Allosteric Interactions Coordinate Catalytic Activity between Successive Metabolic Enzymes in the Tryptophan Synthase Bienzyme Complex†

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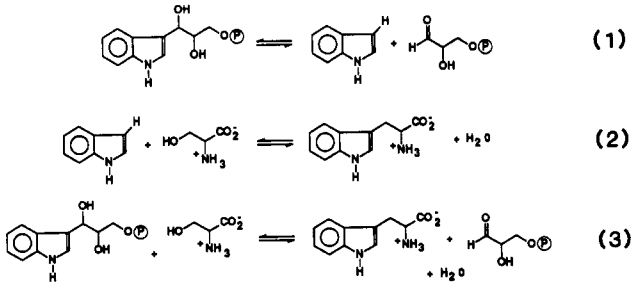
ABSTRACT: Tryptophan synthase from enteric bacteria is an $\alpha_2\beta_2$ bienzyme complex that catalyzes the final two reactions in the biosynthesis of L-tryptophan (L-Trp) from 3-indole-D-glycerol 3′-phosphate (IGP) and L-serine (L-Ser). The bienzyme complex exhibits reciprocal ligand-mediated allosteric interactions between the heterologous subunits [Houben, K., & Dunn, M. F. (1990) *Biochemistry 29*, 2421–2429], but the relationship between allostery and catalysis had not been completely defined. We have utilized rapid-scanning stopped-flow (RSSF) UV–visible spectroscopy to study the relationship between allostery and catalysis in the $\alpha\beta$-reaction catalyzed by the bienzyme complex from *Salmonella typhimurium*. The pre-steady-state spectral changes that occur when L-Ser and IGP are mixed simultaneously with the $\alpha_2\beta_2$ complex show that IGP binding to the α-site accelerates the formation of α-aminoacrylate [E(A-A)] from L-Ser at the β-site. Through the use of L-Ser analogues, we show herein that the formation of the E(A-A) intermediate is the chemical signal which triggers the conformational transition that activates the α-subunit. β-subunit ligands, such as L-Trp, that react to form covalent intermediates at the β-site, but are incapable of E(A-A) formation, do not stimulate the activity of the α-subunit. Titration experiments show that the affinity of G3P and GP at the α-site is dependent upon the nature of the chemical intermediate present at the β-active site. These results show that ligand-dependent allosteric interactions between heterologous subunits in the bienzyme complex serve to coordinate catalytic events at the α- and β-active sites to ensure the efficient synthesis of L-Trp. We propose that these ligand-dependent allosteric phenomena are accompanied by conformational transitions in both the α- and β-subunits between "open" and "closed" conformations that control ligand affinity and catalytic activity.

The $\alpha_2\beta_2$ tryptophan synthase bienzyme complex from enteric bacteria catalyzes the final two reactions in the biosynthesis of L-tryptophan (L-Trp).[1] The α-subunit (MW 29 000) of the bienzyme complex catalyzes the reversible aldolytic cleavage of 3-indole-D-glycerol 3′-phosphate (IGP) to D-glyceraldehyde 3-phosphate (G3P) and indole (α-reaction). The β-subunit (MW 43 000) catalyzes the condensation of indole with L-serine (L-Ser) in a pyridoxal phosphate (PLP)-dependent β-replacement reaction (β-reaction). The physiological $\alpha\beta$-reaction is the combination of the two individual reactions. The indole produced at the α-active site may then be used by the β-active site in the the synthesis of L-Trp. The mechanistic details of the β-reaction are outlined in Scheme I.



The α- and β-subunits are assembled as an almost linear structure in the $\alpha_2\beta_2$ bienzyme complex (Wilhelm et al., 1982; Ibel et al., 1985; Hyde et al., 1988; Hyde & Miles, 1990) with the two α-subunits separated by the β_2-dimer. The elegant crystal structure (Hyde et al., 1988) of the bienzyme complex shows that the α- and β-active sites are connected by a 25–30-Å tunnel of sufficient size to accommodate the direct passage of indole between the heterologous subunits. Recent rapid kinetic studies have demonstrated that the tunnel allows indole to be directly channeled between the α- and β-catalytic centers (see Figure 1; Dunn et al., 1987a,b, 1990, 1991; Lane & Kirschner, 1991; Anderson et al., 1991).

The available evidence indicates that the $\alpha\beta$-dimer forms a functional unit and reciprocal ligand-mediated allosteric interactions are observed between the heterologous α- and β-subunits (Hyde et al., 1988; Houben & Dunn, 1990; Kirschner et al., 1991). Only recently, however, have clues emerged as to the relationship between the observed allosteric phenomena and catalysis (Lane & Kirschner, 1983a,b, 1991; Kawasaki et al., 1987; Kirschner et al., 1991; Anderson et al., 1991; Dunn et al., 1987a,b, 1990, 1991; Houben & Dunn, 1990). The data presented here further delineate the interrelationship between ligand-mediated, heterotropic allosteric interactions and catalysis in the tryptophan synthase system. Herein we show that (1) IGP binding to the α-subunit increases the rate at which L-Ser reacts with the β-subunit; (2) only one discrete covalent intermediate along the β-reaction pathway, the electrophilic α-aminoacrylate [E(A-A)], is ca-

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[1] Abbreviations: PLP, pyridoxal phosphate; L-Ser, L-serine; L-Trp, L-tryptophan; IGP, 3-indole-D-glycerol 3′-phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerol phosphate; 2,3-DAPA, (D,L)-2,3-diaminopropionic acid; L-Cys, L-cysteine; RSSF, rapid-scanning stopped-flow; SWSF, single-wavelength stopped-flow; GPDH, glyceraldehyde 3-phosphate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetate; $\alpha_2\beta_2$, native tryptophan synthase from *S. typhimurium*; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; E(Q_1), E(Q_2), or E(Q_3), quinonoidal intermediates formed in the conversion of L-Ser and indole to L-Trp; E(A_{ex}), aldimine intermediates formed between the substrate amino acids and the PLP cofactor; E(GD), geminal diamine intermediate formed between the PLP cofactor, the amino group of the substrate, and the ϵ-amino group of Lys87.

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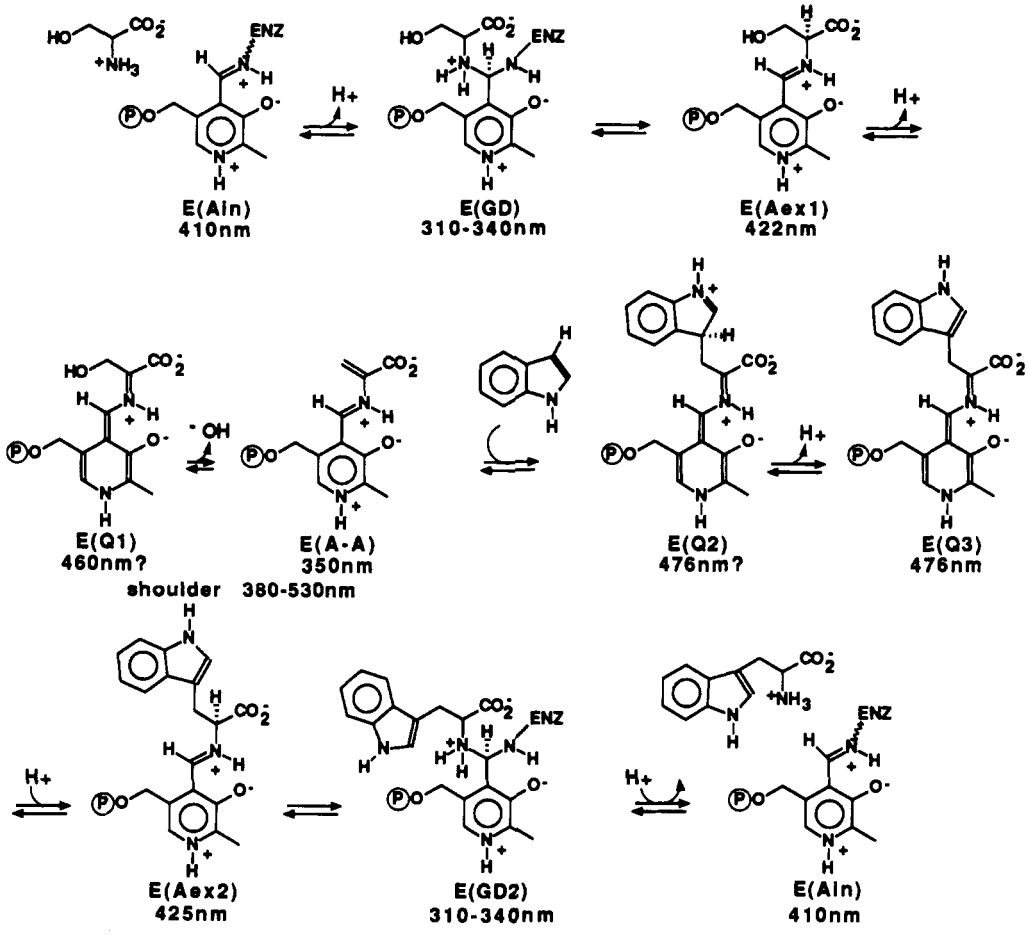
Scheme I: Reaction Mechanism of the PLP-Dependent β-Reaction Showing the Observed or Expected Positions of the UV–Visible Absorption Bands for the Individual Intermediates



pable of stimulating the cleavage of IGP at the α-active site; (3) subsequent catalytic events at the β-active site serve to deactivate the α-subunit; and (4) these findings indicate that the functional role of allosteric interactions in the tryptophan synthase bienzyme complex is to coordinate the catalytic activities of the α- and β-subunits, so that there is a one-to-one correspondence between IGP cleavage and the synthesis of L-Trp. A preliminary account of this work is presented in Dunn et al. (1991) and Brzović et al. (1991).

Materials and Methods

Materials. L-Ser, L-Trp, indole, L-Cys, *S*-Me-L-Cys, *O*-Me-D,L-Ser, β-Cl-L-Ala, 2,3-DAPA, G3P, NAD, and Bicine were purchased from Sigma. D,L-[α-^{2}H]Serine was prepared by the method of Miles and McPhie (1974). IGP was synthesized as previously described (Kawasaki et al., 1987). Purification of wild-type *Salmonella typhimurium* $\alpha_2\beta_2$ tryptophan synthase, determination of protein concentrations, and measurement of enzyme activity have been previously described (Kawasaki et al., 1987; Miles et al., 1987; Miles et al. 1989). GPDH was purchased from Sigma.

Enzyme Assays. The activity of the $\alpha_2\beta_2$ complex in the forward α- and αβ-reactions was monitored by the rate of G3P release in a coupled reaction with GPDH at 25 °C (Creighton, 1970). A second direct assay took advantage of absorbance differences between IGP, indole, and L-Trp at 290 nm. The cleavage of IGP to indole and G3P (α-reaction) is characterized by a decrease in absorbance at 290 mm ($\Delta\epsilon$ = 1.39 mM^{-1} cm^{-1}; Weischet & Kirschner, 1976b). The conversion of IGP to L-Trp (αβ-reaction) is characterized by an increase in absorbance at 290 nm ($\Delta\epsilon$ = 0.56 mM^{-1} cm^{-1}). The β-reaction, in which indole and L-Ser are converted to L-Trp, was also measured by a direct spectrophotometric assay at 290 nm (Miles et al., 1987).

UV–Visible and Fluorescence Titration Measurements. All equilibrium UV–visible spectral measurements were collected with a Hewlett-Packard 8450A diode-array spectrophotometer. Fluorescence titration experiments were performed on a SPEX Fluorolog 2 spectrofluorometer. The binding of L-Trp to the β-site, in the absence or presence of either GP or G3P, was followed by the change in absorbance at 476 nm. This spectral band corresponds to the accumulation of the L-Trp quinonoid. Binding of G3P and/or GP to the α-subunit with L-Ser or L-Trp bound at the β-site monitored the loss of the fluorescence at 506 nm (excitation at 420 nm) or the increase in absorbance at 476 nm, respectively. These spectral signals arise from the redistribution of intermediates at the β-site caused by the binding of ligands at the α-site (Houben & Dunn, 1990). Therefore, the calculated binding constants do not represent true dissociation constants, but only apparent dissociation constants. Binding isotherms were analyzed as described elsewhere (Houben & Dunn, 1990).

Stopped-Flow Kinetic and Spectral Measurements. SWSF and RSSF kinetic studies were performed as previously described (Dunn et al., 1979; Koerber et al., 1983; Drewe & Dunn, 1985, 1986; Brzović et al., 1990). Single-wavelength time courses were fit by nonlinear least-squares regression analysis to the sum of exponentials according to the following equation:

$$A_t = A_\infty \pm \sum_{i=1}^{n} A_i \exp(-t/\tau) \qquad (1)$$

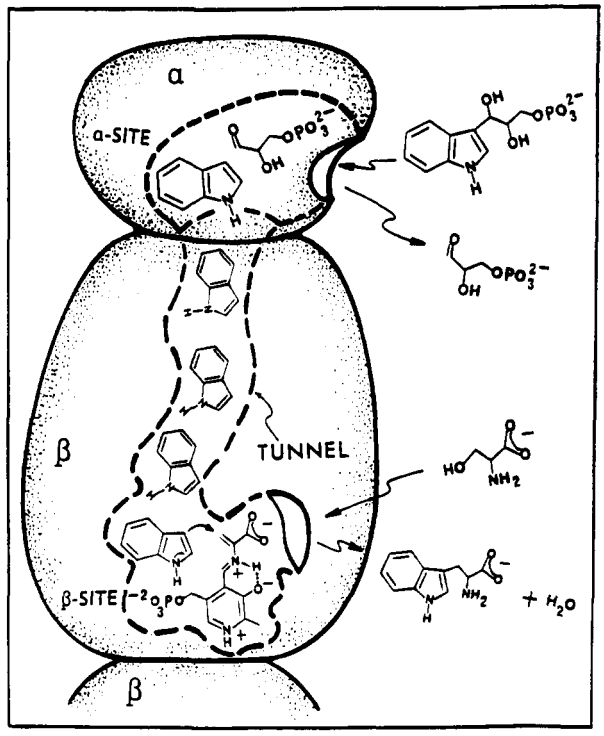


FIGURE 1: Cartoon depicting the orientation of the α- and β-subunits, the relative positions of the α- and β-active sites, and the interconnecting tunnel. IGP binds to the α-site and is cleaved to indole and G3P. L-Ser binds to the β-site and undergoes transformation to the electrophilic E(A-A). Indole, produced at the α-site, travels through the tunnel to the β-site and reacts with E(A-A) to ultimately yield L-Trp. G3P is released directly from the α-active site while L-Trp dissociates into solution from the β-active site.

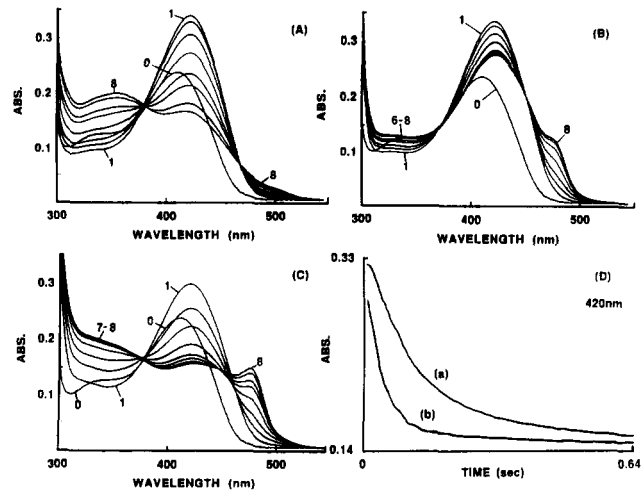


FIGURE 2: Time-resolved rapid-scanning stopped-flow spectra for the reactions of 10 mM $\alpha_2\beta_2$ from *S. typhimurium* with (A) 40 mM L-Ser, (B) 40 mM L-Ser and 0.5 mM indole, and (C) 40 mM L-Ser and 0.22 mM IGP. The native enzyme was in one syringe, the substrates were in the other. Spectra were recorded at 8.54, 25.63, 42.72, 76.9, 128.2, 179.4, 358.9, and 640.1 ms after flow had stopped. Spectrum 0 represents the spectrum of the enzyme in the absence of substrates. (D) Single-wavelength stopped-flow time courses at 420 nm are depicted for the reactions shown in panel A (spectrum a) and panel C (spectrum b). Time courses were derived from 74 successive RSSF spectra for a total acquisition time of 0.64 s with 8.54 ms between each data point. All concentrations refer to conditions immediately after mixing.

RESULTS

RSSF Characterization of the Reactions of L-*Ser, Indole, and IGP with the* $\alpha_2\beta_2$ *Complex.* The RSSF spectral changes that occur during the reaction of L-Ser with the $\alpha_2\beta_2$ complex are shown in Figure 2A (these spectra have been described in detail elsewhere; Drewe & Dunn, 1985; Brzovič et al., 1991, 1992a,b). The native enzyme (spectrum 0) is characterized by an absorbance band at 410 nm with a shoulder at approximately 340 nm. The 410-nm band arises from the internal aldimine Schiff base formed between the PLP cofactor and the ϵ-amino group of Lys87 at the β-active site. Upon mixing with a high concentration of L-Ser, a new intermediate with λ_{max} = 422 nm (spectrum 1) accumulates within the mixing dead time of the instrument ($1/\tau_1$). This highly fluorescent species (Goldberg et al., 1967; Miles & McPhie, 1974; Lane & Kirschner, 1983a; Drewe & Dunn, 1985, 1986) has been identified as the external aldmine [$E(A_{ex1})$] formed in a transimination reaction between the amino group of L-Ser and the enzyme-bound cofactor $E(A_{in})$ (Scheme I; Drewe & Dunn, 1985, 1986). This species subsequently decays in a biphasic process ($1/\tau_2 > 1/\tau_3$) to form the quasistable E(A-A). The final spectrum for the reaction of L-Ser with $\alpha_2\beta_2$ is composed of an absorbance band centered at 350 nm and an extended envelope of absorbance between 380 and 530 nm (Figure 2A, spectrum 8). The UV–visible spectral characteristics of the reaction suggest that the final spectrum is composed of an equilibrating mixture of covalently bound intermediates rather than a single species (Drewe & Dunn, 1985). The equilibrium may be shifted by modulation of the pH of the reaction medium (Mozzarelli et al., 1991) or by the addition of α-subunit-specific ligands such as GP (Dunn et al., 1987, 1990; Houben et al., 1989; Houben & Dunn, 1990; Kirschner et al., 1991; Mozzarelli et al., 1991). The initial rate of the decay of $E(A_{ex})$ ($1/\tau_2$ = 10 s^{-1}), monitored at 420 nm, has been shown to correspond to the rate of formation of the electrophilic E(A-A) (Lane & Kirschner, 1983a; Drewe & Dunn, 1985). The biphasicity of the decay process is thought to arise from the existence of multiple conformational states of the bienzyme complex (Drewe & Dunn, 1985; see Discussion).

Figure 2B shows the spectral changes that occur when the $\alpha_2\beta_2$ complex is mixed simultaneously with L-Ser and indole (Drewe & Dunn, 1985). Again, the external aldimine formed between L-Ser and the PLP cofactor rapidly accumulates. The decay of this species ($1/\tau_2$) is now concomitant with the accumulation of a new species absorbing at 476 nm. There is an apparent isoabsorptive point at 460 nm. The 476-nm band has been shown to correspond to the L-Trp quinonoid $E(Q_3)$ (Miles 1979; Lane & Kirschner, 1981, 1983b, 1991; Drewe & Dunn, 1986; Brzovič et al., 1992a), and this process monitors the rapid formation of a C–C bond between the C-3 carbon of the indole nucleophile and electrophilic β-carbon of E(A-A) (Scheme I). The rate of quinonoid formation under these experimental conditions ($1/\tau$ = 13 s^{-1}) is nearly identical to that of E(A-A) formation when indole is absent. Thus, in this experiment, E(A-A) formation limits the rate at which $E(Q_3)$ accumulates (Drewe & Dunn, 1986).

The amplitude change at 422 nm observed in Figure 2B is less than that observed when indole is absent (compare with Figure 2A). The apparent absorbance maximum undergoes a slight red shift from 422 to 425 nm as the reaction enters the steady state. When the $\alpha_2\beta_2$ complex is preequilibrated with L-Ser and then rapidly mixed with 0.5 mM indole, there is a very rapid increase in absorbance at 476 nm ($1/\tau$ = 150 s^{-1}) corresponding to the formation of $E(Q_3)$ (see Table II; Drewe & Dunn, 1986; Brzovič et al., 1992a). This process is followed by the accumulation of the species with λ_{max} = 425 nm resulting in a steady-state spectrum identical to that shown in Figure 2B. Therefore, the 425-nm band is not due to a stabilization of the L-Ser $E(A_{ex})$ during the β-reaction. In fact,

Table I: Effect of Various β-Ligands on the Steady-State Rate of the α-Reaction

reaction	analogue	concn[a] (mM)	TON[b] (s^{-1})	%
α (forward)	none		0.121	100
	L-His	50	0.117	97
	L-Trp	10	0.103	85
	D-Trp	10	0.080	66
	glycine	400	0.188	155
	reduced $\alpha_2\beta_2$		0.125	103
	reduced $\alpha_2\beta_2$ + L-Ser	40	0.127	105
α (reverse)	none		4.7	100
	L-His	50	6.5	138
	L-Ala	300	4.87	104
	glycine	400	4.03	86
	L-Trp	1	2.83	60
αβ	L-Ser	40	3.35	100
	D,L-[α-^{2}H]Ser	40	2.33	70
	O-Me-D,L-Ser	40	1.60	48
	S-Me-L-Cys	40	0.674	20
	2,3-DAPA	40	0.33	9.9

[a] Concentration of β-ligand in the reaction mixture. [b] Standard error ±10%.

Table II: Summary of Relaxation Rate Constants Observed during the Initial Pre-Steady-State Phase of the Formation of the L-Tryptophan Quinonoid

no.	reaction	rate, $1/\tau_1$[a] (s^{-1})
1	indole + E(A-A)	150
2	indole + E(A-A), GP	13
3	indole + E(A-A), G3P	3
4	IGP + E(A-A)	36

[a] Standard error ±15%. The kinetics of L-Trp quinonoid formation under various experimental conditions was followed by monitoring the rate of absorbance increase at 476 nm. In each experiment, $\alpha_2\beta_2$ premixed with 40 mM L-Ser was placed in one syringe and either indole of IGP was in the other syringe. Concentrations in reactions: (1) 20 μM E(A-A) and 0.5 mM indole; (2) 20 μM E(A-A), 100 mM GP, and 0.5 mM indole; (3) 20 μM E(A-A), 5 mM G3P, and 0.5 mM indole; (4) 20 μM E(A-A) and 0.5 mM IGP. L-Ser and either GP or G3P, when present, were premixed in both syringes in order to maintain constant concentrations of these ligands. All concentrations refer to conditions immediately after mixing.

release of L-Trp has been shown to limit the rate of the β-reaction (Lane & Kirschner, 1981, 1983b). Since L-Ser and L-Trp cannot simultaneously occupy the β-active site, the 425-nm band must represent the accumulation of the external aldimine, $E(A_{ex2})$, of L-Trp (Drewe & Dunn, 1986; Drewe et al., 1989).

To assess the influence of IGP on the reaction of L-Ser at the β-active site, we undertook a RSSF study to characterize the UV–visible spectral changes which occur during the pre-steady-state phase of the αβ-reaction. Mixing the $\alpha_2\beta_2$ complex simultaneously with L-Ser and IGP results in a markedly different set of spectral changes (Figure 2C). The rate of decay of $E(A_{ex_1})$ is increased at least 3-fold ($1/\tau_2 = 30\ s^{-1}$) relative to that observed for the reaction with L-Ser alone or in the β-reaction (Figure 2D). Comparison of panels B and C in Figure 2 shows that the steady-state distribution of accumulated intermediates in the αβ-reaction is altered compared to the β-reaction. The decrease in amplitude at 420 nm is much larger than that observed during the β-reaction, indicating that less $E(A_{ex2})$ accumulates in the steady-state phase of the reaction. There is also a much larger absorbance increase at 350 nm in the αβ-reaction, a process that is concomitant with the decay of $E(A_{ex1})$. Under the experimental conditions shown, the $E(Q_3)$ band at 476 nm accumulates at a rate of $1/\tau = 26\ s^{-1}$. In contrast to the β-reaction, no apparent isoabsorptive point is observed in the 460-nm region during the course of the αβ-reaction. This observation is consistent with the difference in rates observed at 350 nm and 476 nm.

Effect of L-*Ser*, L-*Trp, and Amino Acid Analogues on the Steady-State Rate of both the Forward and Reverse α-Reactions.* The elegant studies of Kirschner et al. (1991) have demonstrated that the binding of L-Ser to the β-active site activates the rates of both the cleavage and the synthesis of IGP catalyzed by the α-subunit. However, from these data it is unclear what physical or chemical event during the reaction of L-Ser with the β-subunit triggers the activation of the α-reaction. In order to identify which catalytic events at the β-site are responsible for the activation of catalysis at the α-site, we have investigated the effects of a series of β-specific ligands on the activity of both the forward and reverse reactions catalyzed by the α-subunit. In our hands, we observed a 27-fold stimulation in the rate of the α-reaction in the presence of L-Ser at 25 °C (Table I) [see also Kirschner et al. (1991) and Kawasaki et al. (1987)]. Table I shows that amino acid substrates such as Gly, L-Ala, L-His, L-Trp, and D-Trp, which react at the β-active site to form Michaelis, E(GD), $E(A_{ex})$, and E(Q), intermediates (Houben et al., 1989; Houben & Dunn, 1990; Miles, 1980; P. S. Brzovič and M. F. Dunn, unpublished observations) *do not* appreciably affect the rate of either the forward or the reverse α-reactions. L-Trp and D-Trp appear to weakly inhibit the α-reaction. These quasi-substrates lack a suitable β-substituent and cannot undergo β-elimination to form E(A-A). UV–visible spectra collected during the steady state of the α-reactions described above show the presence of the same β-subunit-bound PLP intermediates that have been previously described (Dunn et al., 1990; Houben et al., 1989; Houben & Dunn, 1990). $NaBH_4$ reduction of $E(A_{in})$ gives an unreactive pyridoxamine form of the cofactor that is covalently linked to Lys87 in the β-active site (Hathaway et al., 1969). This tetrahedral form of the PLP cofactor does not stimulate the α-reaction, even in the presence of L-Ser. Therefore, activation of the α-subunit cannot be attributed to any other possible allosteric interactions between L-Ser and the $\alpha_2\beta_2$ complex.

Table I shows that the substrate analogues of L-Ser (D,L-[α-^{2}H]Ser; *O*-Me-D,L-Ser; *S*-Me-L-Cys; and 2,3-DAPA) *do* stimulate the rate of the α-reaction. The extent of activation is dependent on the structure of the L-Ser analogue, and we have found that the ability of a particular substrate to enhance the rate of IGP turnover is roughly proportional to the rate at which these substrates react to form E(A-A) (Brzovič et al., 1991). Steady-state experiments that directly monitor the conversion of IGP to L-Trp (see Materials and Methods) failed to detect any significant accumulation of indole when these substrate analogues are utilized for the αβ-reaction. Furthermore, steady-state experiments also establish that activation is dependent upon protein–protein interactions within the αβ-dimer. When channeling is uncoupled and diffusion of indole through solution is compulsory for the synthesis of L-Trp, then L-Ser is unable to enhance the rate of the α-reaction (Yanofsky, 1957; Yanofsky & Rachmeler, 1958; P. S. Brzovič and M. F. Dunn, unpublished results) even though E(A-A) is present in solution.

Influence of GP and G3P on the Rate of Quinonoid Formation from Indole. When 0.5 mM indole is mixed with $\alpha_2\beta_2$ that has been preequilibrated with L-Ser, the rate of quinonoid formation is very rapid ($1/\tau = 150\ s^{-1}$; Table II). *Table II shows that GP and G3P, when bound to the α-active site, inhibit the rate of quinonoid formation by 12-fold and 50-fold,*

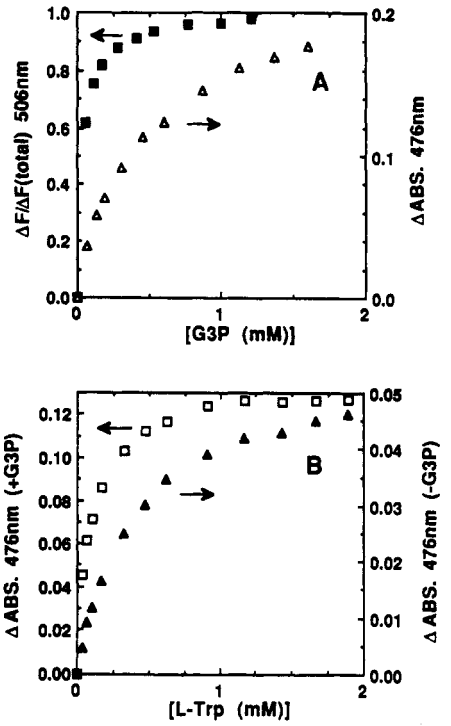


FIGURE 3: (A) Equilibrium binding isotherm for the binding of G3P either to the $\alpha_2\beta_2$–serine complex (left ordinate) or to the enzyme–L-Trp complex (right ordinate). The binding of G3P to the $\alpha_2\beta_2$ complex in the presence of L-Ser monitored the loss in fluorescence at 506 nm (ex 420 nm; see Materials and Methods). $[\alpha_2\beta_2]$ = 3.3 μM, [L-Ser] = 33 mM. The binding of G3P in the presence of L-Trp was monitored by the change in absorbance at 476 nm (see Materials and Methods) $[\alpha_2\beta_2]$ = 26.7 μM, [L-Trp] = 10 mM. (B) Equilibrium binding isotherm for the binding of L-Trp to the $\alpha_2\beta_2$ complex in the absence (left ordinate) or presence (right ordinate) of G3P. In both cases, the binding of L-Trp was monitored by following the change in absorbance at 476 nm. For L-Trp binding in the presence of G3P, $[\alpha_2\beta_2]$ = 13.4 μM, [G3P] = 5 mM. In the absence of G3P, $[\alpha_2\beta_2]$ = 13.4 μM.

respectively. However, when indole is produced from the cleavage of IGP at the α-active site, the rate of quinonoid formation at the β-site is considerably faster (Table II; Lane & Kirschner, 1991; Anderson et al., 1991) and is not subject to the inhibitory effects of G3P. Since indole can react to form a covalent bond with E(A-A) at a rate in excess of 200 s^{-1} (Lane & Kirschner, 1983b; Dunn et al., 1990), the rate of quinonoid formation when indole is derived directly from IGP is likely to be limited by the rate of IGP cleavage at the α-active site (Lane & Kirschner, 1991).

Reciprocal Allosteric Interactions between the α- and β-Subunits Mediated by Ligand Binding. Figure 3 shows that the apparent affinity of ligands at either the α- or β-site is greatly influenced by the nature of the ligand or chemical species present at the heterologous active site. The binding of GP and G3P to the α-site was monitored by following the α-ligand-induced redistribution of PLP intermediates at the β-site (see Materials and Methods). Figure 3A shows that the G3P-induced spectral changes saturate at a much lower G3P concentration when E(A-A) is present at the β-site than when L-Trp-derived intermediates are bound. The apparent affinity of the nonreactive substrate analogue GP to the α-subunit is also greatly dependent on the nature of the chemical species present at the β-site (Table III). Furthermore, Figure 3B shows that the affinity of L-Trp for the β-site, and the accumulation of the L-Trp quinonoid (Houben & Dunn, 1990; Kirschner et al., 1991), is significantly lower in the absence of G3P.

Table III: Summary of Equilibrium Data for the Interaction of G3P, GP, and/or L-Trp with the Tryptophan Synthase Bienzyme Complex[a]

no.	concentrations	ligand varied	K_d(app) (mM)
1	$[\alpha_2\beta_2]$ = 27.4 μM	L-Trp	$K_{d(L\text{-}Trp)}$ = 0.330
2	$[\alpha_2\beta_2]$ = 13.4 μM [G3P] = 5 mM	L-Trp	$K_{d(L\text{-}Trp)}$ = 0.065
3	$[\alpha_2\beta_2]$ = 13.4 μM [GP] = 50 mM	L-Trp	$K_{d(L\text{-}Trp)}$ = 0.026
4	$[\alpha_2\beta_2]$ = 3.33 μM [L-Ser] = 33 mM	G3P	$K_{d(G3P)}$ = 0.038
5	$[\alpha_2\beta_2]$ = 1.33 μM [L-Ser] = 33 mM	GP	$K_{d(GP)}$ = 0.027
6	$[\alpha_\infty\beta_2]$ = 26.7 μM [L-Trp] = 10 mM	G3P	$K_{d(G3P)}$ = 0.430
7	$[\alpha_2\beta_2]$ = 26.7 μM [L-Trp] = 10 mM	GP	$K_{d(GP)}$ = 0.370

[a] Equilibrium data were analyzed as described elsewhere (Houben & Dunn, 1990). Measured equilibrium constants are assumed to be determined with an accuracy of ±25%.

Table IV: Effect of Ligands on the Rate of Quenching of the PLP Cofactor by KOH at 25 °C

reaction	rate[a]
$\alpha_2\beta_2$, L-Ser + indole, KOH	175 s^{-1}
$\alpha_2\beta_2$, L-Trp, GP + KOH	50 s^{-1}
$\alpha_2\beta_2$, L-Ser, GP + KOH	27 s^{-1}

[a] Standard error ±15%. Reaction time courses, derived from successive RSSF spectra, monitored the loss of absorbance at 476 nm. This wavelength corresponds to the λ_{max} of the L-Trp quinonoid and to the broad absorbance band that is characteristic of the E(A-A) spectrum. RSSF spectra (data not shown) show that spectral changes at 476 nm are well resolved from subsequent spectral changes at shorter wavelengths that occur as free PLP is liberated into solution. RSSF spectra do not show large spectral changes occurring within the mixing dead time or any significant light scattering occurring on the experimental time scales. Concentrations of reagents, when present, immediately after mixing: $[\alpha_2\beta_2]$ = 10 μM, [L-Ser] = 40 mM, [L-Trp] = 5 μM, [KOH] = 0.225 N, and [D,L-GP] = 50 mM. Enzyme was present in one syringe and KOH was in the other. L-Ser, L-Trp, and GP, when present, were in both syringes in order to maintain constant concentrations.

The effects of heterologous ligands on the changes in apparent binding affinities are summarized in Table III. The affinity of G3P for the α-site is over 11-fold greater when E(A-A) is present than when L-Trp intermediates are bound at the β-site. Similar results were also obtained for the binding of GP to the α-subunit in the presence of either L-Ser or L-Trp. Furthermore, Table III shows that the apparent affinity of L-Trp at the β-site is decreased over 5-fold from 65 μM in the presence of G3P to 330 μM in the absence of G3P. When GP is the α-subunit ligand, the apparent affinity of L-Trp changes by nearly 13-fold.

Effects of Ligands on the Rate of KOH Quenching. Certain conclusions drawn by Anderson et al. (1991) about the mechanism of indole channeling are dependent upon the assumption that rapid mixing of $\alpha_2\beta_2$-substrate mixtures with 0.2 M KOH gives instantaneous chemical quenching of reactions involving enzyme and substrates. Using RSSF spectroscopy, we undertook a series of KOH quenching studies to determine the rate of KOH-induced alterations of the PLP chromophore spectrum in the presence of various substrates and/or ligands. The results of these studies are reported in Table IV. These data show that at 25 °C the KOH-induced spectral perturbations at 476 nm are slow relative to the experiment dead time (~4 ms), and the observed rates are very dependent both upon the nature of the ligands bound to the $\alpha_2\beta_2$ complex and upon the chemical form of bound PLP. For example, the E(A-A) (GP) complex is quenched at a rate that

is nearly 6-fold slower than is the $\alpha_2\beta_2$–L-Ser–indole system.

Discussion

Protein–protein and ligand–protein interactions in tryptophan synthase are of primary importance for the function of the bienzyme complex. Conformational changes in the subunits which occur upon assembly of the complex (Lane et al., 1984) serve to increase substrate binding affinities, alter the thermodynamic stability of reaction intermediates, and change the rate of chemical steps along the catalytic pathway (Kirschner et al., 1991; Miles & McPhie, 1974; Fader & Hammes, 1970, 1971; Houben et al., 1989; Drewe & Dunn, 1985; York, 1972). The end result is that protein–protein interactions in the assembled bienzyme complex serve to increase the catalytic activities of the individual α- and β-subunits by 100- and 50-fold, respectively. Secondly, the binding of heterologous ligands to the bienzyme complex and the formation of discrete covalent intermediates result in the transmission of allosteric information over a distance of 30 Å and across an enzyme–enzyme interface (Lane & Kirschner, 1983a, 1991; Kirschner et al., 1991; Dunn et al., 1987a,b, 1990; Houben et al., 1989; Houben & Dunn, 1990). As argued below, reciprocal allosteric interactions between the heterologous subunits in the bienzyme complex coordinate the catalytic activites of the individual enzymes for the efficient synthesis of L-Trp.

Stimulation of E(A-A) Formation by IGP. When the $\alpha_2\beta_2$ bienzyme complex is rapidly mixed simultaneously with L-Ser and either indole or IGP, the external aldimine of L-Ser, $E(A_{ex1})$, accumulates as a common intermediate during both the β- and $\alpha\beta$-reactions (compare Figure 2A–C). As described above (see Results), the initial decay ($1/\tau_2$) of this species corresponds to the rate of formation of the highly reactive, electrophilic E(A-A). As shown in Figure 2B, the presence of indole alone does not significantly affect the rate at which L-Ser reacts with the β-subunit. When IGP is mixed simultaneously with L-Ser, there is a 3-fold enhancement in the rate of E(A-A) formation ($1/\tau_2 = 30\ s^{-1}$; Figure 2C,D). Therefore, IGP binding to the α-active site increases the rate of E(A-A) formation from L-Ser at the β-active site. Preequilibration of the $\alpha_2\beta_2$ complex with the α-specific ligand GP results in an 8-fold increase in the rate of E(A-A) formation ($1/\tau_2 = 80\ s^{-1}$) and shifts the final equilibrium distribution of L-Ser reaction intermediates toward E(A-A) (Dunn et al., 1990; Kirschner et al., 1991; Brzovič et al., 1992a,b). It has been shown that α-specific ligands such as G3P, IPP, and GP and presumably IGP induce an isomerization in the α-subunit upon binding (Houben & Dunn, 1990; Dunn et al., 1990; Kirschner et al., 1991; Anderson et al., 1991). This conformational transition increases the apparent affinity of the β-site for L-Ser and alters the equilibrium for the reaction in favor of E(A-A) (Kirschner et al., 1991). This effect increases the fraction of sites in the form of E(A-A), thereby increasing the probability for reaction with indole (Houben & Dunn, 1990).

α-Aminoacrylate Formation Is the Trigger Which Activates IGP Cleavage at the α-Active Site. As shown in Table I, only substrates which can react with the PLP cofactor and, ultimately, undergo β-elimination to form an E(A-A) intermediate are able to stimulate the rate of the α-reaction. During the reaction of L-Ser and indole at the β-active site, at least eight covalent intermediates are formed that differ in chemical structure (Scheme I). Efficient catalysis would seem to necessitate the recognition and stabilization of multiple transition states and intermediates during the course of the β-reaction. This proposal requires that changes in the structure of covalent intermediates be accompanied by corresponding changes in the conformation of the β-active site (Drewe & Dunn, 1986; Dunn et al., 1987a; Roy et al., 1988a,b; Houben & Dunn, 1990). It is reasonable to assume that intermediates formed in the reactions of analogues with the β-subunit elicit similar changes in the conformation of the β-subunit. If activation of the α-reaction occurred at a step preceding E(A-A) formation, then analogues of L-Ser which give a distribution of the Michaelis complex, E(GD), $E(A_{ex})$ and E(Q) intermediates, should stimulate the α-reaction, and the extent of activation should be proportional to the fraction of the $\alpha_2\beta_2$ complex in that conformation which activates the α-reaction. However, L-Ser analogues that are incapable of undergoing β-elimination do not stimulate the α-reaction in either the forward or reverse directions.

Those L-Ser substrate analogues that form E(A-A) *do* stimulate the α-reaction, but to various extents (Table I; Brzovič et al., 1991). Furthermore, we do not detect any accumulation of indole during the course of the $\alpha\beta$-reaction when these L-Ser analogues are used. This finding shows that the rate of the α-reaction is closely coordinated with certain covalent events occurring at the β-active site. Secondly, the overall rate of the $\alpha\beta$-reaction appears to be correlated to the rate of E(A-A) formation (Brzovič et al., 1991; Anderson et al., 1991). Therefore, we conclude that it is only the unique covalent structural changes which accompany E(A-A) formation that trigger the conformational change of the β-active site resulting in the activation of the α-subunit.[2]

Role of G3P in the $\alpha\beta$-Reaction. Lane and Kirschner (1991) have demonstrated that G3P, formed from the cleavage of IGP, is released from the α-active site after the reaction of indole with E(A-A) occurs at the β-site. Therefore, G3P can influence the catalytic events at the β-site during the course of the $\alpha\beta$-reaction. Comparison of the pre-steady-state spectral changes for the β- and $\alpha\beta$-reactions confirms this expectation. The $\alpha\beta$-reaction (Figure 2C) shows an increased accumulation of $E(Q_3)$ (476 nm) relative to $E(A_{ex_2})$ (425 nm) as compared to the β-reaction (Figure 2B). This is consistent with the observation that both GP and G3P, when bound to the α-site, increase the stability of the $E(Q_3)$ relative to other species when L-Trp is bound at the β-site (Houben & Dunn, 1990; Dunn et al., 1990; Kirschner et al., 1991).

Secondly, after IGP is cleaved, bound G3P serves to prevent the dissociation of indole from the α-site into solution. Table II shows that both GP and G3P, when preequilibrated with the $\alpha_2\beta_2$–serine complex, significantly inhibit the rate of L-Trp quinonoid formation in the β-reaction. However, when indole

[2] Implicit in the above arguments is the assumption that allosteric information is transmitted via protein conformational changes. The rate of the protein conformational change and whether this process involves a gross change in the quaternary structure of the $\alpha_2\beta_2$ complex is not clearly defined. Observed changes in the intrinsic protein fluorescence (λ_{ex} = 290 nm, λ_{em} = 340 nm) of the $\alpha_2\beta_2$ complex have been shown to occur at a rate identical to that of E(A-A) formation, indicating a concerted process (Anderson et al., 1991). This is a very attractive hypothesis. However, the interpretation of protein fluorescence changes in tryptophan synthase is complicated both by the absorbance spectra of the cofactor and covalent PLP reaction intermediates at the β-active site (Figure 2A) and by the existence of fluorescence energy transfer between the single tryptophan residue in the β-chain and the PLP cofactor (Lane, 1983). Nonetheless, if initial observations are confirmed, fluorescence studies may provide important information on the role of conformational changes in modulating the function of the tunnel. The single tryptophan residue in the β-subunit is located at the end of α-helix 6 (Hyde et al., 1988). Residues from this helix make structural contributions that form one side of the tunnel wall within the β-subunit. Conformational changes in this region could alter the shape of the interconnecting tunnel so that E(A-A) formation not only activates cleavage at the α-site but also changes the shape of the tunnel, providing for greater access of indole to the β-site.

is produced in situ from the cleavage of IGP, the rate of $E(Q_3)$ formation is much faster. Since the condensation of indole with E(A-A) saturates at a rate of 200 s^{-1} (Dunn et al., 1990), the rate of quinonoid formation in the $\alpha\beta$-reaction is limited by the rate of IGP cleavage at the α-site (Lane & Kirschner, 1991). Thus, the GP- and G3P-induced inhibition is not caused by a ligand-induced conformational change that occludes the tunnel or constricts the β-site. Instead, GP or G3P binding to the α-site alters the conformation of the α-subunit from an open to a closed structure which prevents the direct access of indole into the tunnel and the β-active site via the α-site entrance (Dunn et al., 1990; Brzovič et al., 1992b). This is consistent with the finding of Anderson et al. (1991) that IGP binding to the α-subunit induces a protein isomerization that is necessary for cleavage of IGP. The change from an open to a closed conformation is also important for changing the ground-state stabilities of intermediates at the the β-site (Figures 2C and 3B; Houben & Dunn, 1990; Kirschner et al., 1991; Brzovič et al., 1992b).

Because G3P effectively prevents the entry of indole into the tunnel, the principle of microscopic reversibility demands that bound G3P help prevent indole, sequestered within the $\alpha_2\beta_2$ complex, from escaping into solution from the α-site. Since indole is confined to the α-site, the β-site, and the tunnel, the rate of indole diffusion between active sites, and the subsequent formation of a C–C bond between indole and E(A-A), is likely to be very fast.[3] Therefore, G3P bound to the closed state of the α-subunit provides a physical mechanism which promotes the channeling of indole to the β-active site.

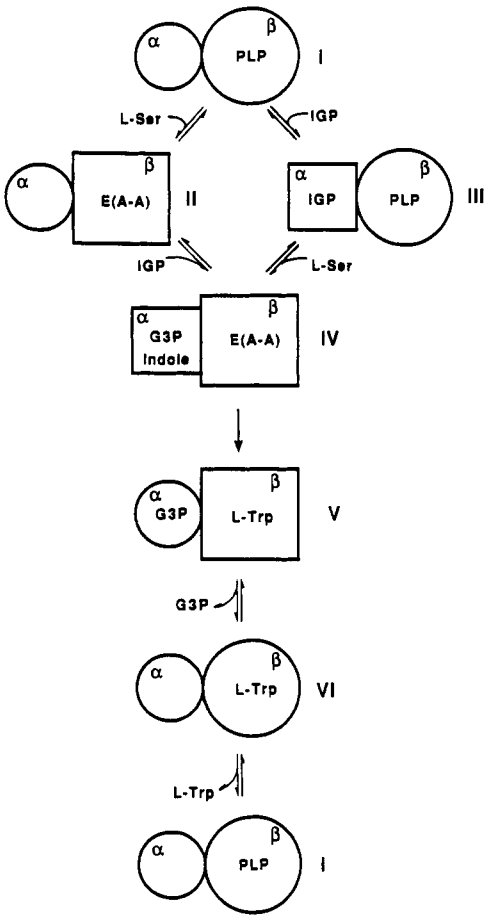
The hypothesis that bound G3P prevents the escape of indole is consistent with the finding that G3P binds very tightly to the E(A-A) complex (Figure 3A; Table III). Figure 3A shows that when L-Trp is bound at the β-site the apparent affinity of G3P for the α-site is decreased by over 11-fold (Figure 3A, Table III).[4] Since L-Trp and L-Trp analogues which form quinonoidal intermediates are incapable of stimulating the α-reaction (Table I), we propose that it is the formation of $E(Q_3)$ that serves as the chemical signal that is transmitted to the α-site and alters the binding affinity for G3P. This protein isomerization would also serve to deactivate the α-subunit. In turn, the dissociation of G3P decreases the apparent affinity of L-Trp at the β-site by at least 5-fold (Figure 3B, Table III). Therefore, G3P, formed from the cleavage of IGP, is bound tightly to the E(A-A) complex until indole has reacted to form $E(Q_3)$. The formation of $E(Q_3)$ induces a conformational change in the $\alpha_2\beta_2$ complex that decreases the affinity of the α-site for G3P and deactivates the α-subunit.

Rapid chemical quench-flow experiments to test the channeling hypothesis have recently been performed on the β- and $\alpha\beta$-reactions (Anderson et al., 1991). These experiments failed to detect measurable quantities of indole during the physiological $\alpha\beta$-reaction, a finding in support of the channeling hypothesis. Analysis of kinetic data for the measured rates of IGP depletion and L-Trp production was accomplished by iterative computer fitting of the observed time courses to an assumed reaction path for the $\alpha\beta$-reaction. As a consequence of this analysis, these authors propose that the efficiency of channeling is primarily a function of a kinetic partitioning due to the rapid rate of diffusion through the channel ($k \approx 1000$ s^{-1}) and the rapid and irreversible reaction of indole with E(A-A) ($k \approx 1000$ s^{-1}) to form tryptophan at the β-site. They hypothesize that both of these processes are faster than the direct release of indole into solution from the α-active site, a rate they were only able to estimate in the absence of L-Ser. However, they offer no explanation for the hypothesized slow rate of indole dissociation. As described above, kinetic factors undoubtedly play an important role in the efficacy of indole channeling, but the model of Anderson et al. (1991) fails to provide a physical basis that rationalizes why channeling is favored over the dissociation of indole into solution. Furthermore, it should be recognized that the value of $k \approx 1000$ s^{-1} proposed for the L-Trp formation at the β-site is based on assumptions which may not be valid. The α- and β-subunits of the bienzyme complex are moderately stable in alkaline solutions (Table IV). Therefore, KOH quenching of reactions occurring at the β-active site is the consequence of nucleophilic attack by hydroxide ion on the PLP cofactor, ultimately liberating free PLP into solution. These processes, contrary to the assumptions of Anderson et al. (1991), should not be instantaneous. Our RSSF studies of the spectral changes that accompany the quenching reaction confirm this prediction (Table IV). It is also well established that ligand binding stabilizes the $\alpha_2\beta_2$ complex and can restrict access of small solute molecules into the β-active site (Lane & Kirschner, 1983c; Lane, 1983; Kawasaki et al., 1987; Dunn et al., 1987, 1990). RSSF studies show that the rate of quenching by 0.225 N hydroxide ion, as measured by the disappearance of spectral bands which characterize covalent PLP reaction intermediates, depends on the presence of bound ligands (Table IV). Therefore, during the course of the $\alpha\beta$-reaction, when ligands are bound at both the α- and β-sites, it appears unlikely that chemical quenching with hydroxide is faster than certain chemical transformations that occur during the β-reaction. These circumstances makes it difficult both to determine by quench-flow methods whether or not indole accumulates in the tunnel during the reaction and to accurately determine the rates of particular catalytic processes. We must emphasize [particularly in light of other recent publications: Dunn et al. (1990) and Lane and Kirschner (1991)] that the above arguments do not affect the qualitative conclusion that indole is channeled between heterologous active sites in the $\alpha_2\beta_2$ complex. However, at present, detailed, quantitative interpretations based upon the determination of reaction rates using rapid-quench data must be approached with some caution. Undoubtedly, these questions will be resolved by further experimentation.

[3] Assuming that the tunnel defines a cylinder approximately 30 Å long and 10 Å in diameter, then the concentration of a single indole molecule within the tunnel is 0.7 M (assuming that the indole molecule diffuses freely within the tunnel). This very high effective concentration would ensure that the rates of chemical processes at the β-active site during the second phase of the β-reaction would be saturated. At the other extreme, if indole is not constrained from escaping into solution from the α-active site, the effective concentration is only equal to the initial concentration of IGP present in the reaction mixture.

[4] These results are in agreement with preliminary, single-turnover SWSF studies which monitored the rate of G3P release from the α-site either from the E(A-A) complex or during the $\alpha\beta$-reaction. G3P dissociation from the α-site of the *Salmonella* enzyme in 50 mM Bicine, pH 7.8, was measured as described by Lane and Kirschner (1991) with the concentration of $\alpha\beta$-sites and L-Ser in excess over either G3P or IGP. The dissociation of G3P from the L-Trp complex was not measured because the apparent K_d is so large (Table III). For the $\alpha\beta$-reaction, we found that G3P release from the α-site occurred at a rate of nearly 5 s^{-1} but was preceded by a pronounced lag phase of approximately 8 s^{-1}. The rate of $E(Q_3)$ formation under these conditions was 26 s^{-1}. Therefore, the reaction of indole with E(A-A) to form $E(Q_3)$ at the α-site is essentially complete before G3P dissociates from the α-site (Lane & Kirschner, 1991). Secondly, G3P dissociation from the E(A-A) complex is much slower (k_{obs} = 0.32 s^{-1}) than observed during the $\alpha\beta$-reaction. These results are consistent with the interpretation that G3P binds tightly to the closed conformation of the α-subunit when E(A-A) is present at the β-site and that formation of $E(Q_3)$ at the β-site induces a conformational change which facilitates the release of G3P from the α-site. A complete account of this work will be presented elsewhere.

Scheme II: Model Depicting Ligand- or Intermediate-Induced Conformational Changes in the $\alpha_2\beta_2$ Complex That Occur during the Course of the $\alpha\beta$-Reaction[a]



[a] Circles indicate open conformational states, and squares indicate closed conformational states.

A second point concerning the interpretation of rapid-quench experiments is that quenching of the β-reaction at any point following nucleophilic attack by indole will almost certainly result in the release of free L-Trp as well as free PLP into solution. The chemical structures of covalent PLP reaction intermediates (depicted in Scheme I) will be labile to quenching in KOH. Therefore, none of the covalent species, which have been shown to accumulate during both the β- and $\alpha\beta$-reactions (Figure 2; Drewe & Dunn, 1985, 1986; Dunn et al., 1987a,b, 1990, 1991; Lane & Kirschner, 1981, 1983a,b, 1991; York, 1972; Faeder & Hammes, 1970, 1971, Goldberg & Baldwin, 1967), would be detected in the experiments reported by Anderson et al. (1991). Thus, the rapid synthesis of L-Trp, as described by Anderson et al. (1991), almost certainly represents the rapid formation of a covalent C–C bond between indole and E(A-A) rather than the complete synthesis of L-Trp.

Coordination of the α- and β-Catalytic Activities by Allosteric Interactions. The compiled data (this manuscript; Brzovič et al., 1991; Dunn et al., 1987a,b, 1990; Houben & Dunn, 1990; Kawasaki et al., 1987; Anderson et al., 1991; Lane & Kirschner, 1983a,b, 1991; Kirschner et al., 1991) provide a fascinating picture of the coordinated functioning of the α- and β-subunits during catalysis. These findings are summarized in Scheme II. Both the α- and β-subunits undergo conformational changes during catalysis and, therefore, must exist in at least two conformational states which we designate as open (circles) and closed (squares) forms. Furthermore, we assume that the open and closed structures may preexist in solution, with the open structures favored in the absence of ligands. This would account for the biphasicity of E(A-A) formation from L-Ser (see Results; Drewe & Dunn, 1985), the biphasicity of IGP synthesis under single-turnover conditions (Anderson et al., 1991), and the biphasic addition of certain nucleophiles to the E(A-A) complex (Dunn et al., 1987a,b, 1991). Secondly, we show a subset of the possible equilibria in Scheme II. We depict only those steps necessary to explain the experimental data presented herein.

Scheme II shows L-Ser and IGP can bind to the $\alpha_2\beta_2$ complex (structure I) in random order. The reaction of L-Ser with the PLP cofactor to form E(A-A) induces a conformational change in the β-subunit (structure II). Through heterotropic interactions, this conformational transition serves to increase the affinity of the α-site for IGP by facilitating the conversion of the α-subunit from the open structure to the catalytically-active, closed conformation. Alternatively, when IGP binds to the α-site (structure III), the reciprocal allosteric interaction stimulates the reaction of L-Ser at the β-site and the formation of the fully closed $\alpha_2\beta_2$ complex (IV). Consequently, structure IV is favored in the presence of both α- and β-subunit ligands.

In the fully closed structure (IV), IGP is rapidly cleaved to G3P and indole. Indole is prevented from escaping the $\alpha_2\beta_2$ complex both by the presence of bound G3P and by the closed conformation of the α-subunit. Instead, indole diffuses rapidly through the tunnel to the β-site where it undergoes the essentially irreversible reaction with E(A-A) to form $E(Q_3)$. (The L-Trp depicted in Scheme II represents L-Trp and L-Trp-derived intermediates at the β-site.) The formation of $E(Q_3)$ at the β-site is accompanied by a protein isomerization that lowers the affinity of G3P for the α-site (structure V). The release of G3P from the bienzyme complex, in turn, facilitates the release of L-Trp from the β-site (structure VI), returning the bienzyme complex to the native state (structure I).

The function of the allosteric interactions described for the tryptophan synthase bienzyme complex provides an interesting contrast with classical examples of ligand-mediated allosteric interactions. Such interactions often involve the cooperative binding of homotropic ligands to an oligomeric protein, as is observed for phosphofructokinase, or the binding of heterotropic ligands to sites distinct from the catalytic center, of which aspartate carbamoyltransferase is an excellent example [see Perutz (1989)]. These allosteric enzymes are usually located at key regulatory points in metabolic pathways, and the allosteric interactions function to control the flux of metabolites along a given pathway. The tryptophan synthase bienzyme complex also exhibits ligand-mediated heterotropic allosteric interactions. However, this allosteric behavior serves to coordinate the activity of sequential metabolic enzymes that comprise the bienzyme complex. Further investigation will undoubtedly determine whether or not the allosteric properties exhibited by tryptophan synthase are an isolated occurrence or rather one example of a more general phenomenon that governs enzyme–enzyme interactions and metabolite channeling.

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