Characterization of the Functional Role of a Flexible Loop in the α-Subunit of Tryptophan Synthase from *Salmonella typhimurium* by Rapid-Scanning, Stopped-Flow Spectroscopy and Site-Directed Mutagenesis

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ABSTRACT: The function of a flexible loop (loop 6) in the α-subunit from the tryptophan synthase $\alpha_2\beta_2$ bienzyme complex has been investigated utilizing rapid-scanning (RSSF) and single-wavelength (SWSF) stopped-flow spectroscopies. Loop 6 is an extended sequence of residues which connects β-strand 6 with α-helix 6 in the β/α-barrel fold of the α-subunit. Substitution of Leu for Arg179 near the base of loop 6 does not significantly affect either the association of the α- and β-subunits to form the bienzyme complex or the kinetics of the reaction of indole with L-serine (L-Ser) to form L-tryptophan (L-Trp), the process catalyzed by the wild-type β-subunit [Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S., & Miles, E. W. (1987) *J. Biol. Chem. 262*, 10678–10683]. However, the α-subunit-specific ligand glycerol phosphate (GP), which is an inhibitor of the wild-type β-reaction, is a much less effective inhibitor of the αR179L-catalyzed β-reaction. Equilibrium titration studies show that the affinity of GP for the α-site when either L-Ser or glycine is bound at the β-site has been reduced by nearly 100- and 200-fold, respectively. SWSF analysis of the reaction of IGP and L-Ser to form L-Trp catalyzed by the bienzyme complex revealed a 15-fold reduction in the binding affinity of the α-site substrate 3-indole-D-glycerol 3′-phosphate (IGP) in the reaction catalyzed by the αR179L mutant as compared to the wild-type enzyme. These studies show that loop 6 is important both for ligand binding to the α-site and for the ligand-induced conformational transition of the α-subunit from an "open" to a "closed" structure. Modeling studies, based on extensive structural homology of the α-subunit with the glycolytic enzyme triosephosphate isomerase (TIM), predict that closure of loop 6 induced by ligand binding at the α-active site would effectively sequester the bound substrate from the solvent and trap indole, produced from the cleavage of IGP, within the confines of the bienzyme complex. This conformational transition would promote the diffusion of indole to the β-active site via the interconnecting tunnel and would help ensure the close coordination of α- and β-subunit catalytic activities.

Surface loops are common structural features of globular proteins. Loops are nonregular segments of protein secondary structure that allow directional changes in the polypeptide chain and serve to link together other regular secondary structural elements. Often loops are found to contribute to the structure of an enzyme active site. Increasingly, it has been recognized that loops may also play essential functional roles both in ligand binding and in enzyme catalysis. An elegant example is the flexible loop found in triosephosphate isomerase (TIM).[1] X-ray crystallographic studies have shown that an extended loop of 11 residues moves by nearly 7 Å from an "open" position to a "closed" conformation upon substrate binding, effectively covering the active site (Banner et al., 1976; Alber et al., 1981; Lolis & Petsko, 1990). Kinetic characterization of mutations in this loop has demonstrated that loop closure serves to stabilize a labile reaction intermediate, preventing an unwanted side reaction and ensuring the effective conversion of substrate to product (Pompliano et al., 1990).

The α-subunit of the tryptophan synthase bienzyme complex from *Salmonella typhimurium* exhibits the same folding topology as that found in triosephosphate isomerase (Hyde et al., 1988; Hyde & Miles, 1990). The α-subunit is an αβ-barrel protein composed of a central core of eight parallel β-strands with eight parallel α-helices that pack around the periphery of the barrel. The α-active site is a largely

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[1] Abbreviations: PLP, pyridoxal phosphate; L-Ser, L-serine; L-Trp, L-tryptophan; IGP, 3-indole-D-glycerol 3′-phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerolphosphate; IPP, 3′-indole-3-propanol phosphate; GPDH, glyceraldehyde-3-phosphate dehydrogenase; BZ, benzimidazole; Leu, L-leucine; Arg, L-arginine; RSSF, rapid-scanning stopped-flow; SWSF, single-wavelength stopped-flow; $\alpha_2\beta_2$, wild-type tryptophan synthase from *S. typhimurium*; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; $E(Q_1)$, $E(Q_2)$, or $E(Q_3)$, quinonoidal intermediates formed in the conversion of L-Ser and indole to L-Trp; $E(A_{ex})$, aldimine intermediates formed between the substrate amino acids and the PLP cofactor; E(GD), geminal diamine intermediate formed between the PLP cofactor, the amino group of the substrate, and the ε-amino group of Lys87; αR179L, αG51L, and αD60Y, mutant $\alpha_2\beta_2$ bienzyme complexes in which single amino acid substitutions of Leu for Arg, Tyr for Asp, and Leu for Gly have been introduced at positions 49, 51, and 60, respectively, in the primary sequence of the α-subunit; TIM, triosephosphate isomerase.

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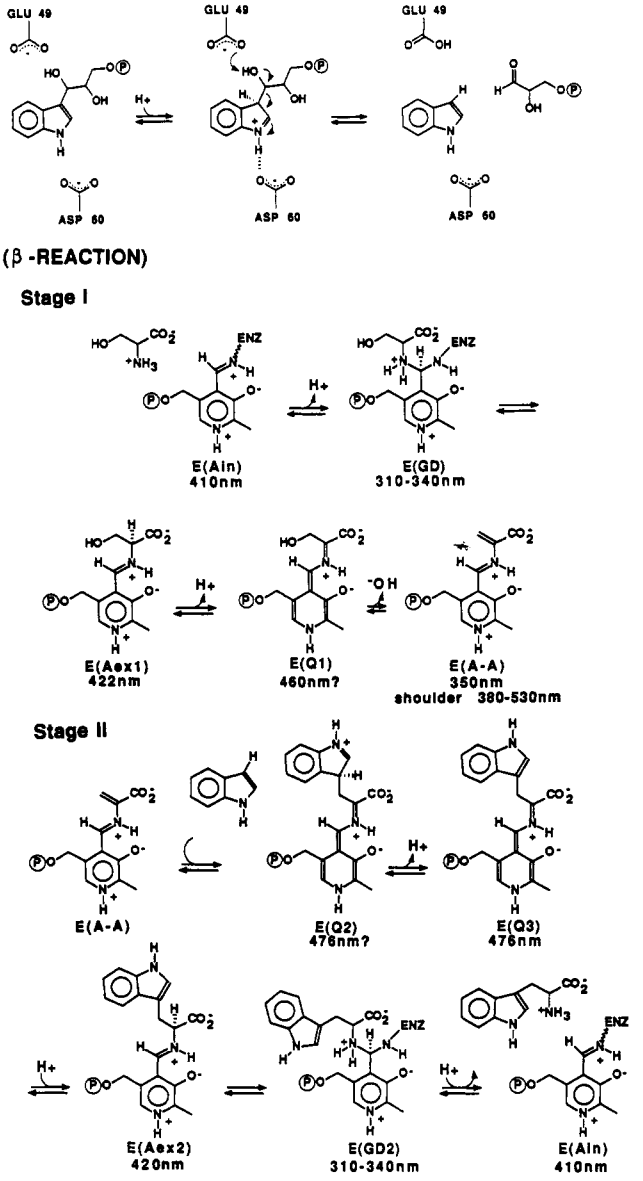
hydrophobic region located at the carboxy-terminal ends of the β-strands that compose the central barrel. Connecting strand 6 and helix 6 is an extended loop that is in an analogous position to the flexible loop found in TIM (Wilmanns et al., 1991). Weak electron density in this region suggests that loop 6 has a high degree of conformational mobility. Furthermore, position 183 of loop 6 has been identified as the site of an inactivating missense mutation (Yanofsky & Crawford, 1972; Yang & Miles, 1992). These observations suggest a functional role for loop 6 in tryptophan synthase catalysis that may involve a conformational change analogous to that observed in TIM.

The α-subunit of tryptophan synthase is part of an $\alpha_2\beta_2$ multienzyme complex that catalyzes the final two reactions of the tryptophan biosynthetic pathway. The α-subunit catalyzes the reversible aldolytic cleavage of IGP to indole and G3P (α-reaction, Scheme I), and the β-subunit catalyzes the PLP-dependent condensation of L-Ser and indole to yield L-Trp (β-reaction, Scheme I). X-ray crystallographic studies (Hyde et al., 1988) and kinetic characterization of reactions catalyzed by the bienzyme complex have demonstrated that indole produced in the α-reaction is channeled to the β-site via an interconnecting tunnel that directly links the α- and β-active centers (Dunn et al., 1990; Lane & Kirschner, 1991; Anderson et al., 1991). Furthermore, the α- and β-subunit catalytic activities are very closely coordinated via an intricate set of reciprocal allosteric interactions. Communication between the heterologous α- and β-sites is mediated both by the formation of discrete chemical intermediates at the β-site and by the binding and dissociation of substrates and products at the α-site (Brzović et al., 1992a; Kirschner et al., 1991; Anderson et al., 1991; Houben & Dunn, 1990).

The α- and β-active sites are separated by nearly 30 Å (Hyde et al., 1988). Communication over such a large distance undoubtedly involves protein conformational changes. However, the relationship between protein structure, conformational change, and catalysis is, at present, poorly understood. Therefore, it is important to understand the role of particular protein structural features, such as the α-subunit loop 6, in enzyme catalysis. Previous work has shown that proteolytic cleavage (Miles, 1991) and mutations within loop 6 (Yang & Miles, 1992) affect both ligand binding to the α-subunit and allosteric interactions between the α- and β-subunits. Secondly, binding of ligands at either the α- or β-sites alters the susceptibility of loop 6 to proteolytic cleavage, providing evidence that loop 6 is involved in conformational changes that occur upon substrate binding (Ruvinov & Miles, 1992). Herein we report rapid-kinetic studies on an α-subunit mutant where arginine 179 in loop 6 has been replaced by leucine. Whereas the conversion of threonine 183 to alanine or the deletion of residues 185–187 in loop 6 drastically reduces or eliminates the catalytic activity of the α-subunit, the αR179L mutant retains significant catalytic activity in reactions catalyzed by the α-subunit (Kawasaki et al., 1987). This permits characterization of the effect of mutations in loop 6 on the overall catalytic cycle of the αβ-reaction. Our results show that the αR179L mutation primarily affects the binding of IGP at the α-site. Most of the experimentally observed allosteric interactions between α- and β-subunits remain intact. The similarity of kinetic behavior between mutations in another extended loop segment of the α-subunit, loop 2 (Brzović et al., 1992b), and that observed for αR179L strongly suggests that both loop regions are directly involved in the transition of the α-subunit to a closed conformation. Steady-state kinetic studies with the inhibitor benzimidazole show that the integrity of loop 6 helps to ensure the close coordination of α- and β-catalytic activities and the effective channeling of indole between active sites within the bienzyme complex.

Scheme I: Outline of the Reactions Catalyzed by the Tryptophan Synthase Bienzyme Complex[a]



[a] The α-reaction is the reversible aldolytic cleavage of IGP to indole and G3P catalyzed by the α-subunit. The PLP-dependent β-reaction consists of two phases. Stage I involves the covalent reaction of L-Ser with the PLP cofactor to produce the quasi-stable E(A-A) intermediate. In stage II, indole reacts with E(A-A) to form L-Trp. Changes in covalent bonding during the course of the reaction produce distinct changes in the UV–visible properties of the cofactor. These spectral changes allow the reaction at the β-active site to be directly monitored. The symbols denoting specific reaction intermediates are referred to throughout the text.

MATERIALS AND METHODS

Materials. L-Ser, L-Trp, indoline, indole, GP, and Bicine were purchased from Sigma. Benzimidazole was purchased from Aldrich.

Enzyme Purification and Enzymatic Assays. Oligonucleotide-directed mutagenesis of *trp*A to produce the αR179L mutant has been presented elsewhere (Kawasaki et al., 1987). Purification of wild-type and α-mutant $\alpha_2\beta_2$ tryptophan

synthase, determination of protein concentrations, and measurement of enzyme activities have been previously described (Nagata et al., 1989; Miles et al., 1987; Miles et al., 1988; Brzović et al., 1992c).

Static UV–Visible and Single-Wavelength Kinetic Measurements. All static UV–visible absorption spectra and equilibrium binding experiments were performed on a Hewlett-Packard 8450A or 8452A diode array spectrophotometer. Binding of glycine to the β-site was observed by monitoring spectral changes at 410 nm (Houben et al., 1989). Binding of GP to the α-subunit when either L-Ser or glycine was bound at the β-site was monitored either by the loss of fluorescence at 506 nm or by the increase in absorbance at 464 nm for L-Ser and glycine, respectively (Brzović et al., 1992a; Houben & Dunn, 1990).

Single-wavelength transient kinetic studies were performed with a Durrum Model D-110 stopped-flow spectrophotometer (20-mm light path) interfaced for on-line computer data acquisition and analysis. All single-wavelength time courses, whether collected with the single-wavelength or the rapid-scanning stopped-flow spectrophotometer, were fit to equation 1 by a nonlinear least-squares regression analysis where A_t represents the absorbance at time t, A_∞ is the final absorbance, A_i is the absorbance change that occurs during each relaxation, and $1/\tau_1$ corresponds to the observed rate for each relaxation. All time courses were collected under pseudo-first-order conditions, and reported rate constants represent the average of at least three separate determinations.

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

Rapid-Scanning Stopped-Flow Spectrophotometry. The RSSF spectrophotometer used in these studies employed elements of the Durrum D-110 rapid-mixing stopped-flow spectrometer and a Princeton Applied Research (PAR) OMA-III multichannel analyzer with a 1463 detector–controller card and a 1214 photodiode array detector. The RSSF system has been described in detail elsewhere (Koerber et al., 1983). For a typical experiment, a 100% transmission spectrum (defined as the light transmitted through the buffer solution used) and the diode array dark current spectrum are first collected and stored. By use of these spectra, the spectra collected at programmed intervals after mixing are converted to absorbance and stored on floppy disk. The experiments reported herein used 512 pixels for a repetitive scan time of 8.544 ms and a wavelength resolution of ±0.5 nm. Collection of single-wavelength time courses from RSSF data was accomplished by single-channel analysis of 200 consecutive 100% transmission spectra collected at intervals of 8.544 ms and converted to absorbance time courses. The total acquisition time was 1.71 s. All concentrations refer to reaction conditions immediately after mixing.

All of the time-resolved RSSF data represented herein were collected utilizing one of two different data collection timing sequences. In each case, spectrum 0 represents the spectrum of the enzyme, or the enzyme–substrate complex, before mixing with the alternate substrate. Scanning was initiated after mixing was complete and flow had stopped. Spectra were recorded and stored at various intervals using the following predetermined timing sequences: Timing sequence 2 collected spectra at 8.53, 17.1, 25.6, 34.1, 42.6, 76.8, 127.9, 255.8, 426.4, and 639.6 ms after flow had stopped. Timing sequence 3 collected spectra at 8.53, 17.1, 25.6, 34.1, 42.6, 76.8, 127.9, 383.8, 852.8, and 1705.6 ms after flow had stopped. Difference spectra were calculated by subtracting either spectrum 0 or spectrum 1 from the remaining data set.

Atomic Modeling of the Flexible Loop Conformation. Since loop 6 in the α-subunit of tryptophan synthase is not visible in the crystal structures seen to date, the two conformations were modeled on the basis of structural homology to the glycolytic enzyme triosephosphate isomerase (TIM) from various species. Both TIM and the α-subunit are 8-fold β/α-barrel proteins, the flexible loop in the α-subunit is in the same part of the structure as the flexible loop in TIM (i.e., follows strand 6) and the loops are approximately the same length. The TIM structure has been solved crystallographically in both "open" and "closed" conformations in the absence and presence of substrate analogs (Alber et al., 1981; Brunner et al., 1976; Wierenga et al., 1991). The two TIM models were superimposed locally in the region of the flexible loop with the tryptophan synthase α-subunit using 11 pairs of analogous Cα positions in barrel strands 6 and 7 and in the loop following strand 7: residues 173–176 and 209–215 in the α-subunit and residues 163–166 and 209–215 in TIM. (The identical sequence numbering in the second peptide segment is fortuitous.) Superpositions of the coordinate sets were made with the program ALIGN by G. Cohen (NIH) to give an rms deviation of 0.64 Å in Cα atom positions. Figure 6 was produced on a Silicon Graphics computer workstation using the program Ribbons by M. Carson (University of Alabama). The two structures used in this study were the tryptophan synthase model with Protein Data Bank designation 1WSY (Hyde et al., 1988) and the trypanosomal TIM model (1) P6TIM (preliminary release). The "A" chain of TIM served as the "open" conformation and the "B" chain, with bound glycerol phosphate, as the "closed" conformation (Wierenga et al., 1991).

RESULTS

Because of the intimate interaction between the α- and β-subunits and the close coordination of the α- and β-activities (Brzović et al., 1992a,b), changes in the catalytic behavior caused by a mutation in one subunit are often reflected by differences in catalytic activity at the heterologous active site. Thus, transient kinetic changes in the αβ-reaction caused by replacement of Arg179 by Leu in loop 6 of the α-subunit may be observed by changes in the pre-steady-state kinetics of reactions at the β-site as compared to the wild-type-catalyzed reactions. The chromophoric properties of the PLP cofactor provide a convenient signal to observe catalytic events at the β-active site (see Scheme I) directly. Secondly, spectral differences induced by the binding of allosteric effectors which correspond to changes in the relative accumulation of reaction intermediates provide a direct measurement of differences in allosteric behavior that are due to the α-subunit mutation.

RSSF Spectra of the Reactions of L-*Ser and Indole with* $\alpha_2\beta_2$. The RSSF time-resolved spectral changes that occur during the reaction of wild-type enzyme with L-Ser have been thoroughly described elsewhere (Drewe & Dunn, 1985; Brzović et al., 1992b). Briefly, as shown in Figure 1A, there is rapid formation of a new spectral band ($1/\tau_1$), with λ_{max} = 422 nm, that accumulates within the mixing dead time of the stopped-flow instrument (typically 3–4 ms). This intermediate corresponds to the external aldimine [$E(A_{ex_1})$, Scheme I] formed between L-Ser and the PLP cofactor (Drewe & Dunn, 1985). The $E(A_{ex_1})$ species decays in a biphasic process ($1/\tau_2 = 10\ s^{-1} > 1/\tau_3 = 1\ s^{-1}$) to give the spectrum of the E(A-A) complex (Figure 1A, spectrum 8). The biphasicity of this process has been interpreted in terms of the existence of

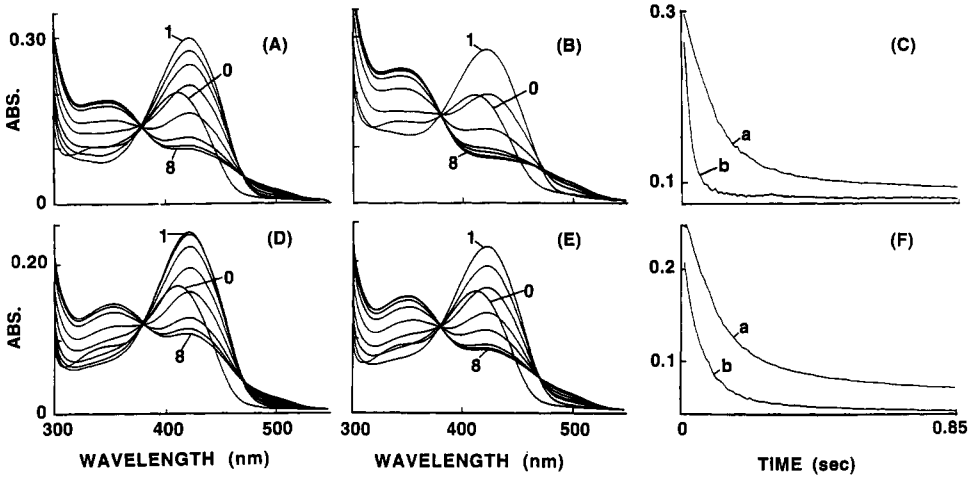


FIGURE 1: RSSF spectra and single-wavelength time courses for the reactions of 10 μM wild-type and 8.3 μM αR179L enzymes with 40 mM L-Ser at pH 7.8 in both the absence and presence of 100 mM D,L-GP. Enzyme was in one syringe and L-Ser was in the other. When present, GP was placed in both syringes. Spectrum 0 represents the spectrum of the enzyme in the absence of substrates. Reactions of L-Ser with (A) wild-type enzyme, (B) wild-type preequilibrated with 100 mM D,L-GP, (D) αR179L, and (E) αR179L preequilibrated with 100 mM D,L-GP. Data were collected utilizing timing sequence 2. (C and F) Time courses at 420 nm for the reaction of L-Ser in either the absence (trace a) or presence (trace b) of 100 mM D,L-GP for the wild-type and αR179L enzymes, respectively.

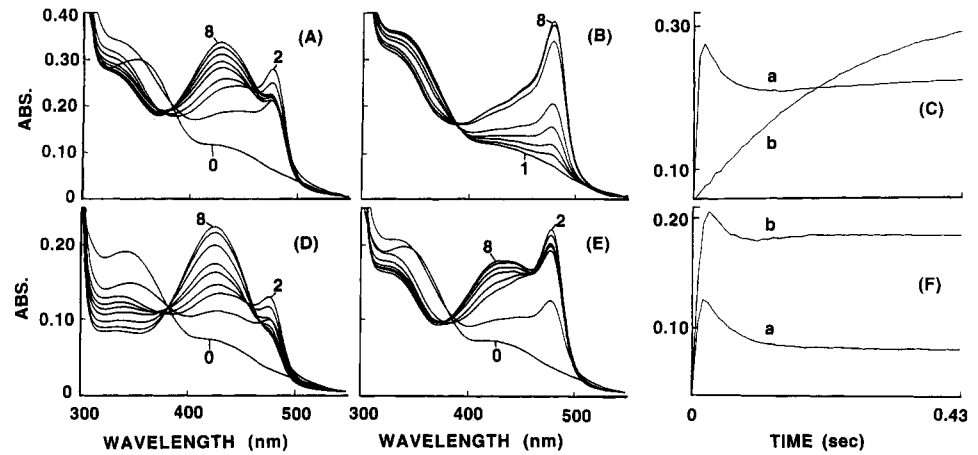


FIGURE 2: RSSF spectra for both the wild-type and $\alpha_2\beta_2$ αR179L-catalyzed β-reaction. Indole (1 mM) was mixed with 10 μM wild-type or 8.1 μM αR179L preequilibrated with 40 mM L-Ser in either the absence or presence of 100 mM D,L-GP at pH 7.8. Enzyme was in one syringe while indole was in the other. In order to avoid unwanted concentration changes in L-Ser and GP during mixing, these ligands, when present, were placed in both syringes. Spectrum 0 represents the spectrum of the enzyme–serine complex in the absence of indole. Reactions of (A) 10 mM wild-type enzyme, (B) 10 μM wild-type enzyme + 100 mM D,L-GP, (D) 8.1 μM αR179L, and (E) 8.1 μM αR179L + 100 mM D,L-GP. Data were collected utilizing timing sequence 2. (C and F) Pre-steady-state courses at 476 nm for the wild-type and αR179L-catalyzed reactions of indole with E(A-A) in either the absence (trace a) or presence (trace b) of 100 mM D,L-GP, respectively.

multiple conformations of the $\alpha_2\beta_2$ bienzyme complex (Drewe & Dunn, 1985). The E(A-A) is a quasi-stable complex composed of an equilibrium distribution of reaction intermediates. The distribution of species may be altered by varying the pH (Mozzarelli et al., 1991) or the temperature, or by adding allosteric effectors, such as GP which binds at the α-active site (Dunn et al., 1987, 1990; Houben et al., 1989; Houben & Dunn, 1990; Kirschner et al., 1991; Mozzarelli et al., 1991). The same intermediates are also observed for the reaction of $\alpha_2\beta_2$ with L-Ser in the presence of GP bound at the α-site (Figure 1B). However, the rate of $1/\tau_2$ (=80 s^{-1}) is increased 8-fold, and the final distribution of species is slightly altered.

The spectral changes and the observed rates of chemical processes for the reaction of L-Ser with the αR179L mutant are nearly indistinguishable from those observed for the wild-type enzyme (Figure 1C). Binding of GP at the α-site also increases $1/\tau_2$, although the effect is not as large. Under the experimental conditions shown, $1/\tau_2$ is increased only 2-fold to 20 s^{-1} in the presence of GP. The final E(A-A) spectrum is nearly identical to that observed for the wild-type enzyme in the presence of GP.

The RSSF spectrum for the reaction of the wild-type E(A-A) complex with indole is characterized by the rapid accumulation ($1/\tau$ = 200 s^{-1}) of a new spectral band with λ_{max} = 476 nm (Figure 2A). This spectral band corresponds to the L-Trp quinonoidal species, E(Q_3) (Miles, 1979; Lane & Kirschner, 1981, 1983b, 1991; Drewe & Dunn, 1986), that accumulates after the formation of a C–C bond between the β-carbon of E(A-A) and indole (Scheme I). Formation of E(Q_3) is followed by the accumulation of another spectral band centered at 425 nm. This species is likely the L-Trp external aldimine, E(A_{ex_2}) (Brzović et al., 1992a). As the reaction approaches the steady state, E(Q_3) is evident as a shoulder at 476 nm adjacent to the larger E(A_{ex_2}) band and is present only as a minor component in the spectrum. GP bound at the α-site significantly inhibits the rate of E(Q_3) formation ($1/\tau$ = 12 s^{-1}; Dunn et al., 1990), but does not affect the position of the spectral band. However, the total amount of quinonoid which accumulates as the reaction

approaches the steady state is much larger (Figure 2B, spectrum 8), and the $E(Q_3)$ band dominates the spectrum as the reaction enters the steady state.

The RSSF spectra of the reaction of indole with the αR179L E(A-A) complex (Figure 2D) in the absence of GP are essentially identical to those observed with the wild-type enzyme. Thus, the position of the observed spectral bands and the rates of accumulation and decay of reaction intermediates at the β-site are not affected by the mutation in the α-subunit. However, in the presence of GP, the RSSF spectra of the αR179L-catalyzed β-reaction are very different compared to those of the wild-type reaction. Although the same intermediates are observed in each case, the rates of formation and decay of the observed intermediates have changed considerably. The most significant difference is that GP reduces the rate of formation of $E(Q_3)$ from indole and E(A-A) in the αR179L-catalyzed reaction by only 2-fold ($1/\tau$ = 90 s^{-1}), while the rate of the same process is reduced nearly 17-fold for the wild-type enzyme ($1/\tau$ = 12 s^{-1}) under the same experimental conditions.

As shown in Figure 2B, GP binding to the α-subunit enhances the accumulation of quinonoidal species when nucleophiles react with E(A-A) at the β-site (Dunn et al., 1987, 1990). In the αR179L-catalyzed β-reaction, the presence of GP does lead to the increased accumulation of $E(Q_3)$ at the β-site, but not to the same extent as that observed for the wild-type enzyme. Furthermore, the formation of $E(Q_3)$ is followed by a small decrease at 476 nm and a corresponding increase of the spectral band at 425 nm, showing that the relative distribution of $E(Q_3)$ and $E(A_{ex_2})$ in the αR179L-catalyzed β-reaction has been altered relative to the wild-type reaction (compare Figure 2B,D). Because turnover in the αR179L-catalyzed β-reaction is rapid, we examined the quinonoid-forming reaction utilizing indoline as a nucleophile in place of indole. Indoline reacts rapidly to form a quinonoidal intermediate which absorbs at 466 nm, but is only very slowly converted to a new amino acid product (Roy et al., 1988). In the absence of GP, the transient spectral changes and the observed rate of quinonoid formation in the reaction of indoline and E(A-A) are essentially identical to those of the wild-type-catalyzed reaction ($1/\tau_1$ = 240 s^{-1}). As is also observed for the pre-steady-state phase of the αR179L-catalyzed β-reaction, GP-induced inhibition of the quinonoid-forming reaction from indoline is greatly diminished. The observed reduction in rate in the presence of 100 mM D,L-GP is only 5-fold for the mutant αR179L ($1/\tau_{1GP}^{\alpha R197L}$ = 50 s^{-1}), while the wild-type reaction is reduced 60-fold ($1/\tau_{1GP}^{Wt}$ = 4 s^{-1}). Nonetheless, GP binding does significantly increase the total accumulation of the indoline quinonoid in the αR179L-catalyzed reaction (data not shown).

Transient Kinetic Characterization of the αβ-Reaction. The RSSF spectral changes for the reaction of IGP with the wild-type E(A-A) complex are shown in Figure 3A. The reaction is characterized by a broad decrease in absorbance below 385 nm, a corresponding increase in absorbance between 385 and 500 nm, and an apparent isoabsorptive point at 385 nm. The difference spectra for the αβ-reaction are shown in Figure 4A. The spectral changes are dominated by the accumulation of a quinonoidal absorbance band at 476 nm that corresponds to the formation of the $E(Q_3)$ intermediate (Scheme I). Under identical experimental conditions, only very small spectral changes occur in the αβ-reaction catalyzed by the αR179L mutant (Figure 3B). Comparison of difference spectra for the β- and αβ-reactions catalyzed by the αR179L mutant indicates that the same intermediates are present in

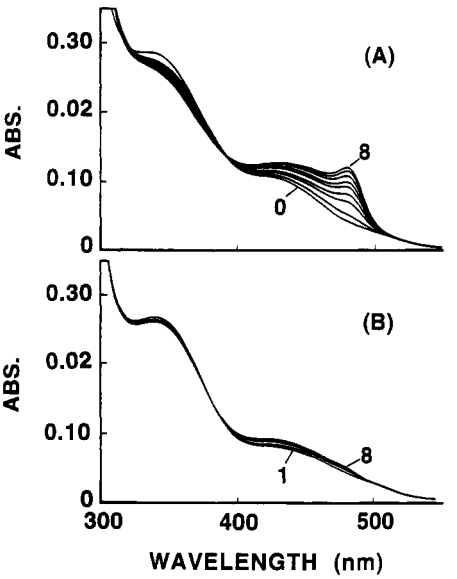


FIGURE 3: RSSF spectra of the (A) wild-type and (B) αR179L-catalyzed αβ-reaction. In each case, 0.2 mM IGP was mixed with either 10 μM wild-type or 8.1 μM αR179L $\alpha_2\beta_2$ preequilibrated with 40 mM L-Ser. Data were collected utilizing timing sequence 2.

both reactions (compare Figure 4B,C). However, comparison of the wild-type and αR179L-catalyzed αβ-reactions (Figure 4A,C) shows that the mutation significantly affects both the accumulation of intermediates and the distribution of species at the β-site. The calculated difference spectral changes of the αR179L αβ-reaction are qualitatively similar to those of the αR179L-catalyzed β-reaction in the absence of a ligand bound at the α-site (compare Figure 4B,C).

The rate dependence of $E(Q_3)$ formation on IGP concentration is shown in Figure 5. The concentration dependence data for both the wild-type enzyme and the αR179L mutant may be adequately fit to a regular rectangular hyperbola (see the Discussion). This analysis shows that the rate of $E(Q_3)$ formation saturates at a 15-fold lower concentration of IGP in the wild-type-catalyzed reaction. The half-saturation value for the αR179L-catalyzed reaction is 300 μM, as compared to the wild-type value of only 20 μM. The rate at saturating IGP concentration ($1/\tau \simeq 40$ s^{-1}) is nearly the same for both enzyme complexes.

Binding of GP to the α-Subunit. Kinetic analysis of the αβ-reaction shows that the affinity of IGP for the E(A-A) complex has been significantly decreased by the αR179L mutation. In order to verify that the αR179L mutant has lower affinity for α-site ligands, equilibrium titration experiments were performed to determine the apparent binding affinity of GP in the presence of the β-site substrates L-Ser and glycine. Because binding of GP to the α-site is monitored by observing changes in the equilibrium distribution of PLP intermediates at the β-site nearly 30 Å away, this analysis does not yield a true dissociation constant for GP at the α-site. Instead, it affords a comparative measure of binding affinity between the αR179L and wild-type enzyme complexes. The results are presented in Table I. In the absence of GP, the αR179L mutation has little effect on substrate binding at the β-site. The apparent affinity of glycine changes by less than 2-fold. Similar results have been obtained for the binding of β-ligands to a modified $\alpha_2\beta_2$ complex in which loop 6 of the α-subunit has been proteolytically cleaved (Miles, 1991). In marked contrast, the relative affinity of GP in the presence of both L-Ser and glycine has been greatly reduced by nearly 100- and 200-fold, respectively. These findings are consistent

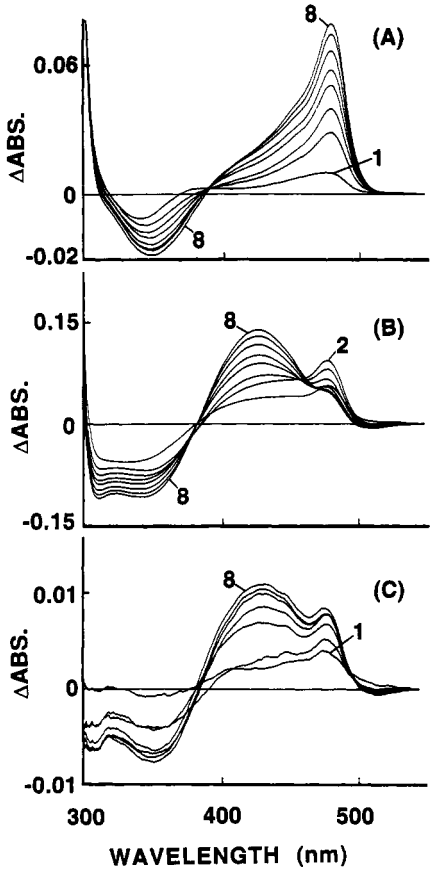


FIGURE 4: Calculated difference spectra for wild-type and αR179L-catalyzed β- and αβ-reactions. (A) Difference spectra calculated from the wild-type catalyzed αβ-reaction shown in Figure 3A. Spectrum 0 of Figure 3A was subtracted from subsequent spectra. (B) αR179L-catalyzed β-reaction Spectrum 0 of Figure 2D was subtracted from subsequent spectra. The spectral changes in this reaction are nearly identical to those of the wild-type-catalyzed β-reaction. (C) Difference spectra of the αR179L-catalyzed αβ-reaction shown in Figure 3B. Spectrum 1, which is nearly identical to the αR179L E(A-A) spectrum, was subtracted from subsequent spectra in the data set.

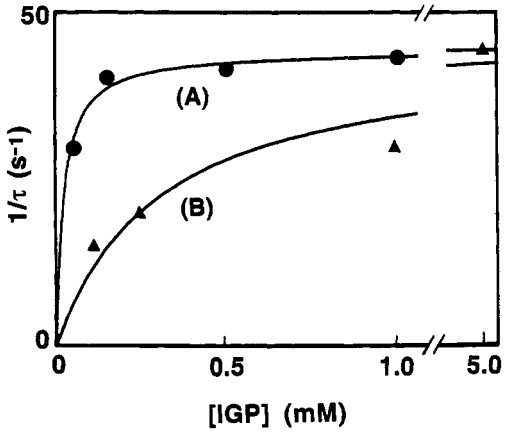


FIGURE 5: Dependence of the observed rate ($1/\tau$) of L-Trp quinonoid formation, $E(Q_3)$, on the concentration of IGP for both the (A) wild-type and (B) αR179L-catalyzed αβ-reactions. Either 7.5 μM wild-type or 25 μM αR179L, preequilibrated with 40 mM L-Ser, was mixed with increasing concentrations of IGP at pH 7.8. The rate constants were determined by analysis of single-wavelength time courses collected at 476 nm according to eq 1. The errors in reported rate constants are estimated to be ±15%.

with previous steady-state kinetic studies, which show that the K_I for IPP binding in the αβ-reaction is increased 25-fold for the mutant enzyme (Kawasaki et al., 1987).

Table I: Summary of Apparent Dissociation Constants for the Interaction of α- and β-Ligands with the Tryptophan Synthase Bienzyme Complex[a]

no.	ligand varied	ligand held constant	$K_{d(app)}$ wild-type (mM)	$K_{d(app)}$ αR179L (mM)	αR179L/wt
1	glycine	none	25.1	47.0	1.9
2	glycine	GP[b] (50 mM)	0.92	11.0	12.0
3	GP	glycine (300 mM)	0.075	16.7	220.0
4	GP	L-serine (40 mM)	0.027[c]	2.6	96.0
5	GP	L-Trp (10 mM)	0.37[c]	19.2	52.0

[a] Equilibrium data were analyzed as described elsewhere (Houben & Dunn, 1990). Measured equilibrium constants are assumed to be determined with an accuracy of ±25%. [b] It is assumed that only the D isomer of GP binds to the α-subunit. [c] Data from Brzović et al. (1992a).

Table II: Benzimidazole Inhibition of the Steady-State αβ-Reactions Catalyzed by Wild-Type and αR179L Bienzyme Complexes[a]

reaction	enzyme	rate of G3P release (s^{-1})[b]	observed rate of L-Trp production (s^{-1})[b]
α-reaction	wt	0.033	
	αR179L	0.033	
αβ-reaction	wt	1.29	1.80
	αR179L	0.50	0.53
αβ-reaction + 4 mM BZ	wt	0.09	0.108
	αR179L[c]	0.05	0.008 (0–30 s)
			0.050 (150–300 s)

[a] G3P formation, produced in the cleavage of IGP at the α-site, was measured in a coupled assay utilizing GPDH and NAD^+. L-Trp production at the β-site was directly assayed by monitoring the change in absorbance at 290 nm as IGP is converted to L-Trp. [b] Standard error ±10%. [c] An initial lag in the production of L-Trp at the β-site during the αR179L-catalyzed αβ-reaction was observed in the presence of BZ. Note the difference in the steady-state rate of L-Trp production when measured during the first 30 s (0–30 s) of the reaction compared to the final rate (measured between 150 and 300 s). No lag was observed either in the αR179L-catalyzed cleavage of IGP at the α-site or in the reactions catalyzed by the wild-type enzyme in the presence of BZ.

Inhibition of the αβ-Reaction by Benzimidazole. Under steady-state conditions, the αβ-reaction may be monitored either by following G3P release in the α-reaction utilizing a coupled enzyme assay with GPDH and NAD^+ or by directly observing the change in absorbance at 290 nm as L-Trp is synthesized at the β-site (Brzović et al., 1992a). With the wild-type enzyme, both assays give identical results for the rate of the αβ-reaction, a finding indicative of the close coordination of α- and β-subunit catalytic activities. Although k_{cat} for the αβ-reaction catalyzed by the αR179L mutant is slightly lower (4-fold: Kawasaki et al., 1987), there is no detectable difference in rates at either the α- or β-sites of this mutant. Benzimidazole (BZ), a nonreactive analog of indole, has previously been shown to be a competitive inhibitor of the β-reaction (Heilmann, 1978; Dunn, et al., 1990). We have found that BZ is also an inhibitor of the αβ-reaction. As shown in Table II, BZ binding to the wild-type $\alpha_2\beta_2$ complex inhibits both the α- and β-reactions to the same extent. This indicates that, even in the presence of BZ, the wild-type α- and β-subunit enzymatic activities are tightly coupled.

BZ binding to the αR179L,E(A-A) complex also inhibits the α-reaction, but not to the same degree as that observed for the wild-type enzyme. Observation of the β-reaction shows a pronounced lag phase in the production of L-Trp at the β-site. No lag is observed for the α-reaction as monitored by G3P release. This finding indicates that BZ binding effectively uncouples the α- and β-reactions of the αR179L bienzyme complex, leading to the accumulation of indole in the reaction

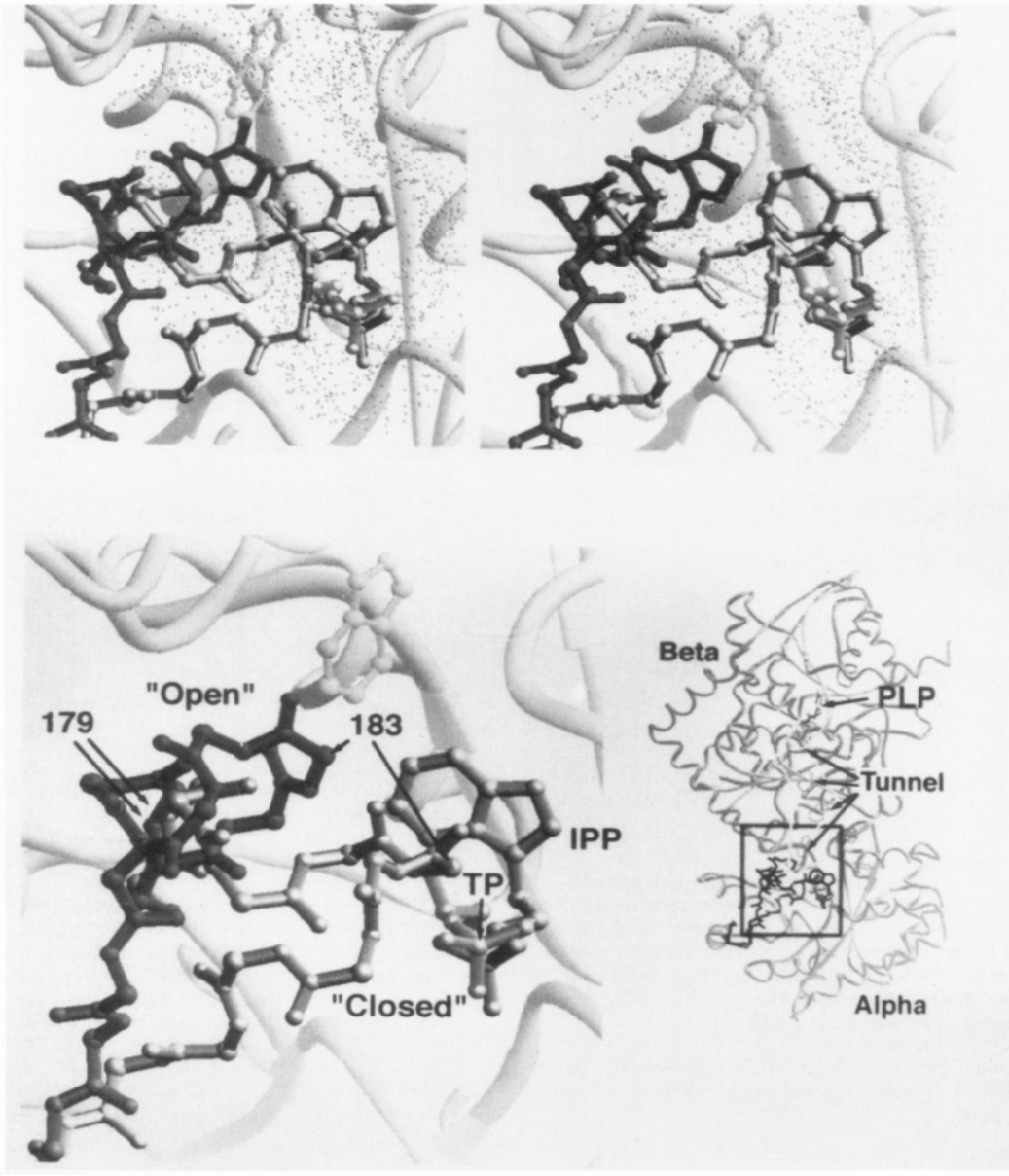


FIGURE 6: Model for the "open" and "closed" conformations of flexible loop 6 of the α-subunit based on structural homology to triosephosphate isomerase. Only the backbone atoms of the loops from TIM are shown. The inset at the lower right shows an $\alpha\beta$-subunit pair from tryptophan synthase: "PLP" marks the location of the coenzyme in the β-subunit active site. The α-subunit active-site region is boxed and shown enlarged to the left and also shown above in stereo. The tunnel and substrate binding pocket are outlined by a dot surface in the stereo figure. The substrate binding site is shown occupied by indolepropanol phosphate (IPP). Also shown is the triosephosphate (TP) analog glycerol phosphate as it binds in the active site of TIM. The propyl phosphate moieties of IPP and TP are nearly superimposable. The position of the mutation R179L is near the C-terminal end of strand 6, adjacent to the base of the flexible loop and near the $\alpha\beta$-subunit interface. Note that, in the "closed" form of the TIM loop, the residue which comes closest to the bound triosephosphate corresponds to position 183 in the α-subunit sequence, the locus of a known missense mutation. The active site is viewed along the path that the substrate must follow to enter the α-active site from the solvent—the indole ring of the substrate is more deeply buried than is the triosephosphate portion. Note that the substrate would likely be completely buried by the flexible loop when locked in the "closed" conformation.

mixture. This situation apparently persists until the concentration of indole is sufficient to displace BZ from the β-active site and the rates of the α- and β-reactions equalize (Table II).

DISCUSSION

The $\alpha\beta$-barrel topology is one of the most frequently encountered folding patterns (Brändén, 1991). It is shared by at least 18 different enzymes, including three successive enzymes in the tryptophan biosynthetic pathway, PRA isomerase, IGP synthase, and the α-subunit of tryptophan synthase (Hyde et al., 1988; Wilmanns et al., 1991). Despite the common folding motif, there is no substantial sequence homology within this class of proteins, making it difficult to discern evolutionary relationships. However, several $\alpha\beta$-barrel proteins share common structural features (Wilmanns et al., 1991). Careful comparison of tertiary folding patterns led to the recognition that the phosphate binding region in several $\alpha\beta$-barrel enzymes is highly conserved. Secondly, an extended loop connecting β-strand 6 and α-helix 6 has been identified in the X-ray structures of TIM, RUBISCO, PRA isomerase, and the α-subunit of tryptophan synthase (Figure 6). In each case, the loop is poorly defined in electron density maps indicating a high degree of conformational flexibility. Mobility in this region of the protein likely has important functional consequences. As discussed in the introduction, the confor-

mational change in the flexible loop from an open to a closed position is critically important for the reaction catalyzed by TIM (Pompliano, 1990).

Previous work with $\alpha_2\beta_2$ complexes that have specific mutations in and around loop 2 of the α-subunit has shown that a conformational transition in the α-subunit from an open to a closed structure is induced by ligand binding to the α-active site (Brzović et al., 1992b). Modeling of the TIM flexible loop onto the analogous position of the α-subunit of tryptophan synthase (Figure 6) suggests that closure of loop 6 may interact both with the bound substrate and with residues in loop 2. Closure of loop 6 would also effectively cover the α-active site and block access from the solvent into the active site and the connecting tunnel between the α- and β-sites [Figure 6; see also Brzović et al. (1992b)]. As described herein, replacement of Arg179 by Leu in loop 6 of the α-subunit primarily decreases the binding affinity of the α-subunit for the substrate IGP. Furthermore, the similarity of the observed transient kinetic behavior in the presence of GP between the αR179L mutant and certain loop 2 mutants is consistent with the hypothesis that both the loop 2 and loop 6 segments of the α-subunit participate in the same ligand-induced conformational change from an open to a closed structure.

Subunit Association and Allosteric Interactions. The activities of both the α- and β-subunits in the bienzyme complex are critically dependent upon subunit interactions. Association to form the bienzyme complex enhances the catalytic activities of both the α- and β-subunits by nearly 100-fold. On the basis of the inferred mobility of loop 6 from X-ray structures and the expected position of Arg179 in the α-subunit, it is not likely that residue 179 should influence the assembly of the bienzyme complex. Kawasaki et al. (1987) have demonstrated that the stability of the αR179L bienzyme complex is similar to that of the wild-type enzyme. Furthermore, both steady-state (Kawasaki et al., 1987) and pre-steady-state kinetic studies (this work) show that the reactivity of the β-subunit, in the absence of α-subunit ligands, is not affected by the αR179L mutation. This is reflected in the RSSF spectral changes during the first and the second stages of the β-reaction (Figures 1 and 2).

Most of the observed allosteric interactions between the α- and β-subunits of the wild-type enzyme are also observed in the αR179L bienzyme complex. The binding of L-Ser and subsequent formation of E(A-A) at the β-site significantly stimulate the turnover of IGP at the α-site (Kawasaki et al., 1987). The rate of E(A-A) formation is enhanced by the presence of α-subunit-specific ligands such as GP (Figure 1C) and IGP (data not shown). GP bound at the α-site increases the amount of quinonoidal species which accumulates in reactions of nucleophiles, such as indole (Figure 2B) and indoline (Figure 3), with E(A-A). GP also increases the apparent affinity of ligands which bind specifically at the β-site (Table I). Therefore, most allosteric interactions between the α- and β-subunits are intact in the αR179L mutant.

The notable exception to these observations is that the quinonoidal species $E(Q_3)$, derived from the binding of L-Trp and the β-site in the reverse reaction (eq 2, Scheme I), does not accumulate to the same extent as that observed for the wild-type enzyme, even in the presence of GP (Kawasaki et al., 1987). Similar behavior was also found in the α-subunit loop 2 mutants, αG51L and αD60Y (Brzović et al., 1992b). These findings suggest that the accumulation of $E(Q_3)$ from L-Trp at the β-site is dependent upon a conformational change that involves both loop 6 and loop 2 of the α-subunit. This conformational change is not necessarily identical to that which produces other GP-induced allosteric effects.

$$E + \text{L-Trp} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(\text{L-Trp}) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E(GD_2) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E(A_{ex_2}) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E(Q_3) \quad (2)$$

Mutation of Arg179 to Leu Changes the Affinity of IGP for the E(A-A) Complex. Previous work with the αR179L bienzyme complex has demonstrated that this mutant retains appreciable activity in the αβ-reaction. Steady-state studies (Kawasaki et al., 1987) have shown that replacement of Arg179 by Leu decreases both the k_{cat} of the αβ-reaction and the K_m for IGP by only 4- and 5-fold, respectively. RSSF spectra of the αβ-reaction (Figures 3 and 4) show large differences in the accumulation of reaction intermediates between the wild-type and αR179L enzymes, although difference spectra (Figure 4) indicate that the same intermediates are present in both cases. Since the αR179L mutation has little effect either on the assembly of the bienzyme complex or on the intrinsic activity of the β-subunit in the absence of α-site ligands, it appears that the primary effect of the mutation is on the reactivity of the α-subunit.

It is possible to observe changes in the reactivity of the α-subunit by following the accumulation of $E(Q_3)$ (λ_{max} = 476 nm) at the β-site during the reaction of IGP and L-Ser with the bienzyme complex. Equation 3 describes the mechanism for the formation of $E(Q_3)$ when IGP is mixed with the $\alpha_2\beta_2$ E(A-A) complex. When the bienzyme complex

$$E(A\text{-}A) + IGP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(A\text{-}A)(IGP) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E(A\text{-}A)(\text{indole,G3P}) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E(Q_3)(G3P) \quad (3)$$

has been preequilibrated with L-Ser, the transient accumulation of $E(Q_3)$ is limited by the rate of cleavage of IGP at the α-site (Lane & Kirschner, 1991; Anderson et al., 1991). The diffusion of indole between the α- and β-sites and the subsequent bond-forming reaction to form $E(Q_3)$ are much faster processes (Dunn et al., 1990; Brzović et al., 1992a). Secondly, synthesis of a covalent C–C bond between indole and E(A-A) is an essentially irreversible reaction (Ahmed et al., 1986). Under these conditions, eq 3 may be simplified to eq 4:

$$E(A\text{-}A) + IGP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(A\text{-}A)(IGP) \overset{k_2}{\rightarrow} E(Q_3)(G3P) \quad (4)$$

The rate dependence for $E(Q_3)$ formation on IGP concentration is then given by

$$k_{(obs)} = k_2[IGP]/(K_d + [IGP]) \quad (5)$$

where k_2 represents the rate of IGP cleavage and K_d is the dissociation constant for IGP (Bernasconi, 1976; Lane & Kirschner, 1991).

Because the overall reaction is irreversible, the hyperbolic curve described by eq 5 should pass through the origin. This expression adequately describes the data shown in Figure 5. Analysis of the data for the wild-type-catalyzed reaction yields K_d(IGP) = 20 μM, a value in very good agreement with those previously reported for both the *Escherichia coli* (Lane & Kirschner, 1983a, 1991) and *S. typhimurium* (Anderson et al., 1991) enyzmes. Application of the same analysis to the αβ-reaction catalyzed by the mutant shows that the K_d of

IGP for the αR179L, E(A-A) complex has increased by 15-fold (K_d(IGP) = 300 μM), consistent with the findings that other α-subunit ligands such as GP and IPP bind with greatly reduced affinity at the α-active site of the αR179L mutant-(Table I; Kawasaki et al., 1987). Figure 5 also shows that the observed rate of E(Q_3) formation saturates at a similar value (~40 s^{-1}) for both enzymes. Thus, the αR179L mutation predominantly affects the binding affinity of IGP for the α-site and not the catalytic cleavage step of the α-reaction.

The modeling studies depicted in Figure 6 show that position R179 is at the base of the flexible loop region and that the Arg side chain probably does not directly interact with the bound substrate. Instead, it is postulated that movement of loop 6 upon substrate binding would place threonine 183 in a position to make direct contact with the glycerophosphate portion of the substrate. It is not immediately clear from the modeling studies why the mutation at position 179 would alter the function of loop 6. Position 179 is relatively buried and is near the interface between the α- and β-subunits. Therefore, R179 probably does not undergo the large conformational changes predicted for other residues in the loop. At least two possibilities can be imagined to explain the experimental results: (1) Analysis of loop closure in TIM suggests that the loop moves as a rigid "lid" attached to the protein at two "hinge" regions (Joseph et al., 1990). R179, positioned at the base of loop 6 in the α-subunit, may actually be part of, or adjacent to, one of the "hinges" necessary for loop closure. Thus, substitution of Arg179 by Leu may interfere with the motion of the flexible loop. (2) Alternatively, the αR179L mutation may affect the overall structure of loop 6, preventing maximum interaction between residues in the closed loop with the bound substrate and/or residues from other regions of the α-subunit.

Functional Role of Loop 6 in Allostery and Catalysis. The analysis of loop 6 mutants, presented here and elsewhere (Yang & Miles, 1992), shows that this region is an important functional element for catalysis at the α-active site. The similarity between loop 6 and the flexible loop in TIM (Wilmanns et al., 1991) provides the basis for modeling studies (Figure 6; Brzović et al., 1992b) to develop a physical mechanism which helps explain our experimental results. Consequently, we postulate that the binding of α-subunit-specific ligands and allosteric interactions between the α- and β-subunits which involve covalent intermediates at the β-site (Brzović et al., 1992a) would induce a conformational change in loop 6 from an open to a closed position. In the open conformation, the α-active site is accessible to the solvent and permits binding of IGP to the α-subunit. Closure of loop 6, induced by substrate binding, effectively covers the α-site and sequesters the bound substrate from solvent. Mutations that either affect direct interactions between residues in loop 6 and substrate functional groups or residues in loop 2 or impair the ability of loop 6 to undergo the conformational change destabilize the closed conformation of loop 6. This results in decreased ligand affinities at the α-site.[2]

This model has other implications for the αβ-reaction catalyzed by tryptophan synthase. Previous rapid kinetic characterization of the β-reaction has shown that the α-site and the interconnecting tunnel are the preferred route by which indole gains access from solution into the β-active site (Dunn et al., 1990, 1991). Ligands which bind specifically to the α-active site inhibit the reaction of large nucleophiles with the E(A-A) intermediate at the β-site, and this inhibition is dependent, in part, on a ligand-induced conformational change (Brzović et al., 1992b). According to the proposed model, if the stability of the closed conformation is decreased by certain mutations, then the ability of α-subunit ligands to inhibit access of nucleophiles into the β-site via the α-site and the tunnel would also be diminished. One of the most significant effects of the αR179L mutation is the loss of GP-induced inhibition of the β-reaction. GP binding to the αR179L α-active site actually stimulates the steady-state rate of the β-reaction (Kawasaki et al., 1987). The inhibitory effect of GP on the pre-steady-state rate of E(Q_3) formation from indole and E(A-A) at the β-site is greatly diminished in the αR179L mutant. Whereas the wild-type reaction is decreased nearly 15-fold, the αR179L reaction is reduced by only 2-fold. These kinetic results are consistent with conclusions based upon our modeling studies (Figure 6). Closure of loop 6 upon substrate or ligand binding would effectively block the access of small molecules into the α-active site and the interconnecting tunnel from the solvent. Conversely, indole, formed during the course of the α-reaction, would be trapped within the confines of the bienzyme complex, increasing the probability of diffusion through the tunnel to the β-active site.

Secondly, it has been demonstrated for the αβ-reaction that G3P release from the α-site occurs after the formation of E(Q_3) at the β-site (Lane & Kirschner, 1991). Brzović et al. (1992a) have shown that G3P binds much more tightly to the E(A-A) complex than to the E(Q_3) complex and that the formation of E(Q_3) acts as a chemical trigger inducing a conformational change which deactivates the α-site and facilitates the release of G3P. One of the effects of the αR179L mutation in loop 6 is to reduce the affinity of α-site ligands for the E(A-A) complex (Table I; Kawasaki et al., 1987).

[2] The data shown in Figure 5 are consistent with either a one-step, concerted mechanism for the binding of IGP to the α-subunit (eq 6) or a two-step process in which the binding of IGP induces a subsequent conformational change in the α-subunit (eq 7):

$$E + IGP \overset{K}{\rightleftharpoons} E^*(IGP) \overset{k_{cat}}{\rightarrow} \text{products} \quad (6)$$

$$E + IGP \overset{K_1}{\rightleftharpoons} E(IGP) \overset{K_2}{\rightleftharpoons} E^*(IGP) \overset{k_{cat}}{\rightarrow} \text{products} \quad (7)$$

In both equations, E represents the open conformation of the α-subunit while E* represents the closed conformation. Movement of loop 6 to cover the α-active site is one component of the overall α-subunit conformational transition which is important both for catalysis and the transmission of allosteric information to the β-subunit. If the two-step mechanism for binding is correct, then the measured K_d(IGP) would be a function of both K_1 and K_2:

$$K_d = K_1/(1 + 1/K_2) \quad (8)$$

where K_1 = [E][IGP]/[E(IGP)] and K_2 = [E(IGP)]/[E*(IGP)]. If E* is favored when IGP is bound, then $K_2 \ll 1$ and $K_d \approx K_1K_2$. Mutational changes, such as those in loop 6 or loop 2 (Brzović et al., 1992b), or even changes in the structure of the ligand which binds to the α-site may affect the equilibrium (K_2) between open and closed forms of the α-subunit. Such a model may be useful for interpreting differences between α-subunit-specific ligands, such as those observed between GP and IPP (Brzović et al., 1992b), in both the transmission of allosteric information and the inhibition of stage II of the β-reaction (see text).

[3] The binding of L-Trp to the β-site is accompanied by extensive conformational changes in the N-terminal domain of the β-subunit (C. C. Hyde, unpublished results). Although residues in loop 6 have not yet been visible in crystal structures of the bienzyme complex, opening and closing of this loop is coupled, either directly or indirectly to long-range conformational changes involving the N-domain of the β-subunit. The proximity of residues surrounding position 179 in the α-subunit to the N-domain of the β-subunit raises the possibility for direct contact between these residues and the N-domain of the β-subunit, providing a possible network of interactions for the transmission of allosteric information between α- and β-subunits in the bienzyme complex.

Destabilization of the closed conformation of the α-subunit would allow for premature dissociation of G3P from the α-site. (This is consistent with the spectral differences shown in Figure 4). Prevention of the covalent reaction between indole and E(A-A) at the β-site by the presence of bound BZ together with an open conformation imposed by the αR179L mutation on the α-subunit would provide one means by which indole could escape from the bienzyme complex into solution. Thus, we conclude that destabilization of the closed conformation of the α-subunit by the αR179L mutation in loop 6 leads to the uncoupling of reactions at the α- and β-sites in the presence of an inhibitor at the β-site.

Inspection of Figure 6 suggests that closure of loop 6 would place residues from this polypeptide segment in contact both with the glycerol phosphate portion of the bound substrate and with amino acid residues in loop 2 [see also Brzović et al. (1992b)]. Specific mutations in both loop segments result in enzyme complexes that display very similar allosteric and kinetic properties, behavior which is consistent with this hypothesis. GP binding to the α-site of both the loop 6 mutant (αR179L) and the loop 2 mutants (αG51L and αD60Y) does not lead to a large enhancement of $E(Q_3)$ at the β-site when L-Trp is the reactant (eq 2).[3] Secondly, the loop 2 and loop 6 mutations each greatly diminish the inhibitory properties of GP and lead to GP-induced stimulation of the steady-state turnover in the αβ-reaction. Because GP-induced inhibition is dependent upon an isomerization in the α-subunit from an open to a closed structure, the observed similarity of loop 6 and loop 2 mutants suggests that both loop segments are components of the same conformational change. Whereas loop 6 is highly flexible, loop 2 is a compound loop of 26 residues containing an extra helical segment and is the most highly conserved region of the α-subunit (Hyde et al., 1988). The catalytic residue Asp60 (Nagata et al., 1989) is part of this polypeptide segment. Although residues 55–58 in this region appear disordered, loop 2 has extensive contacts with the β-subunit (Hyde et al., 1988). Therefore, the possibility of a direct interaction between residues in loop 6 and loop 2 provides one mechanism for the communication of allosteric information from the α-site to the β-subunit.

REFERENCES

Ahmed, S. A., Martin, B., & Miles, E. W. (1986) *Biochemistry 25*, 4233–4240.

Alber, T. C., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Rivers, P. S., & Wilson, I. A. (1981) *Philos. Trans. R. Soc. B293*, 159–171.

Anderson, K. S., Miles, E. W., & Johnson, K. A. (1991) *J. Biol. Chem. 266*, 8020–8033.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., & Wilson, I. A. (1976) *Nature 255*, 609–614.

Bernasconi, C (1976) *Relaxation Kinetics*, Academic Press, New York.

Brändén, C. (1991) *Curr. Opin. Struct. Biol. 1*, 978–983.

Brzović, P. S., Ngo, K., & Dunn, M. F. (1992a) *Biochemistry 31*, 3831–3839.

Brzović, P. S., Sawa, Y., Hyde, C. C., Miles, E. W., & Dunn, M. F. (1992b) *J. Biol. Chem. 267*, 13028–13038.

Brzović, P. S., Kayastha, A. M., Miles, E. W., & Dunn, M. F. (1992c) *Biochemistry 31*, 1180–1190.

Drewe, W. F., Jr., & Dunn, M. F. (1985) *Biochemistry 24*, 3977–3987.

Drewe, W. F., Jr., & Dunn, M. F. (1986) *Biochemistry 25*, 2494–2501.

Dunn, M. F., Aguilar, V., Drewe, W. F., Jr., Houben, K., Robustell, B., & Roy, M. (1987a) *Indian. J. Biochem. Biophys. 24*, 44–51.

Dunn, M. F., Roy, M., Robustell, B., & Aguilar, V. (1987b) in *Proceedings of the 1987 International Congress on Chemical and Biological Aspects of Vitamin B6 Catalysis* (Korpela, T., & Christen, P., Eds.) pp 171–181, Birkhaeuser Verlag, Basel, Switzerland.

Dunn, M. F., Aguilar, V., Brzović, P. S., Drewe, W. F., Jr., Houben, D. F., Leja, C. A., & Roy, M. (1990) *Biochemistry 29*, 8598–8607.

Dunn, M. F., Brzović, P. S., Leja, C., Houben, K., Roy, M., Aguilar, A., & Drewe, W. F., Jr. (1991) *Proceedings of the 8th International Congress on Vitamin B6 and Carbonyl Catalysis* (Wada, H., Soda, K., Fukui, T., & Kagamiyama, H., Eds.) pp 257–267, Pergamon Press, New York.

Heilmann, H. (1978) *Biochim. Biophys. Acta 522*, 614–624.

Houben, K. F., & Dunn, M. F. (1990) *Biochemistry 29*, 2421–2429.

Houben, K. F., Kadima, W., Roy, M., & Dunn, M. F. (1989) *Biochemistry 28*, 4140–4147.

Hyde, C. C., & Miles, E. W. (1990) *Biochemistry 8*, 27–32.

Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857–17871.

Joseph, D., Petsko, G. A., & Karplus, M. (1990) *Science 249*, 1425–1428.

Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S., & Miles, E. W. (1987) *J. Biol. Chem. 262*, 10678–10683.

Kirschner, K., Lane, A. N., & Strasser, A. W. M. (1991) *Biochemistry 30*, 472–478.

Koerber, S. C., MacGibbon, A. K. H., Dietrich, H., Zeppezauer, M., & Dunn, M. F. (1983) *Biochemistry 22*, 3424–3431.

Lane, A. N., & Kirschner, K. (1981) *Eur. J. Biochem.* **120**, 379–387.

Lane, A. N., & Kirschner, (1983a) *Eur. J. Biochem. 129* 561–570.

Lane, A. N., & Kirschner, K. (1983b) *Eur. J. Biochem. 129*, 571–582.

Lane, A. N., & Kirschner, K. (1991) *Biochemistry 30*, 479–484.

Lolis, E., & Petsko, G. A. (1990) *Biochemistry 29*, 6619–6625.

Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127–186.

Miles, E. W. (1991) *J. Biol. Chem. 266*, 10715–10718.

Miles, E. W., Bauerle, R., & Ahmed, S. A. (1987) *Methods Enzymol. 142*, 398–414.

Miles, E. W., McPhie, P., & Yutani, K. (1988), *J. Biol. Chem. 263*, 8611–8614.

Mozzarelli, A., Peracchi, A., Bettati, S., & Rossi, G. L. (1991) *Proceedings of the 8th International Congress on Vitamin B6 and Carbonyl Catalysis* (Wada, H., Soda, K., Fukui, T., & Kagamiyama, H., Eds.) pp 273–275, Pergamon Press, New York.

Nagata, S., Hyde, C. C., & Miles, E. W. (1989) *J. Biol. Chem. 264*, 6288–6296.

Pompliano, D. L., Peyman, A., & Knowles, J. R. (1990) *Biochemistry 29*, 3186–3194.

Roy, M., Keblawi, S., & Dunn, M. F. (1988) *Biochemistry 27*, 6698–6704.

Ruvinov, S. B., & Miles, E. W. (1992) *FEBS 299*, 197–200.

Wierenga, R. K., Noble, M. E. M., Vriend, G., Nauche, S., & Hol, W. G. J. (1991) *J. Mol. Biol. 220*, 995.

Wilmanns, M., Hyde, C. C., Davies, D. R., Kirschner, K., & Jansonius, J. N. (1991) *Biochemistry 30*, 9161–9169.

Yang, X., & Miles, E. W. (1992) *J. Biol. Chem. 267*, 7520–7528.

Yanofsky, C., & Crawford, I. P. (1972) in *The Enzymes*, 3rd ed. (Boyer, P. D., Ed.), pp 1–31, Academic Press, New York.