The Tryptophan Synthase Bienzyme Complex Transfers Indole between the α- and β-Sites via a 25–30 Å Long Tunnel†

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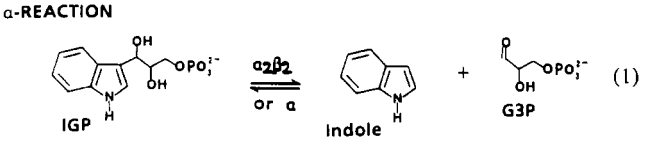
Received April 12, 1990; Revised Manuscript Received June 21, 1990

ABSTRACT: The bacterial tryptophan synthase bienzyme complexes (with subunit composition $\alpha_2\beta_2$) catalyze the last two steps in the biosynthesis of L-tryptophan. For L-tryptophan synthesis, indole, the common metabolite, must be transferred by some mechanism from the α-catalytic site to the β-catalytic site. The X-ray structure of the *Salmonella typhimurium* tryptophan synthase shows the catalytic sites of each α–β subunit pair are connected by a 25–30 Å long tunnel [Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857–17871]. Since the *S. typhimurium* and *Escherichia coli* enzymes have nearly identical sequences, the *E. coli* enzyme must have a similar tunnel. Herein, rapid kinetic studies in combination with chemical probes that signal the bond formation step between indole (or nucleophilic indole analogues) and the α-aminoacrylate Schiff base intermediate, E(A-A), bound to the β-site are used to investigate tunnel function in the *E. coli* enzyme. If the tunnel is the physical conduit for the transfer of indole from the α-site to the β-site, then ligands that block the tunnel should also inhibit the rate at which indole and indole analogues from external solution react with E(A-A). We have found that when D,L-α-glycerol 3-phosphate (GP) is bound to the α-site, the rate of reaction of indole and nucleophilic indole analogues with E(A-A) is strongly inhibited. These compounds appear to gain access to the β-site via the α-site and the tunnel, and this access is blocked by the binding of GP to the α-site. However, when small nucleophiles such as hydroxylamine, hydrazine, or *N*-methylhydroxylamine are substituted for indole, the rate of quinonoid formation is only slightly affected by the binding of GP. Furthermore, the reactions of L-serine and L-tryptophan with $\alpha_2\beta_2$ show only small rate effects due to the binding of GP. From these experiments, we draw the following conclusions: (1) L-Serine and L-tryptophan gain access to the β-site of $\alpha_2\beta_2$ directly from solution. (2) The small effects of GP on the rates of the L-serine and L-tryptophan reactions are due to GP-mediated allosteric interactions between the α- and β-sites. (3) The α-site and the interconnecting tunnel function as a highly preferred route for the transfer of indole and indole analogues such as benzimidazole, indoline, or aniline between solution and the β-catalytic site of E(A-A). (4) GP inhibits the access of these molecules to the β-site by blocking the tunnel opening at the α-site. (5) The entry of small nucleophiles into the β-site of E(A-A) from solution is not blocked by GP. (6) Either GP does not completely block the tunnel opening, or these small nucleophiles have an alternative route of access (perhaps through a leak in the tunnel wall or directly through the β-site).

Virtually all protein function is mediated via specific recognition of one molecular surface by another. X-ray crystallography has shown that these recognition phenomena involve stereochemically well-defined surfaces of the native protein in the form of crevices, clefts, pockets, tunnels, and channels that provide chiral sites complementary to the surface and charge properties of the cognate partners (Imoto et al., 1972; Arone & Perutz, 1974; Murthy et al., 1981; Cedergren-Zeppezauer et al., 1982; Raftery et al., 1983; Creighton, 1983; Jurnak & McPherson, 1987; Dunn et al., 1987a; Hyde et al., 1988; Herve, 1989; Rossman, 1989). In contrast to the specific binding properties and binding function of these channels and sites, there are a wide variety of protein channels which function in vivo as selective vectoral transporters of small molecules and ions. These transport proteins form channels which span membranes (Raftery et al., 1983; Henderson & Unwin, 1975). A spate of recent reports indicates that in vivo the enzymes of intermediary metabolism aggregate as supramolecular complexes and that some of these multienzyme complexes form channels for the efficient transport of metabolite from one enzyme to the next along the pathway (Yanofsky & Rachmeler, 1958; Friedrich, 1984; Srivastava & Bernhard, 1987; Dunn et al., 1987c; Hyde et al., 1988).

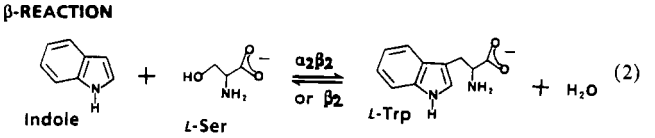
The bacterial tryptophan synthase bienzyme complex (an $\alpha_2\beta_2$ tetramer in most bacteria) catalyzes the last two steps of L-tryptophan (L-Trp)[1] biosynthesis (eqs 1 and 2). Con-

α-REACTION



(1)

β-REACTION



(2)

† Supported by NSF Grants DMB-84-08513 and DMB-87-03697.

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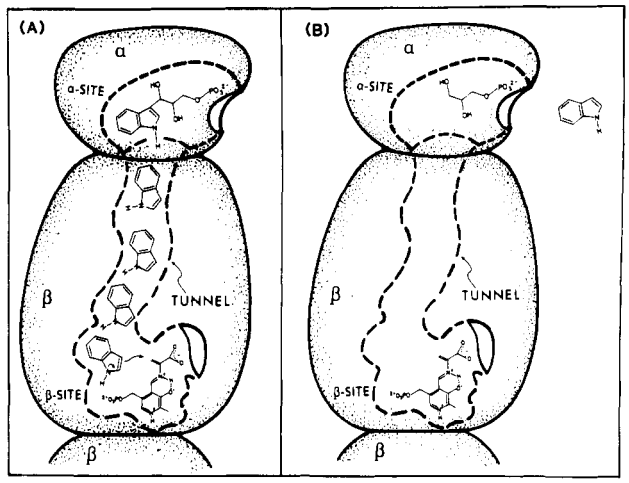
0006-2960/90/0429-8598$02.50/0

Scheme I: Cartoon Based on 2.5 Å Resolution X-ray Crystal Structure of *S. typhimurium* Tryptophan Synthase (Hyde et al., 1988) Depicting α- and β-Catalytic Sites and 25–30 Å Long Interconnecting Tunnel within an $\alpha\beta$ Dimeric Unit of Tetrameric Enzyme[a]



[a] In (A), the substrate 3-indole-D-glycerol 3′-phosphate (IGP) is shown bound to the α-site, and the α-aminoacrylate Schiff base intermediate, E(A-A), is shown bound to the β-site. IGP enters the α-site from the cleft opening on the right. Indole produced by cleavage of IGP diffuses along the tunnel to the β-site where it reacts with E(A-A). L-Ser and L-Trp would enter and leave the β-site from the opening shown on the right of the β-subunit. According to the X-ray structure, the 25–30 Å long tunnel is large enough to accommodate four indole molecules (as shown). In (B), access of indole to the tunnel via the α-site is blocked by the inhibitor α-glycerol 3-phosphate.

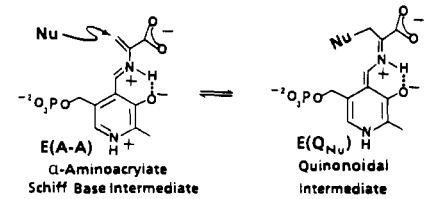
sequently, the product, indole, formed in the α-subunit-catalyzed cleavage of 3-indole-D-glycerol 3′-phosphate (IGP, eq 1) becomes the substrate for the β-subunit-catalyzed conversion of L-serine (L-Ser) to L-Trp (eq 2) (Yanofsky & Crawford, 1972; Miles, 1979, 1986, 1990; Yanofsky, 1987, 1989). Although the separate α- and β-species are catalytically functional, subunit interactions in the native $\alpha_2\beta_2$ complex enhance the catalytic activities described by eqs 1 and 2 by factors of 50–100-fold (Yanofsky & Crawford, 1972; Miles 1979). Evidence for, and proposals concerning, the direct transfer of indole between the α- and β-sites first came from the works of Yanofsky and Rachmeler (1958), DeMoss (1962), Creighton (1970), Matchett (1974), and Kirschner et al. (1975). Together, these studies strongly indicated that, provided there is sufficient IGP and L-Ser to sustain a steady-state rate of synthesis, the indole derived from cleavage of IGP is transferred from the α-site to the β-site and incorporated into L-Trp via a mechanism that does not require dissociation into solution and rebinding. Fluorescence energy transfer experiments (Lane & Kirschner, 1983c) gave the first indication that the α- and β-sites are located about 25–30 Å apart. X-ray and neutron scattering studies (Wilhelm et al., 1982; Ibel et al., 1985) indicated an approximately linear α–β–β–α subunit arrangement. The X-ray crystal structure of the tryptophan synthase complex from *Salmonella typhimurium* (viz., Scheme IA) confirms this architecture and establishes that the active sites of each α–β subunit pair are linked by an interconnecting, 25–30 Å long tunnel (Hyde et al., 1988; Hyde & Miles, 1990). From the molecular architecture and dimensions of the interconnecting tunnel, Hyde et al. hypothesize that indole generated at the α-site reaches the β-site via diffusion along the tunnel rather than by dissociation into solution and rebinding (Scheme IA). Herein, we present a series of kinetic studies that test the channeling hypothesis for the *Escherichia coli* enzyme.

Experimental Procedures

The *E. coli* tryptophan synthase isolation, purification, and assay methodology, the RSSF and SWSF instrumentation, and the sources and purification of all chemicals used have been previously described (Adachi et al., 1974; Tschopp & Kirschner, 1980a,b; Dunn et al., 1979, 1987b,c; Drewe & Dunn, 1985, 1986; Roy et al., 1988; Houben et al., 1989; Houben & Dunn, 1990). All of the experimental work described in the figures presented herein were performed in 50 mM Bicine-HCl buffer, pH 7.8 at 25 °C.

Results

Strategies for Testing Tunnel Function. In previous studies, we have shown that a variety of nucleophilic chemical/structural analogues (Nu) of indole are capable of reacting with E(A-A) to form quasi-stable quinonoid species (Q_{Nu}) (eq 3) (Dunn et al., 1987b,c; Roy, 1987; Roy et al., 1988). The



(3)

species formed in these Nu reactions are identified as quinonoids on the basis of the following criteria: (a) Quinonoids exhibit large apparent ϵ_{max} values (usually 20 000–50 000 M^{-1} cm^{-1}). (b) They have at least two closely spaced long-wavelength transitions consisting of a narrow peak and a prominent shoulder at shorter wavelength with amplitude ratios of ~3:1. These transitions are usually spaced about 1200 cm^{-1} (~27 nm) apart. (c) The most intense band is characterized by a skewness factor in the range of 1.1 for the log-normal distribution curve fit (Kallen et al., 1985; Metzler et al., 1985, 1988). (d) The enzyme-bound species show a close similarity in band shape, intensity, and energy to model PLP quinonoids (Schirch & Slotter, 1966; Abbott & Bobrick, 1973; Sala et al., 1987; Thanassi, 1975; Matusmoto & Matsushima, 1974; Karube & Matsushima, 1977; Benecky et al., 1985; Metzler et al., 1988).

In devising tests of the channeling hypothesis, we use the Q_{Nu}-forming reactions as spectrophotometric probes of tunnel function in three types of transient kinetic measurements. (1) If the tunnel provides a highly preferential route of access for indole and the other nucleophiles into the β-site for reaction with E(A-A) (viz., Scheme IA), then blocking the tunnel entrance with a ligand (of sufficient steric bulk) specifically bound to the α-site should strongly inhibit the rate of reaction of nucleophiles with E(A-A) but not the yield of (quasi-stable) Q_{Nu} formed. As will be shown, the available evidence indicates that the α-site substrate analogue GP is a ligand suitable for this purpose (viz., Scheme IB). The α-catalytic site is the only locus on $\alpha_2\beta_2$ known to bind GP. Heyn and Weischet (1975) have shown that the binding of GP and the binding of D-

[1] Abbreviations: $\alpha_2\beta_2$, native tryptophan synthases from *E. coli* or *S. typhimurium*; L-Trp, L-tryptophan; L-Ser, L-serine; IGP, 3-indole-D-glycerol 3′-phosphate; PLP, pyridoxal 5′-phosphate; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; Q_1, Q_2, and Q_3, quinonoidal intermediates formed in the conversion of L-Ser and indole to L-Trp; Q_{Nu}, quinonoidal species formed in the reactions of nucleophiles with E(A-A); Nu, nucleophiles that react with E(A-A); indoline, 2,3-dihydroindole; G3P, D-glyceraldehyde 3-phosphate; GP, D,L-α-glycerol 3-phosphate; P_i, phosphate ion; IPP, 3-indolepropanol 3′-phosphate; BZI, benzimidazole.

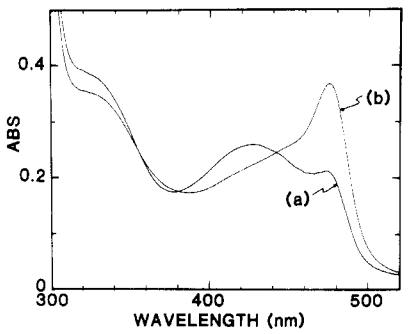


FIGURE 1: Rapid-scanning stopped-flow spectra comparing the effects of D,L-glycerol 3-phosphate (GP) on the steady-state distribution of chromophoric species which accumulate in the *E. coli* tryptophan synthase ($\alpha_2\beta_2$) catalyzed conversion of indole and L-Ser to L-Trp. (a) Spectrum measured 5 s after mixing: [indole] = 1.0 mM; [L-Ser] = 40 mM; [$\alpha_2\beta_2$] = 14.7 μM. (b) Same conditions except that [GP] = 50 mM. The bands located at ~420 and 476 nm correspond, respectively, to the L-Trp Schiff base and L-Trp quinonoidal species.

glyceraldehyde 3-phosphate (G3P) are both strictly competitive with that of the IGP analogue, 3-indolepropanol 3′-phosphate (IPP). In studies to compare the allosteric effects of GP and G3P (P. Brzovič and M. F. Dunn, unpublished results), we have found these two ligands induce remarkably similar effects on reactions catalyzed by the β-subunits of $\alpha_2\beta_2$. X-ray structure studies of the IPP complex with α_2b_2 (Scheme I) have identified a single site on the α-subunit to which IPP is bound, and this site is undoubtedly the α-catalytic site. In this complex, the indole moiety of IPP is positioned at the opening to the tunnel (Scheme I; Hyde et al., 1988). (2) Benzimidazole (BZI) is a close structural analogue of indole that binds tightly to the indole subsite at the β-site when enzyme is in the form of E(A-A). Thus, BZI is a potent, reversible, competitive inhibitor of the reaction of E(A-A) with nucleophiles (eqs 4 and 5) (Heilman, 1978; Roy et al., 1988; Houben et al., 1989; Houben & Dunn, 1990), but BZI does not undergo covalent reaction with E(A-A) (eq 5). As will be shown below, when

$$Q_{Nu} \underset{k_Q}{\overset{k_{-Q}}{\rightleftharpoons}} E(A\text{-}A) + Nu \quad (4)$$

$$E(A\text{-}A) + BZI \rightleftharpoons E(A\text{-}A)(BZI) \quad (5)$$

$$Nu_2 + Q_{Nu_1} \rightleftharpoons Q_{Nu_2} + Nu_1 \quad (6)$$

BZI is mixed with Q_{Nu}, the tight binding of BZI to E(A-A) causes a net shift of the equilibria in eqs 4 and 5 in favor of E(A-A)(BZI).[2] If BZI and Nu both must enter and leave via the tunnel opening, then an α-site-specific ligand such as GP that blocks the opening should inhibit the BZI-mediated displacement of Nu from Q_{Nu}. (3) Since quinonoid formation is reversible (see below), and if nucleophile exchange occurs via the tunnel and the α-site, then the effects of GP on the rate of exchange of one nucleophile (Nu_2) for another (Nu_1) (eq 6) should lend further insight into the properties and functioning of the tunnel.[2]

Allosteric Site–Site Interactions Mediated by D,L-*α-Glycerol 3-Phosphate.*[3] Figure 1 compares the steady-state

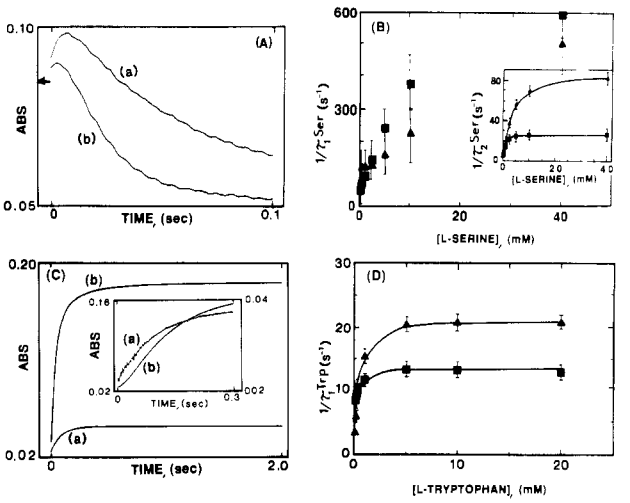


FIGURE 2: Influence of GP on the kinetics of the reactions of L-Ser (A and B) and L-Trp (C and D) with $\alpha_2\beta_2$. (A) Time courses for the L-Ser reaction of 5 mM L-Ser with 10 μM $\alpha_2\beta_2$ measured in the absence (a) and presence (b) of 100 mM GP. The arrow on the ordinate indicates the absorbance of the internal aldimine form of $\alpha_2\beta_2$ expected after mixing but prior to any reaction with L-Ser. (B) Dependencies of $1/\tau_1$ and $1/\tau_2$ (inset) on the concentration of L-Ser in the absence (■) and presence (▲) of 50 mM GP. The large errors in $1/\tau_1$ values reflect the relatively rapid rates and small amplitudes of this relaxation. (C) Time course for the reaction of 20 mM L-Trp with 13.8 μM $\alpha_2\beta_2$ in the absence (a) and presence (b) of 50 mM GP. The inset shows time courses measured for 0.4 mM L-Trp; note the lag phase in trace (b). [The left and right ordinate scales of the inset correspond to traces b and a, respectively.] (D) Dependence of $1/\tau_1$ (see text) on the concentration of L-Trp in the absence (■) and presence (▲) of 50 mM GP. The L-Ser data were measured at 420 nm; the L-Trp data were measured at 476 nm.

RSSF spectra for the $\alpha_2\beta_2$-catalyzed reaction of indole with L-Ser in the presence (b) or absence (a) of 50 mM GP. The binding of GP causes a redistribution of the covalent intermediates that accumulate in the steady state (Dunn et al., 1987b,c; Roy, 1987; Kawasaki et al., 1987; Houben et al., 1989; Houben & Dunn, 1990). As indicated by the larger amplitude of the 476-nm band in Figure 1b, this redistribution increases the amount of L-Trp quinonoid that accumulates.

The synthesis of L-Trp via reaction of indole with L-Ser occurs at the β-site in two "half-reactions" (Yanofsky & Crawford, 1972; Goldberg et al., 1967, 1968; York, 1970, 1972; Faeder & Hammes, 1970, 1971; Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985, 1986). In the first half-reaction L-Ser reacts with PLP [bound as the internal aldimine, E(Ain)], generating as transient intermediates the *gem*-diamine, E(GD), the first external aldimine, $E(Aex_1)$, and the first quinonoid, $E(Q_1)$. Then, $E(Q_1)$ decays to the α-aminoacrylate Schiff base, E(A-A), a quasi-stable species poised for reaction with indole (the initial step of the second half-reaction) (Goldberg & Baldwin, 1967; Goldberg et al., 1968; Faeder

[2] Due to the relatively rapid conversion of the indole quinonoids to species further along the reaction path, we are unable to examine the kinetics of either the BZI displacement or the nucleophile exchange reactions for the indole system. When indole is reacted with $Q_{aniline}$ (viz., eq 6), the presence of 50 mM GP decreases the rate of nucleophile interchange by >100-fold (C. A. Leja and M. F. Dunn, unpublished results), showing that GP also strongly inhibits the rate of this reaction.

[3] In each of the kinetic experiments described in this study, the reaction time courses consist of at least two phases. We do not fully understand the mechanistic origins of these relaxations. Certainly, the fact that some of the processes involve more than one chemical step is consistent with the observation of multiphasic time courses. Also, the existence of ligand-mediated allosteric transitions implies the preexistence of more than one form of the enzyme, a situation that could give rise to more than one kinetic phase. Because the emphasis of this study is on the characterization of the role played by the tunnel in the transfer of ligands from solution to the β-catalytic site, our treatment of the kinetic data and the discussions presented herein are limited to those initial events that appear to be coupled directly to ligand binding steps. Detailed analyses of the subsequent slower processes and the allosteric properties of this enzyme will be treated in subsequent manuscripts.

& Hammes, 1970, 1971; York, 1972; Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985, 1986). The data presented in Figure 2A,B compare the effects of GP on the first half-reaction. Figure 2A shows typical SWSF time courses for the reaction of 5 mM L-Ser with E(Ain) both in the absence (a) and in the presence (b) of 100 mM GP; Figure 2B summarizes the effects of GP on the L-Ser concentration dependencies of the first two relaxations ($1/\tau_1^{Ser} \gg 1/\tau_2^{Ser}$). The L-Ser reaction is characterized by four relaxations (Lane & Kirschner, 1983a; Drewe & Dunn, 1985); the absorbance at 420 nm increases during the first relaxation, and this phase is dominated by the formation of $E(Aex_1)$ and $E(Q_1)$; the absorbance decreases in the second and subsequent relaxations; $1/\tau_2^{Ser}$ is dominated by the conversion of $E(Q_1)$ to E(A-A) (Drewe & Dunn, 1985). The third relaxation is believed to involve interconversion of two forms of the enzyme, while the fourth relaxation is a very slow process of small amplitude that is not on the catalytic path (Lane & Kirschner, 1983a; Drewe & Dunn, 1985). The rate of $1/\tau_1^{Ser}$ is very fast (Lane & Kirschner, 1983a; Drewe & Dunn, 1985), and therefore, the precision with which the apparent rate constants for this process can be measured is poor (viz., Figure 2B). However, the data presented in Figure 2B indicate that GP binding brings about a small reduction in the rate of $1/\tau_1^{Ser}$ (without substantially altering the amplitude; data not shown), while the rate (and the amplitude) of $1/\tau_2^{Ser}$ is increased (Figure 2B, inset). At rate saturation with respect to the concentration of L-Ser, $1/\tau_2^{Ser}$ is increased from $\sim$26 s^{-1} in the absence of GP to $\sim$85 s^{-1} in the presence of 100 mM GP, and the amplitude of this phase is increased about 2-fold. Drewe and Dunn (1985) have shown that the events which occur in $1/\tau_2^{Ser}$, $1/\tau_3^{Ser}$, and $1/\tau_4^{Ser}$ involve the chemical steps subsequent to formation of the external aldimine. Therefore, since the effects of GP on $1/\tau_3^{Ser}$ and $1/\tau_4^{Ser}$ are not relevant to the function of the tunnel, a detailed analysis of the dependencies of these processes on [GP] will be presented elsewhere.

The dependencies of $1/\tau_1^{Ser}$ and $1/\tau_2^{Ser}$ (and $1/\tau_3^{Ser}$) on the concentration of L-Ser tend toward saturated values at high L-Ser concentrations. Over the concentration range 0.5–40 mM L-Ser, the presence of 100 mM GP reduces the value of $1/\tau_1^{Ser}$ by no more than 17%, whereas $1/\tau_2^{Ser}$ is increased approximately 3-fold (Figure 2B) and $1/\tau_3^{Ser}$ (data not shown) is essentially unchanged.

The reaction of L-Trp with E(Ain), Figure 2C,D, yields an equilibrating mixture consisting of the corresponding external aldimine, $E(Aex_2)$, and quinonoidal species, $E(Q_2)$ and/or $E(Q_3)$, together with other species (Lane & Kirschner, 1981; Drewe et al., 1989; Houben & Dunn, 1990). Evaluation of the effects of [GP] on the reaction time course is complicated by the appearance of a lag phase ($1/\tau^*$) in the presence of GP that is not detected in the absence of GP. This lag phase is most noticeable at low L-Trp concentrations (see trace b of the inset in Figure 2C). As the concentration of L-Trp is increased, the rate of $1/\tau^*$ shows an approximately linear dependence, increasing from $\sim$25 s^{-1} at 0.2 mM L-Trp to $\sim$290 s^{-1} at 20 mM L-Trp. Comparison of the effects of L-Trp concentration on $1/\tau_1^{Trp}$ (the first observed phase of increasing amplitude measured at 476 nm after the lag phase, Figure 2D) shows that the observed rate constants for these relaxations saturate with values of $\sim$13 and $\sim$21 s^{-1} respectively, in the absence and in the presence of GP. However, the amplitude of $1/\tau_1^{Trp}$ (viz., Figure 2C) is increased 6–10-fold by the presence of GP. The rates and amplitudes of the second observed phase of increasing amplitude are essentially unaffected by GP (data not shown).

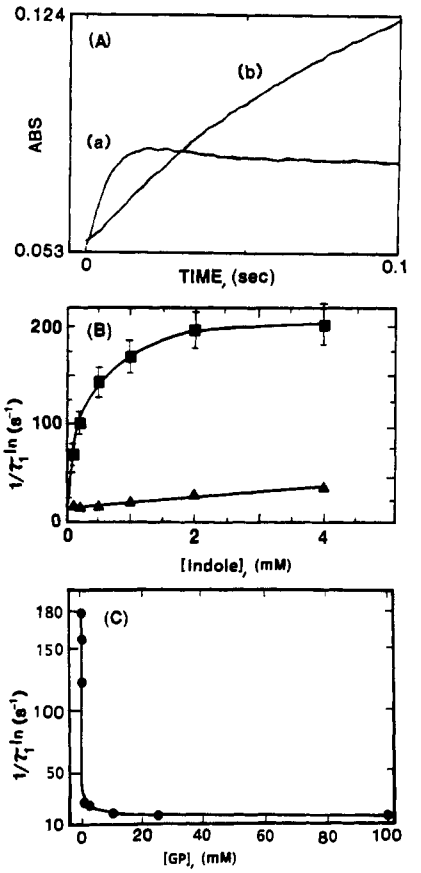


FIGURE 3: Effects of GP on the reaction of indole with the α-aminoacrylate Schiff base intermediate, E(A-A), measured at 476 nm. (A) Time courses for the reaction of 0.5 mM indole with 10.8 μM E(A-A) were measured in the absence (a) and presence (b) of 50 mM GP. (B) Dependencies of $1/\tau_1$ on the concentration of indole measured in the absence (■) and presence (▲) of 50 mM GP, with final concentrations of $\alpha_2\beta_2$ = 9.5 μM and L-Ser = 40 mM. (C) Dependence of $1/\tau_1$ on the concentration of GP: [indole] = 0.5 mM; [L-Ser] = 40 mM; $[\alpha_2\beta_2]$ = 10.4 μM.

From the data presented in Figure 2, it is evident that GP causes relatively modest, albeit interesting allosteric effects on the time courses for the reactions of L-Ser and L-Trp with $\alpha_2\beta_2$. The dominant effect on the L-Ser reaction is the increased rate of $1/\tau_2^{Ser}$, while the dominant effect on the L-Trp reaction is the 6–10-fold increase in the amplitude of the quinonoid absorbance band in $1/\tau_1^{Trp}$. This increased amplitude in the L-Trp reaction is due to the GP-mediated redistribution of covalent intermediates in favor of the quinonoid [see Houben and Dunn (1990)]. The spectra in Figure 1 indicate that GP has a similar effect on the steady-state distribution of covalent species formed in the $\alpha_2\beta_2$-catalyzed reaction of L-Ser and indole to form L-Trp.

Influence of GP on Reaction of E(A-A) with Nucleophiles.[3] In the presence of excess L-Ser, the reaction of indole with E(A-A) gives pre-steady-state time courses that are characterized by a rapid nucleophilic attack of indole on the electrophilic C-β of the α-aminoacrylate Schiff base species. This rapid C–C bond forming step generates quinonoidal species with λ_{max} 476 nm (Faeder & Hammes, 1970, 1971; York, 1970, 1972; Lane & Kirschner, 1938b; Drewe & Dunn, 1986) that decay to a steady-state mixture of intermediates along the reaction path (viz., Figure 1). The effects of GP on a typical SWSF time course measured at 476 nm are shown in Figure 3A. It is clear from inspection of this figure that the time courses are quite different. In the absence of GP (trace a), there are two rapid phases ($1/\tau_1^{ln} > 1/\tau_2^{ln}$) of increasing amplitude ($A_1 \gg A_2$) followed by a phase ($1/\tau_3^{ln}$) of de-

creasing amplitude and then a final phase ($1/\tau_4^{In}$) of increasing amplitude prior to attainment of the steady state ($A_1 \gg A_3$, A_4). In the presence of a saturating concentration of GP (trace b), the time course is changed considerably; there is a single, slow relaxation $1/\tau_1^{In}$ with increasing amplitude in the pre-steady-state phase. Figure 3B shows the effect of indole concentration on the fastest phase of quinonoid formation detected both in the absence and in the presence of GP. In the absence of GP, the increase in $1/\tau_1^{In}$ from ~70 to 200 s^{-1} follows a hyperbolic isotherm (with increasing indole concentration). In the presence of GP, $1/\tau_1^{In}$ increases linearly from ~13 to ~37 s^{-1} as indole is increased from 0.1 to 4 mM.

The dependence of $1/\tau_1^{In}$ on the concentration of GP is given in Figure 3C. This isotherm is biphasic: at low GP concentrations (0–2 mM), $1/\tau_1^{In}$ decreases sharply (from 180 to 20 s^{-1}); at higher GP concentrations (2–30 mM), there is a gradual further decrease in $1/\tau_1^{In}$ (from ~20 to ~14 s^{-1}). At concentrations greater than 30 mM, $1/\tau_1^{In}$ becomes independent of [GP]. The reactions of the indole analogues indoline and aniline with E(A-A) (Roy 1987; Dunn et al., 1987b,c) respond similarly to the presence of GP. *The large GP-mediated inhibition of reaction rate documented in Figure 3 is in marked contrast to the small rate effects on the* L-*Ser reaction (Figures 2A–C), to the enhancement of rate found in the* L-*Trp reaction (Figure 2D–F), and to the small rate effects on small nucleophiles (see below).*

Small nucleophiles such as hydroxylamine, hydrazine, and their mono *N*- and *O*-methyl derivatives have been shown to react with E(A-A), yielding quasi-stable quinonoids with λ_{max} 454–468 nm (Dunn et al., 1987b,c). The effects of [GP] on the reaction of *N*-methylhydroxylamine (NMHA) with E(A-A) are described by the data presented in Figure 4. Reaction of NMHA with E(A-A) gives a biphasic time course for the appearance of the NMHA quinonoid. In the presence of 50 mM GP, the rates and amplitudes of $1/\tau_1^{NMHA}$ and $1/\tau_2^{NMHA}$ are only slightly affected (Figure 4A). Figure 4B shows that over the concentration range studied (1–150 mM NMHA) both relaxations are apparent second-order processes (first order with respect to [NMHA] and first order with respect to [α-aminoacrylate Schiff base]). The slopes of these curves both are ~220 M^{-1} s^{-1}. The extrapolation of the curves to [NMHA] = 0 gives *y* intercepts of ~12 and ~5 s^{-1}, respectively, in the absence and in the presence of 50 mM GP. It is likely that the difference in these intercept values is due to the allosteric effect of GP on k_{-Q} (eq 4). The GP effect is constant over the NMHA concentration range studied (Figure 4C). Thus, in contrast to the behavior of indole, the rates and amplitudes that characterize the reaction of NMHA with E(A-A) are only slightly perturbed by the presence of GP. We have found that GP affects the reactions of hydroxylamine, hydrazine, and *O*-methylhydroxylamine similarly (data not shown).

Influence of GP on Benzimidazole-Mediated Reversal of Quinonoid Formation.[2,3] The indole analogues indoline, aniline, and phenylhydrazine (and other structurally related compounds) react rapidly and reversibly with E(A-A), yielding quasi-stable quinonoid species. The quinonoid formed in the indoline reaction slowly undergoes conversion to form a new, novel amino acid, dihydroiso-L-tryptophan (Roy et al., 1988). We have been unable to detect the formation of a new amino acid from the aniline quinonoid. ^1H NMR studies (W. Kadima and M. F. Dunn, unpublished results) indicate that the phenylhydrazine system gives trace amounts of the corresponding new amino acid.

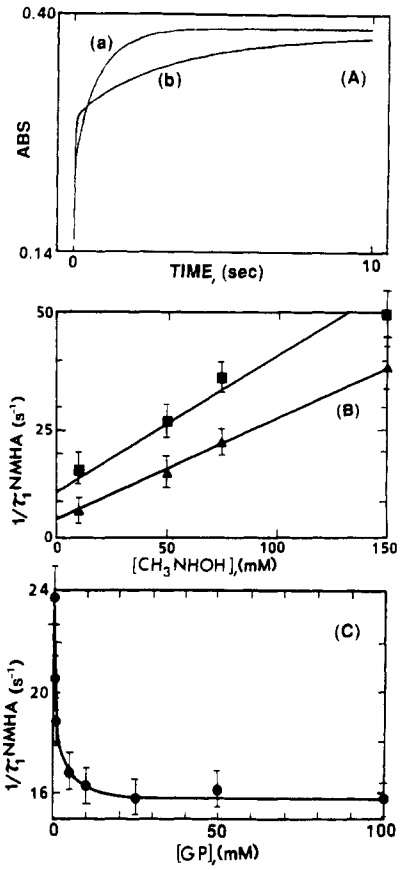


FIGURE 4: Effects of GP on the reaction of *N*-methylhydroxylamine (NMHA) with E(A-A) measured at 456 nm. (A) Time courses for the reaction of 150 mM NMHA with 2.5 μM E(A-A) measured in the absence (a) and presence (b) of 50 mM GP. Prior to mixing in (a) and (b), both syringes of the stopped-flow apparatus contained identical concentrations of 40 mM L-Ser and one contained $\alpha_2\beta_2$ and the other contained 300 mM NMHA. In (b), both syringes also contained identical concentrations of GP. (B) Dependencies of $1/\tau_1$ on the concentration of NMHA measured in the absence (■) and presence (▲) of 50 mM GP. (C) Dependence of $1/\tau_1$ on the concentration of GP: [NMHA] = 50 mM; [L-Ser] = 40 mM; $\alpha_2\beta_2$ = 2.5 μM.

These quasi-stable quinonoids are formed rapidly and reversibly, and the position of the Nu + E(A-A) $\rightleftharpoons$ Q_{Nu} equilibrium is sensitive to the concentrations of nucleophile, E(A-A), and any inhibitors that bind reversibly to the indole subsite of the β-subunit. As is evident from the data given in Figure 5, competition of the indole analogue benzimidazole (BZI) for the indole subsite in the absence of GP (trace a) causes a rapid displacement of the equilibrium in favor of the E(A-A)(BZI) complex. At high BZI concentrations (viz., Figure 5A), this conversion is essentially complete. In the presence of GP, the rate, *but not the extent*, of this displacement is strongly inhibited (Figure 5A, trace b and inset). The data given in Figure 5B show that the magnitude of the faster relaxation ($1/\tau_1^{BZI}$) measured in the absence of GP increases linearly as the concentration of BZI is increased (slope = 3.2×10^3 M^{-1} s^{-1}). In the presence of 50 mM GP, $1/\tau_1^{BZI}$ is essentially independent of [BZI] over the concentration range investigated (0–40 mM). When the concentration of GP is varied (Figure 5C), the rate of the BZI-mediated displacement drops sharply in the region 0–2 mM, changing the observed rate from 180 to ~0.25 s^{-1}. Between 2 and 30 mM, there is a further decrease in rate to 0.18 s^{-1}, and above 30 mM the displacement rate is independent of GP concentration. This GP concentration dependence is similar to that found for the reaction of indole with E(A-A) (Figure 3C).

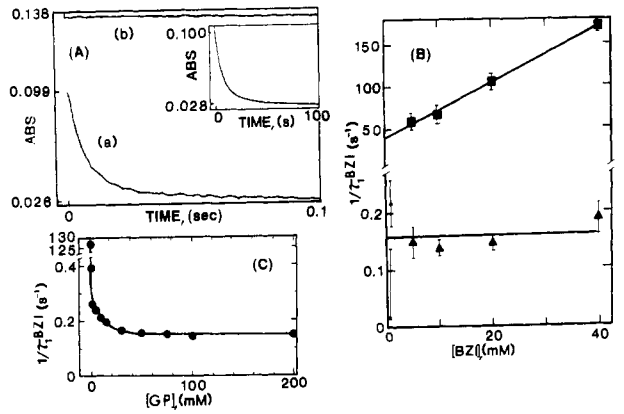
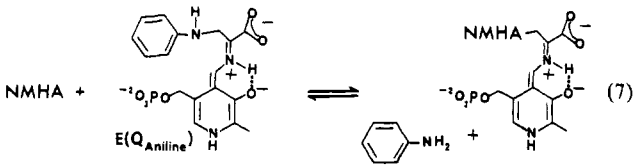


FIGURE 5: Influence of GP on the BZI-mediated displacement of aniline from $Q_{aniline}$ (viz., eqs 4 and 5; text) measured at 466 nm. (A) Time courses for the disappearance of $Q_{aniline}$ measured in the absence (a) and presence (b) (see also the inset) of 50 mM GP. In both (a) and (b), the same concentrations of aniline and L-Ser were present in both syringes of the stopped-flow apparatus, while one syringe contained $\alpha_2\beta_2$ and other BZI. In trace b, the same concentration of GP was also present in both syringes. Concentrations after mixing: [aniline] = 10 mM; [L-Ser] = 40 mM; $[\alpha_2\beta_2]$ = 5 μM; [BZI] = 10 mM. (B) Dependencies of $1/\tau_1$ measured in the absence (■) and in the presence (▲) of 50 mM GP. (C) Dependence of $1/\tau_1$ on the concentration of GP: [aniline] = 10 mM; [L-Ser] = 40 mM; $[\alpha_2\beta_2]$ = 5 μM; [BZI] = 10 mM.

Influence of GP on Kinetics of Nucleophile Exchange.[3] The data presented in Figure 6 describe the effects of GP on the kinetics of nucleophile exchange (eq 6). Figure 6A compares typical time courses for the exchange of aniline with *N*-methylhydroxylamine (NMHA) both in the presence and in the absence of 50 mM GP (eq 7). Bonding in Q_{NMHA} could



(7)

occur via either N or O; the available evidence does not distinguish between these two possibilities. Inspection of Figure 6A shows that preincubation of GP with $Q_{aniline}$ has very little effect on the rates of the biphasic time course (compare traces a and b), while the effect on amplitudes is larger. Figure 6B shows that, both in the absence and in the presence of 50 mM GP, $1/\tau_1^Q$ increases linearly with increasing concentration of NMHA and that over the concentration range studied GP has only a small effect on the value of $1/\tau_1^Q$. Both curves give slopes of $\sim$50 M^{-1} s^{-1} with y intercepts of $\sim$10 and 7 s^{-1}. These differences in intercept values reflect the allosteric effect of GP on the reverse process of eq 7. When $Q_{aniline}$ is preincubated with different GP concentrations ranging from 0 to 200 mM (Figure 6C), reaction with 50 mM NMHA gives rates for the faster relaxation that decrease by less than a factor of 2, changing from $\sim$18 s^{-1} in the absence of GP to a limiting value of $\sim$10 s^{-1} in the presence of 200 mM GP. This small GP effect is essentially independent of the concentration of NMHA over the range studied.

Discussion

Quinonoid-Forming Reactions Are Sensitive Probes of Tunnel Function. The *Salmonella* and *E. coli* enzymes have highly similar amino acid sequences ($\sim$80%) and therefore must have nearly identical three-dimensional structures (Miles, 1986, 1990). Solution studies of the two enzymes indicate highly similar catalytic behavior and response to allosteric

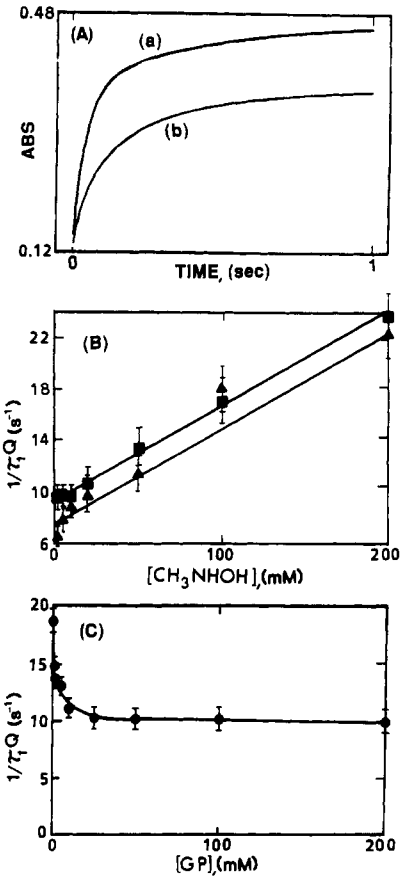
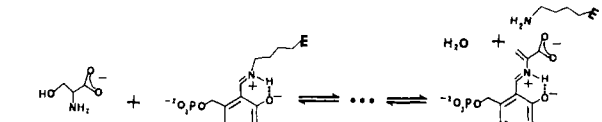


FIGURE 6: Influence of GP on the interconversion of $Q_{aniline}$ and Q_{NMHA} measured at 456 nm (viz., eqs 6 and 7). (A) Time courses for the reaction of NMHA with $Q_{aniline}$ measured in the absence (a) and presence (b) of GP. In (b) both syringes also contained 50 mM GP. Conditions after mixing: [aniline] = 10 mM; [L-Ser] = 40 mM; $[\alpha_2\beta_2]$ = 2.5 μM; [NMHA] = 50 mM. (B) Dependencies of $1/\tau_1$ on the concentrations of NMHA measured in the absence (a) and presence (b) of 50 mM GP. (C) Dependence of $1/\tau_1$ on the concentration of GP.

effectors (Miles, 1986; P. Brzovič, E. Miles, and M. F. Dunn, unpublished results). Therefore, in the discussion which follows, we assume that the structural information for the *Salmonella* enzyme concerning the location of active sites and the geometry of the interconnecting tunnel applies to the *E. coli* enzyme.

Our tests of the channeling hypothesis are based on the following experiments: The PLP-requiring β-subunits catalyze replacement of the β-hydroxyl of L-Ser with indole via a multistep sequence of two half-reactions. In the first half-reaction (eq 8), L-Ser and E(Ain) are converted to the Schiff



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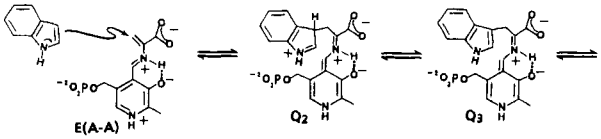

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base of α-aminoacrylate, E(A-A), via the Michaelis complex, (L-Ser)E(Ain), *gem*-diamine, E(GD), external aldimine E-(Aex_1), and quinonoidal (Q_1) intermediates, all tightly bound to the β-site (Yanofsky & Crawford, 1972; Yanofsky & Ra-

chmeler, 1958; Lane & Kirschner, 1983a,b,c; York, 1970, 1972; Goldberg et al., 1968; Faeder & Hammes, 1971; Drewe & Dunn, 1985). Formation of the C–C bond between indole and the α-aminoacrylate intermediate occurs in the second half-reaction by nucleophilic attack of the electron-rich C-3 of indole on the electrophilic C-β of E(A-A) (Yanofsky & Crawford, 1972; Miles, 1979; Drewe & Dunn, 1986) (eq 9). The quinonoid intermediate formed in this step (Q_2) undergoes deprotonation to another quinonoid (Q_3, eq 9; Drewe & Dunn, 1986) and then conversion to L-Trp via the L-Trp Schiff base and *gem*-diamine intermediates. A variety of chemical and/or structural nucleophilic analogues of indole can be substituted for indole in the reaction with E(A-A) (eq 3). These nucleophiles include 2,3-dihydroindole (indoline), aniline, phenylhydrazine, hydroxylamine, hydrazine, and various alkylated derivatives (Dunn et al., 1987b,c; Roy et al., 1988).

The results described herein establish that the properties of the Q_{Nu} intermediates provide sensitive kinetic/spectroscopic signals for monitoring access to the β-site. The quinonoids generated from nucleophile attack are characterized by π–π^* transitions with λ_{max} 452–468 nm and $\epsilon_{max} \simeq 40\,000\ M^{-1}\ cm^{-1}$. These reactions (eq 3) are relatively rapid, reversible, and kinetically well separated from the rates of Q_{Nu} decay (Dunn et al., 1987b,c). Some of the Q_{Nu} species undergo further reaction; indoline gives a new, novel amino acid (dihydroiso-L-tryptophan) (Roy et al., 1988), while hydroxylamine, *N*-methylhydroxylamine, and hydrazine ultimately convert enzyme-bound PLP to the corresponding oxime and hydrazone derivatives (Dunn et al., 1987b,c).

Because nucleophile and the α-site-blocking ligand (GP) do not bind to the same site, inhibition is not a simple competitive interaction; i.e., once a nucleophile gains access to the tunnel, both the nucleophile and the blocking ligand can be bound simultaneously. If entry into the tunnel through the α-site is the obligatory route of access to the β-site and if GP binding completely blocks the tunnel opening (Scheme IB), the plots of $1/\tau$ vs [GP] should extrapolate to a rate of zero at infinite [GP]. Since these rates actually approach finite, limiting values of >0 (Figures 3–6), we conclude either that the tunnel opening is only partially blocked or that there is an alternative but less favorable route of access. Both of these possibilities predict a saturating concentration of the blocking ligand will reduce the rate of nucleophile reaction to a lower, but finite, limiting value characteristic of reaction via the alternative route. If the tunnel is an unimportant route of access for indole and the indole analogues, then blocking the tunnel at the α-suite should have little effect (ignoring allosteric interactions) on the reaction of nucleophiles with E(A-A). This appears to be the case for the small nucleophiles (hydroxylamine, hydrazine, and their *O*- and *N*-methyl derivatives) (compare Figures 3 and 5 with Figures 4 and 6).

Allosteric Effects Are Distinguishable from Tunnel Function. To distinguish between the effects of tunnel blocking and allosteric effects on the conformation of the β-site, mediated by ligand binding to the α-site, we also have undertaken studies of the influence of phosphate ion (P_i) on the $\alpha_2\beta_2$ system (Houben & Dunn, 1990; Dunn et al., 1987b,c). The allosteric effects of GP and P_i are similar, but not identical. Phosphate ion does not inhibit the reactions of nucleophiles with E(A-A) (Dunn et al., 1987b,c; Kawasaki et al., 1987; Houben & Dunn, 1990), nor does GP inhibit the reaction of L-Trp with $\alpha_2\beta_2$ (Figure 2C,D), and the reaction of indole with E(A-A) is essentially unchanged by the presence of phosphate ion. There is some synergy between the two effectors, indicating the presence of two types of allosteric phosphoryl sites (Houben et al., 1989; Houben & Dunn, 1990). Preliminary accounts of some of this work have been presented elsewhere (Dunn et al., 1987b,c).

If GP binds only to the α-site, then GP inhibition of the reactions of indole and indole analogues (Figure 3 and eq 3) with E(A-A) and the inhibition of the BZI displacement of aniline (Figure 5 and eqs 4 and 5) cannot be due to any direct interaction with the β-subunit. Consequently, only two possible explanations remain: inhibition is due either to (a) the blocking of the tunnel (via a direct steric occlusion or a conformation change which closes the tunnel; Scheme IB) or to (b) an allosteric effect which either alters the catalytic properties of the β-site [and/or the reactivity of E(A-A)] or alters the dimensions of the tunnel. We conclude that GP both blocks the tunnel and, via site–site interactions, exerts allosteric effects on the β-subunit. However, these effects appear to be separable. The allosteric effect primarily stabilizes the quinonoidal species, while the inhibitor effect primarily slows the nucleophile binding step. GP, P_i, IPP, and other phosphorylated ligands which bind to the α-site alter the distribution of covalent intermediates bound to the β-site (Lane & Kirschner, 1981, 1983c; Dunn et al., 1987b,c; Kawasaki et al., 1987; Roy, 1987; Houben et al., 1989; Houben & Dunn, 1990; Brzovič and Dunn, unpublished results). For example, L-Trp, L-His, and Gly react at the β-site to form equilibrating mixtures of tetrahedral, external aldimine and quinonoidal intermediates (Houben et al., 1989; Houben & Dunn, 1990). The quinonoids are minor components of both systems. When GP or P_i binds, the equilibrium distribution of species is perturbed such that the amount of quinonoidal intermediate is increased and the external aldimine is decreased. This allosteric effect increases the amount of the L-Trp quinonoid ~7-fold and the amount of the L-His quinonoid ~5-fold. Nevertheless, the rates of the relaxations for the reaction of L-Ser, L-Trp, and L-His (or Gly) with $\alpha_2\beta_2$ are not significantly changed by preincubation of the enzyme with high concentrations of GP or P_i (Houben et al., 1989; Houben & Dunn, 1990). Lane and Kirschner (1981) have reported that the rate of binding of L-Trp to $\alpha_2\beta_2$ in phosphate ion buffer is slowed about 5-fold by the presence of bound IPP. In our studies (viz., Figure 2D) (Brzovič and Dunn, unpublished results), we have found that the GP effect on $1/\tau_1^{Trp}$ in Bicine buffer is less than 2-fold and that GP actually speeds up some of the relaxations detected in the binding of L-Trp (Figure 2D) and in the conversion of L-Ser to E(A-A) (Figure 2A,B). Consequently, GP, P_i, and IPP exert interesting allosteric effects on the relative ground-state stabilities of covalent intermediates on the β-subunit catalytic pathway. However, we conclude that the GP effects on the rates of those reactions which almost certainly occur via entry directly from solution into the β-catalytic site (and, therefore, do not involve the tunnel) are small and seem to bear no relationship to the large GP-induced decreases in rate observed for the reactions of indole and the indole analogues (indoline, aniline, and phenylhydrazine) and E(A-A). In this context, it is important to note that although P_i mimics the above-described allosteric effects exerted by GP, *there is no significant P_i inhibition of Nu reaction with E(A-A)* (Dunn et al., 1987b,c). Therefore, we conclude that the stabilizing effects of GP and P_i and the inhibition of the rate of reaction of large nucleophiles by GP are fundamentally different phenomena.[4] The increased yields of Q_{Nu} attained in the

[4] A further indication that the long-range allosteric effects of GP can be distinguished from the blocking interaction at the tunnel opening in the α-site is the finding of Kawasaki et al. (1987) that, in the α-mutant R179L, quinonoid stabilization is not obligatory for rate reduction by GP.

presence of GP (Figure 1) or P_i [see Houben and Dunn (1990)] no doubt reflect an allosteric interaction between the α-site and the β-site mediated by GP or P_i binding to the phosphoryl subsite of the α-site, whereas the decreased rates observed for the indole, indoline, aniline, and benzimidazole systems (Figures 3 and 5) in the presence of GP must be due to the blocking of the tunnel by GP.[2,5] Although P_i triggers the allosteric transition that stabilizes quinonoids, apparently this ion is too small to interfere with access to the tunnel and, therefore, does not block the opening or significantly alter the dimensions of the tunnel. Since increasing GP concentration decreases both the rate of indole reaction and the BZI displacement rate to lower, but finite, limiting values (viz., Figures 3 and 5), we conclude that when GP is bound, there must be some alternative but much less favorable route of access to the β-site.

Evidence for Alternative Pathways into the β-Site. If the only route of access into the β-site were via the α-site and the tunnel and if bound GP completely blocks this opening (Scheme IB), then the binding and reaction of L-Ser and L-Trp should be strongly inhibited by GP, and the reactions of indole, the indole analogues indoline, aniline, and phenylhydrazine, the small nucleophiles, and the BZI-mediated displacement reactions all would exhibit observed rates that extrapolate to zero as the concentration of GP is increased. Although some of these reactions are strongly inhibited by GP, none of these systems extrapolate to a rate of zero at infinite GP concentration.

The rate data for L-Ser and L-Trp reactions (Figure 2) are consistent with reaction paths that involve entry into the β-site directly from solution through an opening in the β-subunit. The reactions of the small nucleophiles are consistent with a pathway for entry into the β-site via a small, simple pore. This opening could be (a) directly in through the β-site, (b) through a hole in the tunnel wall (i.e., a leak), or (c) via the α-site and the tunnel (in the case where GP does not completely block the opening to the tunnel).

The reactions of the large nucleophiles with E(A-A) and the BZI-mediated displacement reaction exhibit phenomenologically different behavior.[2] The observed rates decrease sharply and then saturate with finite, limiting values as the concentration of GP is increased (viz., Figures 3C and 5C). In the presence of 50 mM GP, the rate of the reaction of indole with E(A-A) is nearly independent of the concentration of indole (Figure 3B), and the rate of the BZI-mediated displacement reaction shows no dependence on the concentration of BZI (Figure 5B). Thus, in both reactions, the binding of GP to the α-site alters the nature of the rate-limiting process for each of the respective initial steps of these reactions. In the absence of GP, the rate-limiting step is either a bimolecular process or a step tightly coupled to a bimolecular process (viz., the concentration dependencies shown in Figures 3B and 5B). In the presence of 50–100 mM GP the rate-limiting step has become a unimolecular process (independent of the concentration of indole or BZI; respectively Figures 3B and 5B). Since the spectral changes establish that the chemistry of each net transformation is unchanged, we conclude that the rate-limiting processes induced by GP binding must be unimolecular transformations of the E(A-A)(GP) complex in the indole reaction and of the $E(Q_{Nu})(GP)$ complex in the BZI reaction. Because these processes are zeroth-order with respect to GP (at high GP concentration), we conclude that the unimolecular process is not simply the dissociation of GP. *We propose these processes most likely involve a rate-determining protein conformational transition that creates an opening into the tunnel.* The X-ray structure of $\alpha_2\beta_2$ shows that one wall of the tunnel consists of a thin covering of hydrophobic side-chain residues that screen the tunnel interior from bulk solution. A conformational transition or breathing mode that creates a fissure in this wall could limit the rate at which indole or BZI gains entry into the tunnel when the α-site opening is blocked by GP. Alternatively, entry could be gained via a conformational change that creates an opening into the tunnel along the α–β subunit interface or by opening a "flap" or "lid" that covers the entrance to the tunnel through the α-site. The kinetic studies of Lane et al. (1984) appear to rule out the possibility that entry is gained via subunit dissociation to form $\alpha + \alpha\beta_2$. Their measured rate constants for this process are too slow by at least 1 order of magnitude to account for our observed rates.

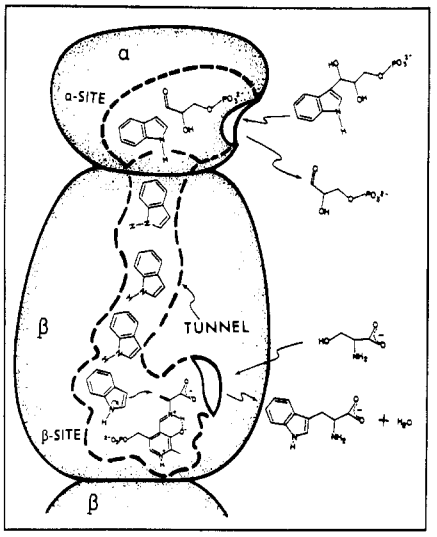
The finding that the rate-limiting, unimolecular process at high [GP] has a value of $\sim 13\ s^{-1}$ in the indole–E(A-A) system (Figure 3B,C) and a value of $0.18\ s^{-1}$ in the BZI–$E(Q_{Nu})$ system (Figure 5B,C) can be explained in several ways. The allosteric properties of the tryptophan synthase system indicate that the strength of α–β site–site interactions is dependent on the nature of the covalent structure of the PLP intermediate bound to the β-site (Faeder & Hammes, 1970, 1971; Lane & Kirschner, 1983b; Lane et al., 1984; Houben & Dunn, 1990). Thus, the energetics of a conformational transition that opens a pore into the tunnel could depend on the structure of the PLP derivative. Certainly, the strength of ligand-dependent subunit–subunit interactions is well-known to reflect the allosteric form of the protein and to influence access of ligands to cryptic sites within the oligomer, viz., hemoglobin (Perutz, 1978), glyceraldehyde-3-phosphate dehydrogenase (Osborne & Hollaway, 1975), and phosphorylase (Barford et al., 1988).

A second factor that should influence rate has its origins in mechanistic differences between the two processes. The dimensions and geometry of the tunnel (Hyde et al., 1988) suggest that, in order for BZI to displace the equilibrium of eq 3 toward the E(A-A)(BZI) Michaelis complex when GP blocks the tunnel entrance at the α-site, first nucleophiles the size of aniline or indoline must be released into solution via the opening created by the protein conformational transition and then BZI must enter the tunnel via the same route and diffuse to the β-subunit indole subsite of the E(A-A) complex. Accordingly, the protein transition that creates the opening will limit *both* the rate of release of these nucleophiles from the tunnel and the rate of entry of BZI into the tunnel.[2] However, this process will limit only one step of the nucleophile reaction with E(A-A), the entry of nucleophile into the tunnel.

Because the rate of reaction of the small nucleophiles (i.e., hydroxylamine and *O*- or *N*-methylhydroxylamine) with the E(A-A) (eqs 6 and 7) are only slightly influenced by binding of GP, we conclude that GP is ineffective in hindering access of these compounds to the β-site. These findings establish two points of interest: (1) Since these rates are not significantly affected, the intrinsic chemical reactivity of E(A-A), as measured by the rate of quinonoid appearance, is not significantly changed by the binding of GP. (2) Either the tunnel is not completely blocked when GP binds, or these small molecules gain access directly to the β-site from solution. Since

[5] Our unpublished studies (P. Brzovič and M. F. Dunn) show that G3P, like GP, is effective in blocking the diffusion of indole and the indole-like nucleophiles from external solution into the β-site. If G3P dissociation is slower than the reaction of indole with E(A-A), then in the physiological reaction the indole produced via cleavage of IGP (eq 1) will be constrained to react with E(A-A) (eq 2) because its escape into external solution will be blocked by bound G3P (viz., Scheme II).

Scheme II: Cartoon Depicting Tryptophan Synthase Bienzyme Complex Catalytic Cycle[a]



[a] IGP enters the α-site via the cleft opening (upper right). Catalysis converts IGP to indole and G3P at the α-site. Indole diffuses along the tunnel into the β-site, while G3P eventually dissociates from the α-site via the cleft opening. Meanwhile, at the β-site, L-Ser enters directly from solution via the β-site cleft opening (lower right) and is converted to the α-aminoacrylate Schiff base and reacts with indole to yield L-Trp. L-Trp and the water molecule generated by the L-Ser reaction dissociate into solution via the β-site cleft opening.

the observed rate of *N*-methylhydroxylamine exchange for aniline is only weakly inhibited by GP (Figure 6), we conclude that the >1000-fold inhibition of the BZI displacement reaction is not due simply to a corresponding reduction in the intrinsic rate constant (k_{-Q}, eq 4) for the conversion of the aniline quinonoid back to E(A-A). Although stabilization of the quinonoid via the allosteric effects of GP binding must alter k_{-Q}, the *N*-methylhydroxylamine data indicate the GP effect on this rate constant is no more than a factor of 2 or 3 (viz., the *y* intercepts of Figures 4B and 6B). The fact that the slopes of the plots shown in Figures 4B and 6B are not much affected by GP while the *y* intercepts are decreased indicates that the stabilizing effects of GP are manifest in slightly decreased rates of cleavage (e.g., k_{-Q}) of the scissile bond between Nu and the β-carbon of the quinonoid. Furthermore, the small effect indicates that, in the presence of GP, the escape of aniline from the tunnel into solution is not rate limiting for the *N*-methylhydroxylamine exchange reaction. Hyde et al. (1988) report that the tunnel is long enough to accommodate at least four indole molecules (viz., Scheme I); hence if aniline is displaced into the tunnel, then the rate of nucleophile interchange in this system would not necessarily be limited by release of aniline into solution. These conclusions are further supported by the finding that the rate of exchange of indole for the aniline quinonoid is reduced ~50-fold in the presence of 50 mM GP (data not shown).

Substrate Channeling in the $\alpha_2\beta_2$ Complex. We favor a model for the functioning of the $\alpha_2\beta_2$ complex that involves the vectoral movement of indole from the α-site to the β-site via the tunnel as shown in Scheme II. It seems plausible and efficient that, rather than traverse the tunnel, L-Trp is released directly from the β-site into solution. It follows that there must be at least three routes which provide access to the indole subsite of the β-subunit; the tunnel from the α-site, direct entry from solution through the β-site opening, and a slow conformational transition that creates an opening from solution into the tunnel. The β-site must accommodate the direct access of both L-Ser and L-Trp from solution (vida infra). Once covalent reaction between L-Ser and PLP occurs to form E(A-A), enough room may remain to allow the unimpeded entry of the small nucleophiles, but due to steric restrictions and protein conformational changes, entry via this route for indole and the indole structural analogues is too slow to be significant.

We conclude from these experiments that the tunnel functions as a highly efficient conduit for the transfer of indole from the α-site to the β-site. In the physiological reaction (eqs 1 and 2), the common metabolite indole is constrained (probably via steric interactions) to the tunnel by bound G3P[5] (viz., Scheme II). The allosteric transition mediated by liganding to the phosphoryl subsite of the α-subunit stabilizes the quinonoidal species formed at the β-site. Hence, steric and allosteric interactions within the $\alpha_2\beta_2$ complex exert control over the covalent transformations which take place at the α- and β-catalytic sites of this bienzyme complex such that L-Trp synthesis is favored.

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