Intermediate Trapping via a Conformational Switch in the Na^+-Activated Tryptophan Synthase Bienzyme Complex†

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Received January 22, 2002; Revised Manuscript Received May 29, 2002

ABSTRACT: The tryptophan synthase bienzyme complex channels substrate indole between the α- and β-sites via a 25 Å long interconnecting tunnel. Channeling efficiency is dependent upon a conformational switch in αβ-dimeric units between open conformations of low activity to which substrates bind and closed conformations of high activity wherein substrates react. In experiments designed to gain a better understanding of the linkage between chemical steps and conformational transitions in the catalytic cycle, the novel amino acid dihydroiso-L-tryptophan (DIT) was used as an analogue of L-Trp. In the forward reaction (indoline + L-Ser) to synthesize DIT, the quinonoid species, $E(Q)_{indoline}$, is formed quickly, while in the reverse reaction (DIT cleavage), the accumulation of $E(Q)_{indoline}$ occurs very slowly. Nevertheless, when the α-site substrate analogue α-D,L-glycerol phosphate (GP) is bound, DIT cleavage was found to give a rapid formation and dissipation of $E(Q)_{indoline}$ followed by a very slow reappearance of $E(Q)_{indoline}$. This result led to the conclusion that the reaction of DIT proceeds quickly through the quinonoid state to give indoline and the α-aminoacrylate Schiff base, E(A-A), both in the absence and in the presence of GP. In the absence of GP the slow conversion of E(A-A) to pyruvate and ammonium ion limits the rate of accumulation of free indoline and therefore the rate of buildup of $E(Q)_{indoline}$. However, when GP is bound to the α-site, the indoline generated by DIT cleavage in the first turnover is trapped within the enzyme complex, shifting the equilibrium distribution strongly in favor of $E(Q)_{indoline}$ as a consequence of the high local concentration of sequestered indoline. This sequestering is the result of a switching of αβ-subunit pairs to a closed conformation when GP binds to the α-site and E(A-A) and/or $E(Q)_{indoline}$ is formed at the β-site, thereby trapping indoline inside. The decay of the transiently formed $E(Q)_{indoline}$ occurs due to leakage of indoline from the closed system.

Tryptophan synthase has been well studied for many years and serves as the paradigm for allosteric regulation of substrate channeling in multienzyme complexes. The $\alpha_2\beta_2$ tryptophan synthase bienzyme complex from *Salmonella typhimurium* catalyzes the final two steps in the biosynthesis of tryptophan (*1*−*3*). The enzyme consists of two αβ-dimers joined β-end to β-end in a nearly linear αββα fashion (*4*−*6*). The α and β active sites in the bienzyme complex are shown by the crystal structure to be connected by a 25−30 Å tunnel (*5*). The α-subunit catalyzes the cleavage of 3-indole-D-glycerol 3′-phosphate (IGP)[1] to indole and D-glyceraldehyde 3-phosphate (G3P) (eq 1), while the β-subunit catalyzes the condensation of the indole generated in the α-reaction with L-serine (L-Ser) to form L-tryptophan (L-Trp) (eq 2). The combined α- and β-reactions are designated as the αβ-reaction (eq 3). Rapid kinetic studies have shown that indole is channeled through the connecting tunnel between the α- and β-sites (*7*−*12*).

$$\text{IGP} \rightleftharpoons \text{indole} + \text{G3P} \quad (1)$$

$$\text{indole} + \text{L-Ser} \rightleftharpoons \text{L-Trp} \quad (2)$$

$$\text{IGP} + \text{L-Ser} \rightleftharpoons \text{G3P} + \text{L-Trp} \quad (3)$$

Scheme 1 shows the mechanistic details of the αβ-reaction. The α-reaction consists of a reversible aldolytic cleavage of IGP to G3P and indole. Indole is then made available, via diffusion along the tunnel, to the β-site for reaction with L-Ser. The β-reaction occurs in two stages. In the first stage, L-Ser enters the β-site and reacts with the internal aldimine species, E(Ain), of PLP, which is linked to the enzyme via the ε-amino group of βLys87. Stage I of the β-reaction path involves geminal diamine, $E(GD_1)$, external aldimine, $E(Aex_1)$, and quinonoid, $E(Q_1)$, intermediates and halts at the quasi-stable α-aminoacrylate Schiff base species, E(A-A). In stage II, the indole formed in the α-reaction is transferred via the tunnel to the β-site where it performs a nucleophilic attack on the electrophilic β-C of E(A-A) to form a C−C bond

† Supported by NIH Grant GM55749.

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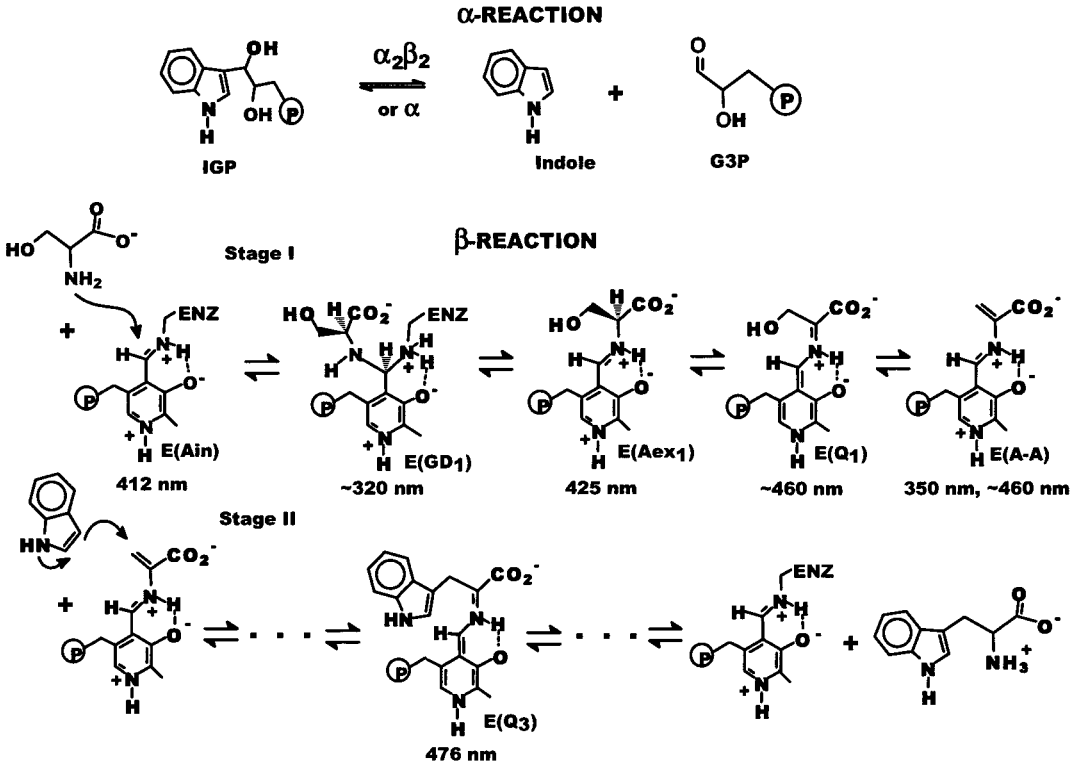
[1] Abbreviations: PLP, pyridoxal phosphate; L-Ser, L-serine; L-Trp, L-tryptophan; DIT, dihydroiso-L-tryptophan; IGP, 3-indolyl-D-glycerol 3′-phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerol phosphate; NMHA, *N*-methylhydroxylamine; D305A, $\alpha_2\beta_2$ complex of tryptophan synthase where aspartate 305 in the β-subunit has been replaced by alanine; RSSF, rapid scanning stopped flow; SWSF, single wavelength stopped flow; NMR, nuclear magnetic resonance; $\alpha_2\beta_2$, native tryptophan synthase from *Salmonella typhimurium*; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; $E(Q_1)$, $E(Q_2)$, $E(Q_3)$, $E(Q)_{indoline}$, or $E(Q)_{NMHA}$, quinonoid intermediates; $E(Aex_1)$ or $E(Aex_2)$, external aldimine intermediates; $E(GD_1)$, $E(GD_2)$, or $E(GD_3)$, geminal diamine intermediates formed between the PLP cofactor, the amino group of the substrate, and the ε-amino group of βLys87; TEA, triethanolamine buffer; CD, circular dichroism.

10.1021/bi0255672 CCC: $22.00 © 2002 American Chemical Society
Published on Web 06/29/2002

Scheme 1: Reactants, Products, and Key Intermediates in the α- and β-Reactions Catalyzed by the Tryptophan Synthase Bienzyme Complex



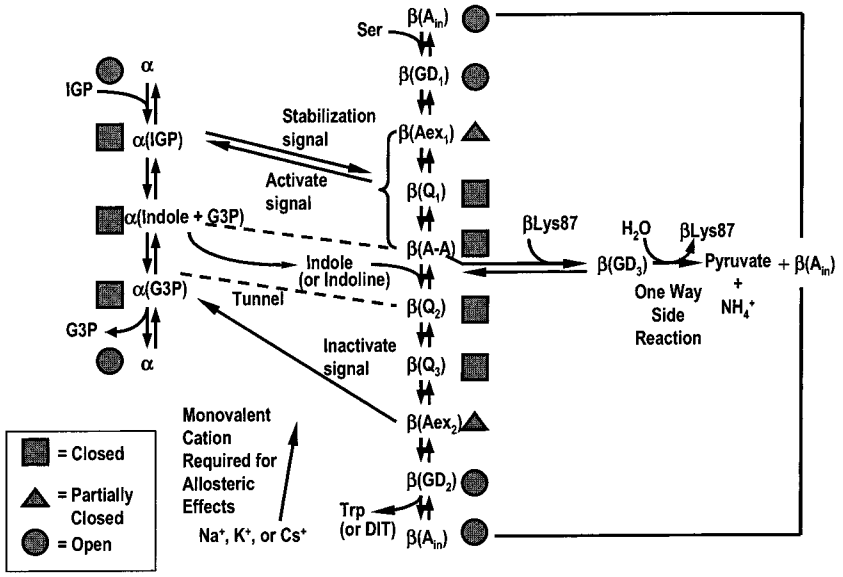
between the indole C-3 and the E(A-A) β-C to give $E(Q_2)$. The reaction then proceeds through $E(Q_3)$, an L-Trp external aldimine species, $E(Aex_2)$, and another geminal diamine species, $E(GD_2)$, to regenerate the internal aldimine species, E(Ain), and release L-Trp.

A set of allosteric communications between the subunits controls the overall αβ-reaction. Scheme 2 presents a schematic representation of the allosteric signals mapped onto the αβ-reaction. When IGP (or an IGP analogue) binds to the α-site, a conformation change is induced in the α-subunit (*9*, *11*−*13*). This conformational transition has been demonstrated by fluorescence energy transfer methods to significantly increase the rigidity of the β-subunit (*13*) and to alter the equilibrium distribution of intermediates in the β-reaction in favor of E(A-A) (*14*, *15*). The reaction of L-Ser at the β-site sends an allosteric signal to the α-subunit, which results in the activation of the α-site by 25−30-fold (*11*, *15*, *16*). The covalent switch that activates the α-site was established to be the conversion of $E(Aex_1)$ to E(A-A) at the β-site, a process that moves the flexible loop (loop 6) in the α-subunit to a closed position (*15*, *17*, *18*). The L-Ser reaction also causes a conformation change in the β-subunit to a closed state, thus trapping the indole generated from the α-reaction within the doubly closed conformation of the αβ-complex. Once indole is cleaved from IGP, it migrates through the tunnel where it can only react with the quasi-stable α-aminoacrylate Schiff base, E(A-A) (stage II of the β-reaction). In stage II, the conversion of $E(Q_3)$, to $E(Aex_2)$, transmits a deactivation signal to the α-site (*19*) and switches the α-subunit to an open configuration which then releases G3P. Allosteric linkages that are involved in this site-to-site communication include salt-bridging interactions between βLys167 and αAsp56 or βAsp305 and between βAsp305 and βArg141, interactions identified through mutation studies and inspection of the three-dimensional structures of enzyme complexes. These interactions have been identified through kinetic studies of the mutant enzymes generated from point mutations at these loci (*20*). The entire set of allosteric signals is also dependent on the binding of a monovalent cation cofactor to a site on the β-subunit (*20*−*24*). The catalytic efficiency of the tryptophan synthase αβ-reaction is a consequence of structural and kinetic constraints that switch αβ-dimeric units between open and closed conformations, thus preventing the escape of indole, and by providing allosteric signals that synchronize the catalytic cycles of the α- and β-subunits (*25*). The closure of the β-site also protects the E(A-A) from reaction with water to form pyruvate, an irreversible side reaction.

The novel amino acid dihydroiso-L-tryptophan (DIT, Chart 1) was first synthesized in 1988 (*26*) by incubating indoline and L-Ser with tryptophan synthase. Indoline emulates indole except that the nucleophilic reaction with E(A-A) occurs through the ring nitrogen instead of the C-3 carbon. When wild-type E(A-A) reacts with indoline, the quasi-stable $E(Q)_{indoline}$ is rapidly formed. Unlike the indole quinonoid species, which is rapidly converted to L-Trp, conversion of $E(Q)_{indoline}$ to DIT occurs very slowly. The tryptophan synthase mutant, βD305A, was found to produce DIT from L-Ser and indoline much more efficiently than does wild-type enzyme (*27*), and this mutant was used to facilitate the synthesis of DIT in amounts sufficient for use in these mechanism studies.

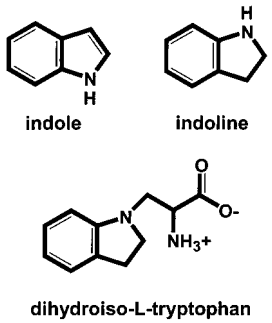
The purpose of this work is to further study the tryptophan synthase catalytic mechanism by using DIT as an L-Trp analogue to investigate β-site catalysis in the reverse reaction. In marked contrast to the reaction of indoline with the (GP)E-(A-A) complex, the results show that reaction of DIT with the (GP)E(Ain) complex gives a rapid transient formation

Scheme 2: Schematic Representation of the Allosteric Signals between the α- and β-Sites[a]



[a] When IGP binds to the α-site, the α-subunit converts to a closed conformation. The closed conformation of the α-site then stabilizes the L-Ser reaction at the β-site. When L-Ser reacts at the β-site, the conversion of E(Aex$_1$) to E(A-A) switches the β-site to the closed conformation and sends an activation signal to the α-site. Once the α-site is activated, IGP is cleaved into G3P and indole; G3P remains bound to the α-site, and the indole is free to migrate along the tunnel to the β-site. Indole is trapped within the closed conformation of the bienzyme complex and can only react with the E(A-A) at the β-site. The reaction of indole with E(A-A) gives two quinonoid intermediates, the L-Trp external aldimine and the geminal diamine species, and then the enzyme is cycled back to E(Ain) with the release of L-Trp. During the transformation of the L-Trp quinonoid species to the external aldimine species, both the α- and the β-sites are converted from the closed to the open conformation releasing G3P, and an inactivation signal is sent to the α-site. The quasi-stable E(A-A) at the β-site can react with water through an irreversible side reaction to form pyruvate and ammonium ion and regenerate E(Ain), but this side reaction occurs at a much slower rate than the indole reaction. All of the allosteric communications between the α- and β-subunits require a monovalent cation bound to the β-subunit. If indoline is used instead of indole, DIT is produced instead of L-Trp.

Chart 1: Structures of Indole, Indoline, and Dihydroiso-l-tryptophan



and decay of E(Q)$_{indoline}$ that is followed by a much slower buildup and decay of this same species. It will be shown that this transient trapping of E(Q)$_{indoline}$ is caused by allosteric interactions within the bienzyme complex that switch the subunits from an open to a closed conformation state.

MATERIALS AND METHODS

Materials. Indoline, L-serine, and α-D,L-GP were purchased from Sigma. The indoline was further purified by vacuum distillation and converted to the hydrochloride salt by bubbling HCl gas through a diethyl ether solution of indoline followed by drying the ether-insoluble salt under vacuum. All reactions were performed in 50 mM triethanolamine (TEA) buffer brought to a pH of 7.8 by the addition of HCl.

Both the wild-type and D305A mutant tryptophan synthases from *S. typhimurium* were purified from *Escherichia coli* expression systems by methods previously described (*16*, *28*, *29*). All experiments with the wild-type enzyme were performed in the presence of 100 mM Na^+ to ensure that the enzyme was in the Na^+-activated form. The D305A mutant enzyme was activated with 100 mM Cs^+ for use in the synthesis of DIT.

Enzymatic Synthesis and Purification of Dihydroisotryptophan (DIT). The protocol used was a modification of one described previously (*26*). Due to light sensitivity, all solutions of indoline and DIT were protected from light as much as possible during the synthesis, purification, and storage. The enzymatic reaction solution consisted of 20 mM indoline, 400 mM L-Ser, 100 mM Cs^+, and 12.5 μM D305A mutant tryptophan synthase, all in TEA buffer. These solutions of indoline, L-Ser, and Cs^+ were prepared in bulk and frozen in aliquots of ∼10 mL. To synthesize DIT, aliquots were thawed, and the D305A mutant was added. The course of the reaction was followed by monitoring the change in absorption at 306 nm using a 600 μL aliquot placed in a 0.2 cm path-length cuvette. The reaction reached equilibrium in about 30 min and was then halted by addition of 600 μL of glacial acetic acid. The contents were then wrapped in foil and stored frozen at −20 °C until the separation step.

The DIT was recovered and purified by thawing the reaction mixture, pelleting the denatured enzyme with a benchtop centrifuge, loading the supernatant on a Sephadex G-15 superfine column (0.8 × 50 cm), and eluting with water. The fractions, which eluted at about 26 h, were assayed for absorbance at 298 nm. The DIT spectrum is similar to that of indoline but is red shifted by ∼8 nm (*26*).

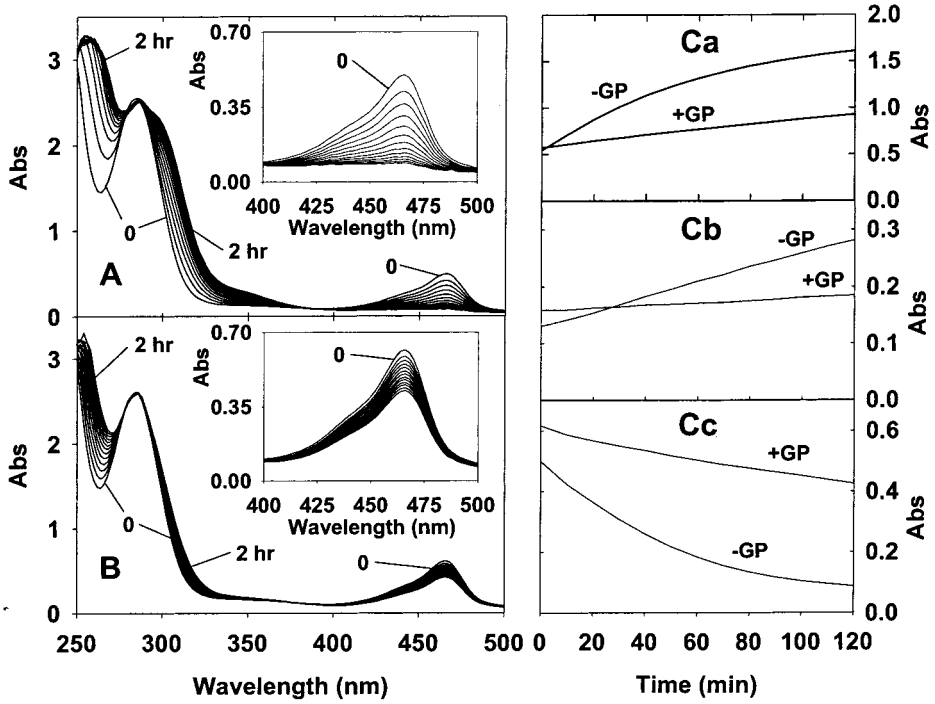


FIGURE 1: Time-resolved spectra for the reaction of $\alpha_2\beta_2$ with indoline and L-Ser without (A) and with GP (B). Indoline and L-Ser react quickly at the β-sites to form $E(Q)_{indoline}$ (λ_{max} = 466 nm). Spectra were measured at 10 min intervals. Insets show expansions of the quinonoid region. The panels in part C show the time courses for the reactions at 310 nm (DIT production) (Ca), 350 nm (pyruvate production) (Cb), and 466 nm [$E(Q)_{indoline}$ production and decay] (Cc). Concentrations: [$\alpha_2\beta_2$] = 10 μM, [Na^+] = 100 mM, [GP] = 50 mM, [L-Ser] = 40 mM, and [indoline] = 1 mM.

The DIT fractions were pooled, lyophilized, and reconstituted in TEA buffer at concentrations ranging from 4 to 12 mM.

Optical Spectroscopy. Absorbance measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer. All of the reactions were performed at 23 ± 2 °C and protected from light as much as possible to protect the photosensitive DIT and indoline.

Rapid Scanning and Single Wavelength Absorbance Measurements. The RSSF and SWSF measurements were carried out as previously described (*30*−*32*). The enzyme and Na^+ were placed in one syringe at concentrations of 40 μM ($\alpha\beta$-sites) and 100 mM, respectively. The DIT and Na^+ were placed in the other syringe at concentrations of 5 mM and 100 mM, respectively. When the contents of the two syringes were mixed, the final concentrations of the enzyme and DIT were cut in half, but the Na^+ concentration remained the same. When the flow was stopped, the time course of the reaction was observed by monitoring the light intensity transmitted through the observation chamber. The production of $E(Q)_{indoline}$ was observed by monitoring the absorbance at 466 nm. Data analysis was preformed using the software Peakfit, version 4 (Jandel Scientific), and Sigma Plot, version 4 (SPSS).

NMR Spectral Measurements. NMR spectra were measured with a Varian 500 MHz spectrometer using ^1H-pulsed FT 1-D methods with aqueous samples in 10% D_2O. ^1H NMR was originally used by Roy et al. to identify DIT (*26*) and was used in this study to confirm DIT production and to assay for L-Ser contamination of the DIT sample by comparing the spectrum of a pure sample of L-Ser to the spectrum of the DIT sample. The DIT samples were found to contain no greater than 1.5% L-Ser to DIT content.

RESULTS

GP Effects on Indoline Reactions with Na^+-Activated Enzyme. When indoline and L-Ser are used as substrates in the tryptophan synthase reaction, a quasi-equilibrium[2] is set up among all of the intermediates in stage I (Scheme 1) plus $E(Q)_{indoline}$. Figure 1A shows that $E(Q)_{indoline}$ (λ_{max} = 466 nm) is the dominant constituent within a few milliseconds after mixing. Then $E(Q)_{indoline}$ slowly decays to DIT. When the reaction is run with GP added, more $E(Q)_{indoline}$ accumulates, and this species persists for a longer time (Figure 1B). Both of the reactions show a red shift in the 300−320 nm region of the absorption spectrum, indicating the production of DIT. This red shifting of the spectrum is more pronounced in the absence of GP. As evidenced by the spectral changes between 340 and 360 nm (Figure 1A), pyruvate generation from the E(A-A) side reaction is also stimulated in the Na^+-activated enzyme. In the Na^+ system, the $E(Q)_{indoline}$ absorption band nearly disappears within 2 h. The production of DIT occurred more slowly in the presence of GP (Figure 1B). Figure 1C shows time courses measured in the absence and in the presence of GP at three different wavelengths. The 310 nm curves are dominated by the appearance of DIT, the 350 nm curves are dominated by the appearance of pyruvate, and the 466 nm curves follow the disappearance of $E(Q)_{indoline}$. The addition of GP stabilizes $E(Q)_{indoline}$, thus inhibiting the production of DIT and pyruvate.

DIT Reactions with and without GP. When DIT is used as a substrate in the tryptophan synthase reaction (the reverse

[2] Owing to the steady-state rate process in which the E(A-A) intermediate is converted to pyruvate and ammonium ion, the reactions of L-Ser and L-Ser plus indoline never attain a true equilibrium.

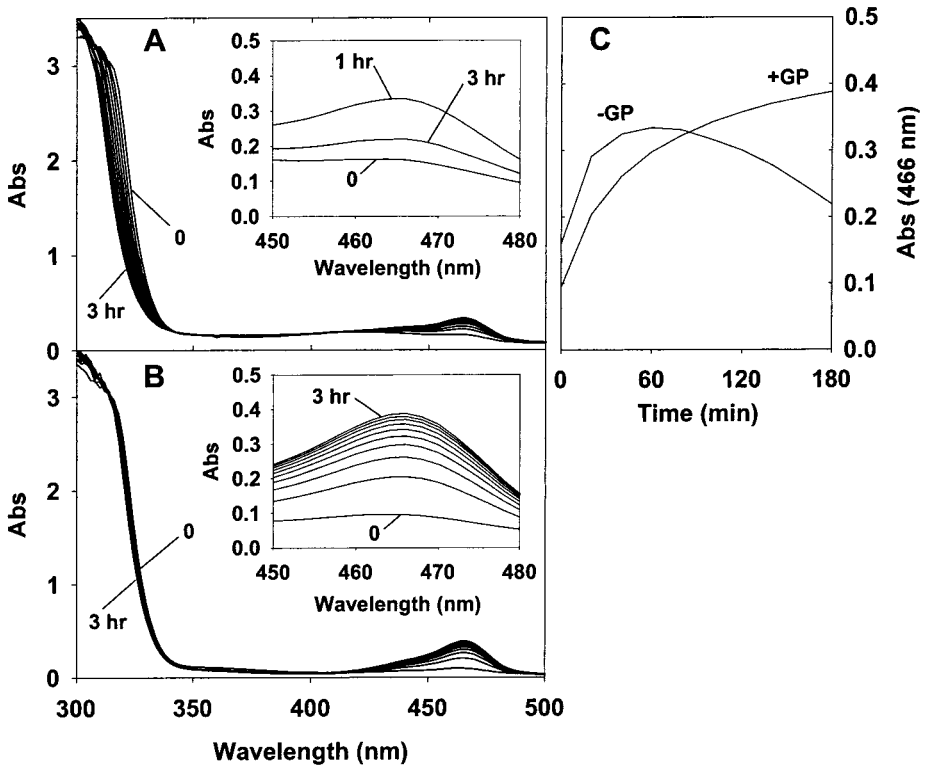


FIGURE 2: Time-resolved spectra for the reaction of $\alpha_2\beta_2$ with DIT without (A) and with GP (B). DIT slowly forms $E(Q)_{indoline}$ ($\lambda_{max} = 466$ nm). Spectra were measured at 20 min intervals. Insets show expansions of the quinonoid region. Panel C shows the single wavelength time courses for the reactions at 466 nm for $E(Q)_{indoline}$. Concentrations: $[\alpha_2\beta_2] = 10\ \mu M$, $[Na^+] = 100$ mM, [GP] = 50 mM, and [DIT] = 5 mM. The spectra designated time 0 were collected approximately 5−10 s after mixing.

of the indoline reaction), a different initial equilibrium involving the intermediates in the reaction is established (see Scheme 1). The effects of GP on the DIT reaction are presented in Figure 2. Reaction both in the presence and in the absence of GP shows a very slow formation of $E(Q)_{indoline}$ at 466 nm and a very slow blue shifting of the indoline ring absorption spectrum (300−320 nm region) as DIT is converted to indoline. Figure 2C shows the time courses for $E(Q)_{indoline}$ production by monitoring the $E(Q)_{indoline}$ absorption band at 466 nm both in the presence and in the absence of GP. The formation of $E(Q)_{indoline}$ reaches a maximum and then slowly decays away as substrate is consumed via conversion of E(A-A) to E(Ain), pyruvate, and ammonium ion. Again, the presence of GP gives the greatest yield of $E(Q)_{indoline}$, and this species persisted for a longer time. The accumulation of $E(Q)_{indoline}$ peaked at about 1 h in the Na^+ reaction and then decayed away nearly back to zero in the next 2 h, whereas the reaction peaked at about 4 h in the presence of Na^+ and GP.

Single Wavelength and Rapid Scan Stopped-Flow Studies of the DIT Reactions. The DIT reactions with tryptophan synthase with and without GP were examined using SWSF and RSSF. The SWSF time courses were found to be triphasic, as determined by the Peakfit software, with the first two phases exponential and the third phase effectively linear (for up to 10 min).

The RSSF instrumentation allowed scanning a wide range of wavelengths simultaneously during reaction, but data could be collected only for a relatively limited time intervial due to restrictions imposed by available computer memory. Therefore, to follow the reaction of DIT with the enzyme, the RSSF data were collected for 48 s. The time-resolved spectra showed only minor changes in absorbance over the wavelength range from 320 to 500 nm with the largest changes occurring in the vicinity of 350, 412, and 466 nm. The slight absorbance increase in the 340−350 range is due to the formation of both E(A-A) ($\lambda_{max} \cong 350$ nm) and pyruvate (320−340 nm). The slight absorbance increase centered at 466 nm is due to the appearance of $E(Q)_{indoline}$. The decrease in absorbance at 412 nm is due to the conversion of E(Ain) ($\lambda_{max} = 412$ nm) to the other intermediates. The single wavelength time course at this wavelength is shown in Figure 3. The curve is biphasic with both phases decreasing in absorbance. Using an exponential to fit each phase, the values of the relaxation rates ($1/\tau$) were found to be $1/\tau_1 = 1.85 \pm 0.2\ s^{-1}$ and $1/\tau_2 = 0.33 \pm 0.05\ s^{-1}$.

In the absence of GP, the DIT reaction shows a rate of $E(Q)_{indoline}$ production that increases as the concentration of added indoline is increased (Figure 4A). Since there is no L-Ser initially present, the formation of $E(Q)_{indoline}$ must be due either to the conversion of DIT to $E(Q)_{indoline}$ or to the reaction of indoline (derived from cleavage of DIT) with E(A-A) (see Scheme 2). In the presence of 0.1 mM indoline, there is only a slight increase in the rate of $E(Q)_{indoline}$ formation. In the presence of 5.0 mM indoline, $E(Q)_{indoline}$ formation is very rapid with most of the change occurring within the stopped-flow dead time. The spectra presented in Figure 4B show that the reaction with 0.1 mM indoline behaves at longer times much like the DIT reaction without indoline (compare with the inset to Figure 2A). The spectra in Figure 4C show that when a high concentration of indoline is used, the reaction behaves at longer times much as the L-Ser and indoline reaction (compare with the inset to Figure 1A).

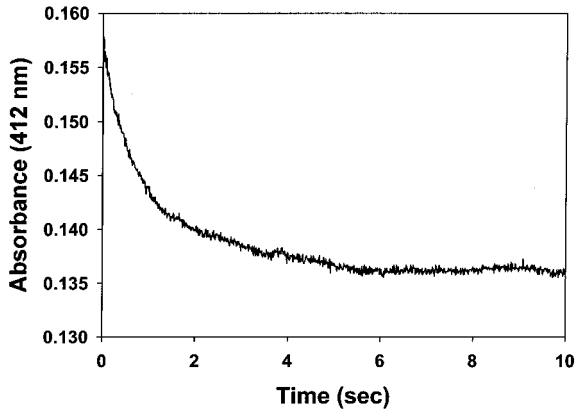


FIGURE 3: Stopped-flow time course for the disappearance of E(Ain) (λ_{max} = 412 nm) during reaction of DIT with $\alpha_2\beta_2$. The curve is biphasic (both decreasing absorbance), and assuming that each phase is exponential, the values of the relaxation rates ($1/\tau$) were found to be $1/\tau_1 = 1.85 \pm 0.2$ s^{-1} and $1/\tau_2 = 0.33 \pm 0.05$ s^{-1}. Concentrations (upon mixing): [$\alpha_2\beta_2$] = 10 μM, [Na^+] = 100 mM, and [DIT] = 2 mM.

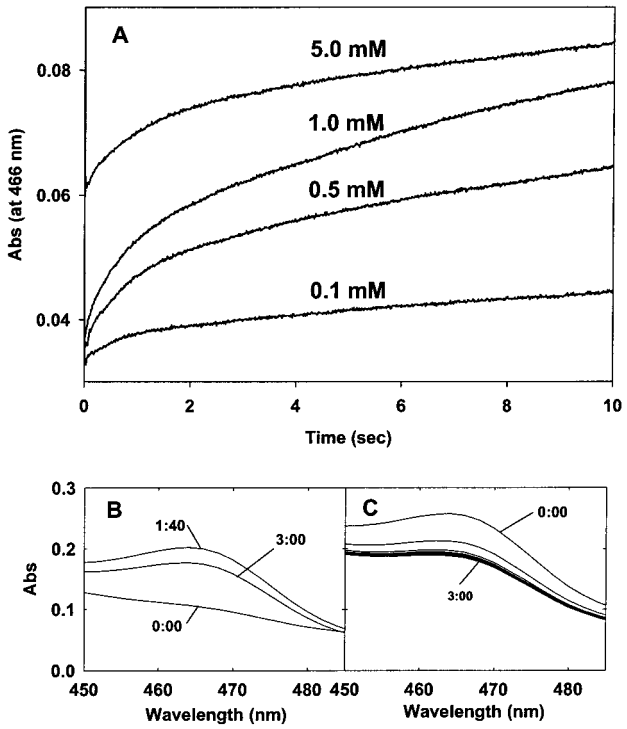


FIGURE 4: Stopped-flow time courses for $E(Q)_{indoline}$ formation in the reaction of DIT with $\alpha_2\beta_2$ and various amounts of free indoline. (A) The rate of quinonoid formation increases as more free indoline is added to the solution. (B) Spectra over time for 4.9 mM DIT reacting in the presence of 0.1 mM indoline (compare to Figure 2A inset). (C) Spectra for 4.9 mM DIT reacting in the presence of 9.8 mM indoline (compare to Figure 1A inset). Concentrations (upon mixing): [$\alpha_2\beta_2$] = 10 μM, [Na^+] = 100 mM, [DIT] = 2 or 4.9 mM, and [indoline] = 0.1, 0.5, 1.0, 5.0, and 9.8 mM.

The reaction of the (GP)E(Ain) complex with DIT resulted in a more interesting time course for $E(Q)_{indoline}$ formation and decay (Figure 5A). There is a strong, rapid initial rise ($1/\tau_1 = 2.65 \pm 0.20$ s^{-1}) and fall ($1/\tau_2 = 0.47 \pm 0.05$ s^{-1}) in the amount of $E(Q)_{indoline}$ that was not detected on slower time scales (viz. Figure 2C). This transient event is followed by the same very slow appearance of $E(Q)_{indoline}$ as seen in Figure 2C. The rapid formation was reduced in amplitude when a lower GP concentration (5 mM) was used, and no

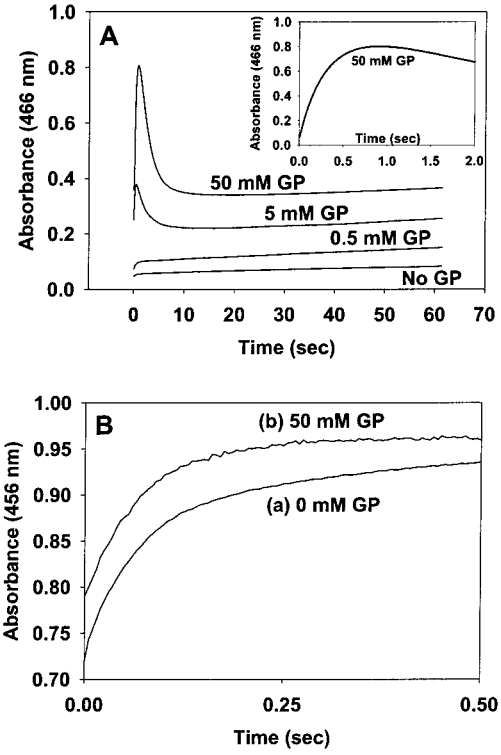
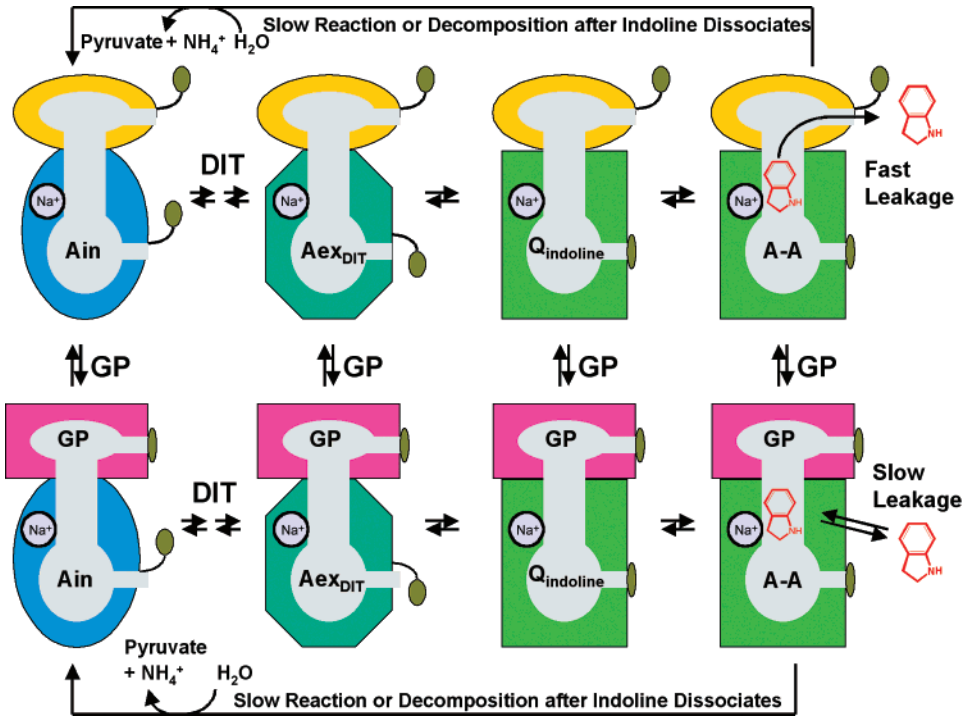


FIGURE 5: (A) Influence of GP concentration on the stopped-flow time courses for formation and decay of the DIT quinonoid intermediate. The curve is biphasic (one ascending phase, $1/\tau_1$, and one descending phase, $1/\tau_2$), and assuming that each phase is exponential, the values of the relaxation rates measured with 50 mM GP were found to be $1/\tau_1 = 2.65 \pm 0.20$ s^{-1} and $1/\tau_2 = 0.47 \pm 0.05$ s^{-1}. The values of the relaxation rates measured with 5 mM GP were found to be $1/\tau_1 = 6.9 \pm 0.7$ s^{-1} and $1/\tau_2 = 0.44 \pm 0.05$ s^{-1}. The inset shows the relaxation time course for the 50 mM GP case expanded to 2 s. Concentrations (upon mixing): [$\alpha_2\beta_2$] = 20 μM, [Na^+] = 100 mM, [DIT] = 2 mM, and [GP] = 0, 0.5, 5.0, or 50 mM. (B) Influence of GP on the time courses for the exchange of the indoline and *N*-methylhydroxylamine (NMHA) moieties during the interconversion of $E(Q)_{indoline}$ and $E(Q)_{NMHA}$ at the β-site of tryptophan synthase. The experiments were carried out in the absence (a) and presence (b) of 50 mM GP by generating $E(Q)_{indoline}$ in one syringe of the stopped-flow apparatus and reacting with NMHA in the other syringe of the apparatus. Absorbance was measured at 456 nm, a wavelength where the extinction coefficients of the two quinonoid species are different. The relaxations are single exponential in form. Analysis of the time courses gave the following relaxation rates: (a) $1/\tau = 8.39 \pm 0.81$ s^{-1} and (b) $1/\tau_{GP} = 13.69 \pm 0.67$ s^{-1}. Conditions: (a) syringe 1, [$\alpha_2\beta_2$] = 20 μM, [indoline] = 5 mM, [L-Ser] = 40 mM, and [Na^+] = 100 mM; syringe 2, [NMHA] = 100 mM, [indoline] = 5 mM, [L-Ser] = 40 mM, and [Na^+] = 100 mM. (b) The conditions were identical to (a) except that each syringe contained 50 mM GP.

transient formation of $E(Q)_{indoline}$ was seen with 0.5 mM or lower GP concentrations. Owing to the short duration of the rapid transient formation and decay of $E(Q)_{indoline}$ (about 8 s), these processes were not detected when using the static spectrophotometer where the sample loading time was of the same order.

To examine the influence of GP on the kinetics of the cleavage of the $E(Q)_{indoline}$ C−N bond to give E(A-A) and indoline, the kinetic time courses for the interconversion of $E(Q)_{indoline}$ and the *N*-methylhydroxylamine quinonoid species, $E(Q)_{NMHA}$, in the absence and presence of GP were determined (Figure 5B) following the experimental procedure used by Dunn et al. (*9*) to determine the influence of GP on nucleophile exchange rates. As Dunn et al. showed, the

Scheme 3: Cartoons Depicting the Open (Ellipse), Closed (Square), and Partially Closed (Octagonal) Conformations for Key Intermediates of the Catalytic Cycle of the $\alpha\beta$-Reaction[a]



[a] See refs *22*, *23*, *25*, and *33*. The small, tethered ellipses represent the structures that act as lids to the α- and β-sites. The upper row represents the reaction intermediates without GP bound, while the bottom row has bound GP. When GP binds to the α-site, the α-subunit adopts a closed configuration (square). When GP dissociates, the α-subunit opens (ellipse). When DIT reacts with the E(Ain) in the β-site, it forms an external aldimine, $E(Aex)_{DIT}$, which partially closes the β-subunit. The $E(Aex)_{DIT}$ then transitions through $E(Q)_{indoline}$, closing the β-subunit (square), and on to cleavage into indoline and E(A-A). The indoline is then trapped within the bienzyme complex where it can slowly leak out or wait for GP to dissociate and escape through the open α-subunit. After indoline escapes, the E(A-A) is slowly reset to E(Ain) via the side reaction that generates pyruvate and ammonium ion.

difference in absorption spectra of the two quinonoid species can be used to follow the kinetics of the exchange process. Figure 5B shows the time courses for the reaction of $E(Q)_{indoline}$ with NMHA to give $E(Q)_{NMHA}$ both in the absence and in the presence of 50 mM GP. Comparison of these time courses shows that the rate of nucleophile exchange is fast and only slightly influenced by the binding of GP ($1/\tau$ = 8.39 ± 0.81 s^{-1} and $1/\tau_{GP}$ = 13.69 ± 0.67 s^{-1}).

DISCUSSION

Allosteric Regulation of Substrate Channeling. As seen in Scheme 2, the production of L-Trp from its precursor substrates is a highly ordered process wherein the activities of the α- and β-subunits are synchronized by allosteric signals that regulate both the production of indole at the α-site and the channeling of indole directly to the β-site. Previous work (*9*, *15*, *17*, *18*, *20*, *33*, *34*) has shown that the active sites of both subunits of the enzyme have "lids" that "close" and "open" under allosteric control during the progression of the reaction. These conformational changes function to trap indole within the $\alpha\beta$-dimeric unit until reaction with E(A-A) occurs and to regulate release of G3P and L-Trp. The synchronization of the α- and β-site activities ensures that IGP is not cleaved until E(A-A) formation has taken place at the β-site and that the lids remain closed until indole has reacted to form $E(Aex_2)$ at the β-site.

Comparison of the Indoline and DIT Reactions. In the papers of Woehl and Dunn (*21*−*23*) it was shown that tryptophan synthase requires the binding of a monovalent cation for allosteric communication. All of the experiments involving either the indoline or DIT reactions in the present work were investigated with the Na^+-activated form of the enzyme. It is interesting to note that when indoline reacts with E(A-A) in the absence of GP, $E(Q)_{indoline}$ is formed rapidly (within milliseconds). As shown by the red shift to 306 nm that occurs over longer time intervals (Figure 1), some DIT and some pyruvate are subsequently produced.

In the reverse direction, DIT appears to be converted to $E(Q)_{indoline}$ very slowly (requiring >30 min) (Figure 2). As indicated by the spectral changes at 306 nm, some cleavage of DIT occurs. The very different apparent rates of $E(Q)_{indoline}$ formation in the indoline and DIT reactions were first thought to be due either to the weak binding or to the slow reaction of DIT. However, the time course presented in Figure 3 shows the occurrence of a moderately rapid biphasic transformation ($1/\tau_1$ = 1.85 ± 0.2 s^{-1} and $1/\tau_2$ = 0.33 ± 0.05 s^{-1}) in the DIT reaction that is complete in about 4 s, indicating that the initial transformation of E(Ain) is relatively rapid. Therefore, both of these possibilities seem to be ruled out. Consequently, Scheme 3 is proposed as a rationale for the slow accumulation of $E(Q)_{indoline}$ in the DIT reaction, and as discussed below, it will be shown that the results presented in Figures 1−5 are consistent with this scheme.

According to the upper part of Scheme 3, DIT rapidly binds to the β-site of E(Ain), passes quickly through the $E(GD_2)$, $E(Aex_2)$, and $E(Q)_{indoline}$ intermediates, and then is cleaved to form indoline and E(A-A). The slow accumulation

of $E(Q)_{indoline}$ is due to the slow buildup of free indoline and the resulting mass action effect of that buildup which gradually shifts the distribution of species from E(A-A) plus indoline back to $E(Q)_{indoline}$. Since the turnover rate for indoline production depends on the slow conversion of E(A-A) to E(Ain), pyruvate, and ammonium ion, the accumulation of $E(Q)_{indoline}$ is correspondingly slow and accounts for the slow buildup of the 466 nm spectral band (Figure 2). As predicted by Scheme 3, when the concentration of indoline is made sufficiently high, reaction with E(A-A) quickly forms $E(Q)_{indoline}$ (Figure 1). The slow decay of $E(Q)_{indoline}$ occurs due to DIT depletion. As DIT is converted to E(A-A) and free indoline, a mass action from the high indoline concentration shifts the distribution of species in favor of $E(Q)_{indoline}$, but a small amount of the E(A-A) intermediate is converted irreversibly to E(Ain), pyruvate, and ammonium ion, thus consuming DIT.

GP Binding Traps the $E(Q)_{indoline}$ during the First DIT Turnover Cycle. As previously shown (*9*, *15*, *17*, *18*), the binding of GP to the α-site strongly inhibits the rate but not the yield of $E(Q)_{indoline}$ formed in the reaction of indoline with E(A-A) at the β-site. GP acts as an α-site G3P analogue and, when bound to the α-site, causes it to adopt the closed form (*9*, *34*). Consequently, with both the α- and the β-sites switched to the closed conformation, the rate at which indoline gains access to the tunnel and the β-site is strongly inhibited, whereas the amount of $E(Q)_{indoline}$ formed is enhanced. Because the rates of reaction of small nucleophiles, such as NMHA, with E(A-A) are not much affected by the binding of GP, it appears that the entry of these small molecule nucleophiles into the β-site is not hindered by conversion of the protein to the closed conformation (*9*). As shown herein, GP binding also slows the decay of $E(Q)_{indoline}$. This decay process is largely due to the pyruvate side reaction, and stabilization of the closed conformation of the αβ-dimeric unit slows this process.

In contrast to the results obtained in the absence of GP, when DIT is reacted with the GP complex of E(A-A), the stopped-flow time course shows a relatively rapid rise and decay of $E(Q)_{indoline}$ during the first 8 s of reaction in the presence of 50 mM GP (Figure 5A). The appearance of the $E(Q)_{indoline}$ absorbance band is essentially complete within 0.8 s ($1/\tau_1 = 2.65 \pm 0.20\ s^{-1}$). This transiently formed $E(Q)_{indoline}$ then decays away nearly completely within 8 s ($1/\tau_2 = 0.47 \pm 0.05\ s^{-1}$).

We have considered two mechanisms to account for the accumulation of $E(Q)_{indoline}$ when GP is bound to the α-site. In the first mechanism (eq 4), if GP binding stabilizes $E(Q)_{indoline}$ by slowing the rate of cleavage of the C−N bond as $E(Q)_{indoline} \rightleftharpoons E(A\text{-}A) + indoline_{free}$, then the transient accumulation of $E(Q)_{indoline}$ simply arises as a consequence of the altered rate of C−N bond cleavage. In the second mechanism (eq 5), the accumulation of $E(Q)_{indoline}$ is the consequence of the GP-mediated switching of the enzyme to the closed conformation, and the slow step is release of sequestered indoline as $[(GP)E(A\text{-}A) + indoline]_{closed} \rightleftharpoons [(GP)E(A\text{-}A)]_{closed} + indoline_{free}$.

$$DIT + E \rightleftharpoons E(DIT) \rightleftharpoons E(Aex)_{DIT} \rightleftharpoons E(Q)_{indoline} \rightleftharpoons E(A\text{-}A) + indoline_{free} \quad (4)$$

$$DIT + (GP)E \rightleftharpoons (GP)E(DIT) \rightleftharpoons (GP)E(Aex)_{DIT} \rightleftharpoons [(GP)E(Q)_{indoline}]_{closed} \rightleftharpoons [(GP)E(A\text{-}A) + indoline]_{closed} \rightleftharpoons [(GP)E(A\text{-}A)]_{closed} + indoline_{free} \quad (5)$$

As previously demonstrated for a similar system (*9*), the data presented in Figure 5B show that the rate of C−N bond cleavage, as implied by the rate of nucleophile exchange, is very rapid and reversible and very little influenced by the binding of GP. This rate is 18-fold faster than the observed rate of decay of $E(Q)_{indoline}$ in Figure 5A. Hence, the first explanation for the GP-mediated accumulation of $E(Q)_{indoline}$ in the DIT reaction must be discarded. Therefore, we propose that the transient formation and decay of $E(Q)_{indoline}$ have its origins in the switching of the β-subunit between open and closed conformations during the first turnover cycle of the DIT reaction as indicated in eq 5. With GP bound, the α-site has the closed conformation. In the first turnover, as DIT reacts to give the $E(Q)_{indoline}$ and E(A-A) intermediates with the production of indoline, the closing of the β-site is triggered and the indoline produced is trapped within the closed αβ-dimeric unit (eq 5 and Scheme 3, lower row). The sequestering of indoline within the closed αβ-dimeric unit creates a very high local concentration of indoline,[3] thereby establishing $E(Q)_{indoline}$ as the dominant intermediate owing to the mass action effect. The decay of $E(Q)_{indoline}$ over the first 8 s (Figure 5A) then is due to the sharp drop in the effective concentration of indoline as indoline leaks out of the closed αβ-dimeric unit into the solution. As the concentration of trapped indoline decreases (eq 5), the distribution of enzyme forms shifts in favor of E(A-A). The pyruvate side reaction then converts E(A-A) to E(Ain), pyruvate, and ammonium ion, allowing additional cycles of DIT turnover to occur at a rate determined by the rate of the pyruvate reaction. As this cycling occurs, the free indoline concentration in solution slowly increases, and the distribution of enzyme-bound species is again shifted to the quinonoid intermediate.

There are at least two possible routes for indoline leakage. First, the indoline escapes slowly from the GP-bound closed αβ-dimeric unit through a "pore" in the tunnel wall or through a pore at either the α- or the β-site that does not involve interconversion between open and closed subunit conformations. Alternatively, leakage occurs as a result of the switching of either site to the open conformation, allowing the indoline to dissociate quickly into solution. Because the dynamics of the interconversion between open and closed forms of the enzyme is dependent on the binding of GP, the amount of $E(Q)_{indoline}$ that accumulates is strongly influenced by the concentration of GP.

The essential role played by GP in stabilizing the closed conformation is illustrated in Figure 5A. This figure shows that the amount of $E(Q)_{indoline}$ trapped is much lower when the GP concentration is reduced from 50 to 5 mM. When the GP concentration is decreased to 0.5 mM, there is almost no difference from the case with no GP. The reason that the

[3] If one molecule of indoline is trapped within the tunnel and the tunnel is assumed to have a cylindrical shape with a height of 25 Å and a diameter of 8 Å, then the concentration of indoline within each individual tunnel would be ~1.3 M. When these trapped molecules escape, the concentration drops to a value that is no greater than the concentration of β-sites (~40 μM β-sites in Figure 5A).

$E(Q)_{indoline}$ formed in the first turnover cycle does not persist is twofold: (a) the indoline produced leaks out, and (b) the recycling of E(Ain) is limited by the much slower rate of conversion of E(A-A) to pyruvate. Because the pyruvate reaction is so slow, only a small fraction of the enzyme sites exist in the quinonoid state after the initial rise and drop, leaving most of the enzyme in the form of E(A-A). The cycling of DIT to indoline, pyruvate, and ammonium ion via conversion of E(A-A) to E(Ain) causes a slow buildup of indoline free in solution. This slow buildup causes the distribution of enzyme-bound species to slowly shift toward $E(Q)_{indoline}$.

The appearance of $E(Q)_{indoline}$ in the absence of GP is slow for the same reason. Because $\alpha\beta$-dimeric units have an open α-site under these conditions, the indoline cleaved from DIT is free to migrate through the tunnel and the α-site out into solution (Scheme 3). Figure 4 shows that when the amount of indoline free in solution is increased, the yield and the rate of $E(Q)_{indoline}$ accumulation are both increased. The mass action effect causes a shift in distribution toward $E(Q)_{indoline}$. Evidence that DIT reacts quickly with the E(Ain) in the first turnover is also seen in Figure 3, which shows that the absorbance at 412 nm due to E(Ain) decreases in the first four seconds, as DIT forms intermediates with E(Ain).

In summary, the behavior of DIT as a substrate provides new insights concerning the functioning of the connecting tunnel and the allosteric communications between the α- and β-sites. These studies show that the binding of GP to the α-site stabilizes the closed configuration of the α-subunit and acts as a "plug" at the α-site opening to the tunnel. Consequently, when GP is bound to the α-site, indoline cleaved from DIT at the β-site is trapped within the tunnel and forces the distribution of species in favor of $E(Q)_{indoline}$. When GP is absent, the α-subunit has the open conformation and indoline cleaved from DIT is able to escape into solution via the tunnel and the α-site. Therefore, the slow rate of appearance of $E(Q)_{indoline}$ is dependent upon the accumulation of free indoline to drive the distribution of enzyme-bound species to $E(Q)_{indoline}$ via the mass action effect of the free indoline concentration.

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BI0255672