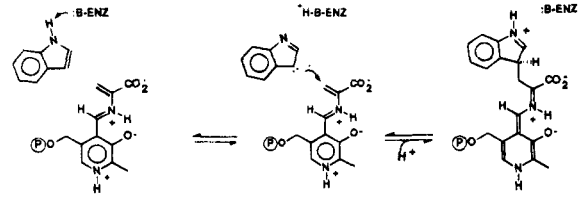
Inferences Concerning Transition States for Nucleophile Reaction with E(A-A). According to current theory, secondary kinetic isotope effects result from the differences in zero-point vibrational energies between the ground state and the transition state. Inferences drawn from these secondary isotope effects provide interesting insights into the way that tryptophan synthase has evolved to favor indole as a nucleophile. In the transition state, for efficient catalysis, the enzyme site should be complementary in structure to the structure of the activated complex and, therefore, interactions between the enzyme site and the activated complex should be optimized (Pauling, 1948). Because indole is a relatively poor nucleophile ($pK_a = -3.5$; Hinman & Lang, 1964), it is reasonable to assume that the enzyme site must chemically activate indole for the nucleophilic attack on E(A-A) to achieve this optimization.

The KIEs on the indole reaction appear to be largely due to effects on the rate constant for bond formation, k_2. For indoline and β-ME, the KIEs appear to be dominated by effects on the reverse process (k_{-2} of eq 8). We speculate that these differences arise from alterations in transition-state structure as a consequence of the large differences both in nucleophilicity and in the chemical nature of the nucleophiles used. For indole, the transition state appears to be well along the reaction coordinate so that the out-of-plane bending vibration of the β-C−H bond more nearly resembles an sp^3 center than an sp^2 center, whereas for stronger nucleophiles, like indoline ($pK_a = 5$) and β-ME ($pK_a = 8.5$), the activated complex appears to more closely resemble a structure with an sp^2-like, out-of-plane bending vibration similar to that of the sp^2 ground state.

Since indoline and β-ME are much stronger nucleophiles than indole, these substrates may not require activating interactions. It has been proposed (Walsh, 1979; Phillips et al., 1984; Miles, 1991; Brzovic' et al., 1992c) that activation of indole occurs either via an electrostatic strain-distortion interaction that enhances the enamine character of the indole ring system via coulombic interaction with the carboxylate of a Glu or Asp residue at the site (eq 12) or by abstraction of the indole ring NH proton by a basic group (eq 13).



(12)

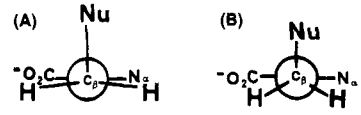


(13)

The enzyme X-ray structure indicates that the carboxylate of βGlu 109 is located in a region of the β-active site that makes up the indole ring subsite (C. C. Hyde, personal communication) and may be positioned to play one or the other of the roles depicted in eqs 12 and 13. The mutant, βE109D, exhibits altered kinetic behavior in stage II of the β-reaction (Brzovic' et al., 1992c); the affinity for indole is decreased, the rate of steady-state turnover is decreased to 3.7% of wild-type enzyme, and there is no detectable accumulation of quinonoidal species when L-Ser and indole are reacted with βE109D. In contrast, the rate of indoline turnover is enhanced, the apparent affinity for indoline is increased, and indoline gives a significant amount of quinonoidal species when reacted with L-Ser and βE109D. These findings are not inconsistent with the above-postulated role for βE109 in catalysis.

Mechanistic Inferences for the Reactions of Indole and Indole Analogues with E(A-A). The Michael addition of nucleophiles to acrylic acid derivatives has received much attention in the physical organic and bioorganic mechanistic literature [see Osman et al. (1988), Pardo et al. (1993), Bernasconi (1989), and Bernasconi et al. (1986, 1987)]. The structure of the activated complex is believed to retain considerable sp^2 character for the β-carbon to the acryloyl moiety when the attacking nucleophile is fluoride ion or nitrogen (a primary or secondary amine). For example, when fluoride ion is the attacking nucleophile, *ab initio* calculations (Osman et al., 1988) indicate that the transition state is reached when the F−β-C distance is 1.951 Å, and the two H−C−C bond angles at the β-C have been distorted in the direction of sp^3 hybridization by only 0.7° and 2.1°, respectively, an activated complex with sp^2-like hybridization. This description is consistent with our conclusion that only the reverse step, cleavage of the bond to the nucleophile, shows a significant α-secondary KIE when indoline or β-ME is the nucleophile. Chart 1A depicts such a transition state: the H−C−C bond angles at the β-carbon are near 120°, the attacking nucleophile (Nu) approaches the β-C along an angle

Chart 1: Neuman Projections of the Proposed Transition States for Nucleophilic Attack at the β-Carbon of E(A-A)[a]



[a] (A) An sp^2-like geometry for C-β in which the H-C-C band angles are near 120° with the attacking nucleophile (Nu) approaching along an angle of incidence of ~115°. The acrylate moiety is slightly perturbed from planarity. (B) An Sp^3-like geometry for C-β in which the bond angles are more nearly tetrahedral.

of incidence of approximately 115°, and the acrylate moiety is slightly perturbed from planarity.

Base catalysis of proton transfer from the attacking nucleophile during a Michael addition is an important component of the activation energy (Bernasconi, 1989; Bernasconi et al., 1986, 1987; Pardo et al., 1993). These results suggest that the action of a basic group (e.g., βE109) could greatly assist the nucleophilic attack of indole on the α-aminoacrylate β-carbon. By partial analogy to the *ab initio* treatment of Pardo et al. (1993), when there is base assistance of the nucleophilic attack, the transition state for the reaction lies farther along the reaction path and the β-C of E(A-A) would have a greater amount of sp^3 character, as depicted in Chart 1B. The very different properties of an enamine carbon as the attacking nucleophile also favor a sp^3-like transition state.

In the indoline system, it appears that base assistance is not available, presumably because the basic group which provides this assistance to indole (likely βE109) is positioned too distant to directly assist the removal of the N−H proton from indoline. For the β-ME system, if the attacking nucleophilic is the thiolate anion (not the thiol), then there would be no need for a base-assisted attack. The relatively high polarizability of S^- makes the thiolate anion a relatively effective nucleophile toward carbon centers in Michael addition reactions (Dunn & Bernhard, 1969). Therefore, an early transition state (viz. Chart 1A) with an sp^2-like structure is not unexpected for these nucleophiles.

GP Effects on the Michael Additions to E(A-A). As is evident from inspection of Figures 2, 4, and 5 and in agreement with the findings of Dunn et al. (1990) and Brzovic' et al. (1992b, 1993), GP strongly inhibits the reactions of indole, indoline, and aniline with E(A-A). The lack of a detectable secondary KIE (Figure 2) and the striking change in concentration dependencies brought about by GP on these Michael addition reactions (Figures 2B and 5) establish that GP binding changes the rate-limiting step for nucleophile addition. Dunn et al. (1990) and Brzovic' et al. (1992b, 1993) have argued that this change in rate-limiting step involves the GP-mediated conversion of the enzyme to a closed conformation which prevents indole and indole analogues from reaching the β-site via the α-site and tunnel. The work presented herein is fully consistent with this interpretation.

Covalent Trigger for Deactivation of the α-Site. While Brzovic' et al. (1992a) established that conversion of $E(Aex_1)$ to E(A-A) in stage I of the β-reaction is the covalent process which triggers the allosteric transition that activates the α-site,[2] the process which deactivates the α-site was not established. Their studies with L-Ser analogues and with D- and L-Trp provided strong evidence showing that formation of external aldimine species, e.g., $E(Aex_1)$ and $E(Aex_2)$ (Scheme 1), does not activate the α-site-catalyzed cleavage of IGP. However, from the data presented, it was unclear whether or not quinonoidal species derived from L-Trp activate or deactivate the α-site. Owing to the relatively rapid rate of conversion of $E(Q_2)$ and $E(Q_3)$ to $E(Aex_2)$ in the β-reaction and to the relatively low fraction of quinonoid formed at equilibrium from L-Trp, the question of whether or not $E(Q_2)$ or $E(Q_3)$ activates the α-site was ambiguous. Kirschner et al. (1991) showed that indole derivatives with bulky substituents at the 6-position are reasonably good substrates for the α-site, but these do not bind or react at the β-site. Using this innovation and the finding that certain nucleophiles form quasi-stable quinonoids upon reaction with E(A−A) (Dunn et al., 1987, 1990; Roy et al., 1988), we have been able to investigate the effects of $E(Q)_{Nu}$ formation at the β-site on catalysis at the α-site. The data presented in Table 2 unequivocally show that the α-site-catalyzed cleavage of 6-nitro-IGP is activated by conversion of $E(Aex_1)$ to E(A-A) and that the quasi-stable quinonoids formed from the reactions of methoxylamine, indoline, or aniline with E(A−A) also activate the α-site, whereas the reaction of L-Trp, which predominantly gives $E(Aex_2)$, does not. From these studies, we conclude that the chemical switch which activates the α-site is conversion of $E(Aex_1)$ to E(A-A),[2] while the chemical switch which deactivates almost certainly is the conversion of $E(Q_3)$ to $E(Aex_2)$ (viz. Scheme 1).

Allosteric Regulation of Substrate Channeling in Tryptophan Synthase. Formation of the α-aminoacrylate Schiff base intermediate from $E(Aex_1)$ has been shown to be the covalent trigger both for activation of the α-site and for shifting the conformational equilibria in favor or a closed conformation (Brzovic' & Dunn, 1993; Brzovic' et al., 1993, 1992a,b; Lane & Kirschner, 1991; Anderson et al., 1991; Dunn et al., 1990; 1994). Dunn et al. (1987, 1990, 1994) and Brzovic' et al. (1992a,b, 1993) have proposed that, in the $\alpha\beta$-reaction, the binding of G3P provides an additional thermodynamic drive to a conformation in which both the α- and β-subunits assume closed conformations, thereby trapping indole within the confines of the α- and β-sites and the tunnel so that reaction with E(A-A) is assured. These studies establish that conversion of $E(Q_3)$ to $E(Aex_2)$ provides the allosteric trigger both to deactivate the α-site and to shift the α-subunit conformational equilibrium in favor of the open form. It, therefore, is inescapable to conclude that the changes in stereoelectronic effects that accompany these conversions between sp^2 and sp^3 centers of the reacting substrate at the β-site provide weak bonding interactions which are linked to the allosteric transition that switches the α-site between inactive (open) and active (closed) conformations. Efforts to identify the network of weak bonding interactions that mediate communciation between the α- and β-sites are now underway and involve single-crystal X-ray diffraction studies to determine high-resolution structures of enzyme−substrate intermediates, site-directed mutagenesis (C. C. Hyde and E. W. Miles, personal communication), and rapid kinetic studies to examine the relationship between structure and function for selected residues and the monovalent metal ion cofactor site, which modulate the behavior of the α- and β-subunits (Woehl, Pan, Miles, and Dunn, work in progress).

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