Evidence of a Low-Barrier Hydrogen Bond in the Tryptophan Synthase Catalytic Mechanism†

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ABSTRACT: In the absence of other substrates, L-Ser reacts rapidly with the tryptophan synthase $\alpha_2\beta_2$ bienzyme from *Salmonella typhimurium* at pH 7.8 and 25 °C to give an equilibrating mixture of species dominated by comparable amounts of the L-Ser external aldimine Schiff base, E(Aex_1), and the α-aminoacrylate Schiff base, E(A-A). The D-isomer of Ser is unreactive toward $\alpha_2\beta_2$, and therefore, D,L-Ser can be used in place of L-Ser for investigations of catalytic mechanism. Due to the equilibrium isotope effect, when α-^{2}H-D,L-Ser is substituted for α-^{1}H-D,L-Ser, the position of equilibrium is shifted in favor of E(Aex_1). On a much slower time scale, the ^{2}H sample undergoes the exchange of enzyme bound ^{2}H for the ^{1}H of solvent water and is converted to a distribution of E(Aex_1) and E(A-A) identical to that obtained with the ^{1}H sample. This slow exchange indicates that the proton abstracted from the α-carbon of E(Aex_1) is sequestered within a solvent-excluded site in E(A-A). Analysis of the UV/vis spectra gave an isotope effect on the equilibrium distribution of E(Aex_1) and E(A-A) of $K^H/K^D = 1.80 \pm 0.18$. This large equilibrium isotope effect is the consequence of an unusual isotope fractionation factor of 0.62 for the residue which functions as the base to deprotonate and protonate the α-carbon proton in E(Aex_1). A fractionation factor of 0.62 qualifies as evidence for the involvement of a low-barrier H-bond (LBHB) in this equilibration. Since this effect arises from abstraction of the α-proton from E(Aex_1), the LBHB must be associated with the E(A-A) species. In contrast to weak H-bonds with energies of 3−12 kcal/mol, LBHBs are proposed to exhibit energies in the 12−24 kcal/mol range [Frey, P. A., Whitt, S. A., & Tobin, J. B. (1994) *Science 264*, 1927−1930]. Possible roles for this LBHB both in the chemical mechanism and in the stabilization of the closed conformation of E(A-A) are discussed.

The $\alpha_2\beta_2$ tryptophan synthase[1] from *Salmonella typhimurium* is a bienzyme complex which catalyzes the final two steps (eqs 1 and 2) in the biosynthesis of L-Trp (Yanofsky & Crawford, 1972; Miles, 1979, 1991a).

$$\text{3-indole-D-glycerol 3′-phosphate} \overset{\alpha}{\rightleftharpoons} \text{indole} + \text{D-glyceraldehyde 3-phosphate} \quad (1)$$

$$\text{L-Ser} + \text{indole} \xrightarrow{\beta_2} \text{L-Trp} + H_2O \quad (2)$$

Catalysis at the α- *and* β*-Catalytic Sites*. The α-subunit catalyzes the reversible aldolytic cleavage of IGP to G3P (designated the α-reaction, eq 1; Scheme 1). The indole thus produced then is transferred via an interconnecting tunnel (Hyde et al., 1988; Dunn et al., 1990; Lane & Kirschner, 1991; Anderson et al., 1991) to the β-subunit 25 Å away (Scheme 2) where the PLP-requiring β-site carries out a β-replacement reaction between indole and L-Ser to give L-Trp (designated the β-reaction, eq 2; Scheme 1). The overall process (the sum of eqs 1 and 2) catalyzed by αβ-subunit pairs of the bienzyme complex is designated as the αβ-reaction. The chemical species presented in Scheme 1 have been shown to be intermediates in the PLP-requiring β-reaction [see Drewe and Dunn (1985, 1986) and Miles (1991a) and references cited therein]. The β-reaction occurs in two stages; in stage I, L-Ser reacts with the PLP cofactor to form a Schiff base (external aldimine) designated as E(Aex_1). Abstraction of the α-proton of E(Aex_1) gives an unstable quinonoid, E(Q_1), which rapidly eliminates hydroxide ion to give a quasistable, electrophilic α-aminoacrylate Schiff base species, E(A-A) (Drewe & Dunn, 1986). In stage II, indole enters the β-site via the tunnel (Dunn et al., 1987b, 1990) and C−C bond formation occurs via nucleophilic attack of the C-3 carbon of indole on the electrophilic C-β of E(A-A) to give a quinonoid species, E(Q_2). Deprotonation of this quinonoid gives E(Q_3), and reprotonation of C-α gives the L-Trp external aldimine, E(Aex_2), which then is converted to L-Trp via a transimination involving the geminal diamine species, E(GD_2).

Although chemical modification studies (Miles & Kumagai, 1974; Miles, 1979, 1991a), site-directed mutagenesis work (Kawasaki et al., 1987; Nagata et al., 1989; Miles et al., 1989; Miles, 1979, 1986, 1991a,b; Brzovic′ et al., 1992a,

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[1] Abbreviations: $\alpha_2\beta_2$, the tryptophan synthase bienzyme complex; β_2, the β-subunit dimer species; IGP, 3-indole-D-glycerol 3′-phosphate; IPP, 3-indolepropanol 3′-phosphate; G3P, D-glyceraldehyde 3-phosphate; GP, α-glycerolphosphate, L-Ser and D,L-Ser, L-serine and D,L-serine, respectively; L-Trp, L-tryptophan; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5-phosphate; LBHBs, low-barrier hydrogen bonds; KIE, kinetic isotope effect; SWSF, single-wavelength stopped-flow; RSSF, rapid-scanning stopped-flow. The various covalent forms of PLP-bound serine at the β-site (Scheme 1) are designated as follows: E(Ain), the internal aldimine (Schiff base); E(GD_1), the first gem diamine species; E(Aex_1), the L-Ser external aldimine (Schiff base); E(Q_1), the L-Ser quinonoid; E(A-A), the α-aminoacrylate Schiff base; E(Q_2), the quinonoid formed initially in the Michael addition of indole with E(A-A); E(Q_3) the quinonoid formed by abstraction of a proton form E(Q_2); E(Aex_2), the L-Trp external aldimine (Schiff base); E(GD_2), the L-Trp gem diamine.

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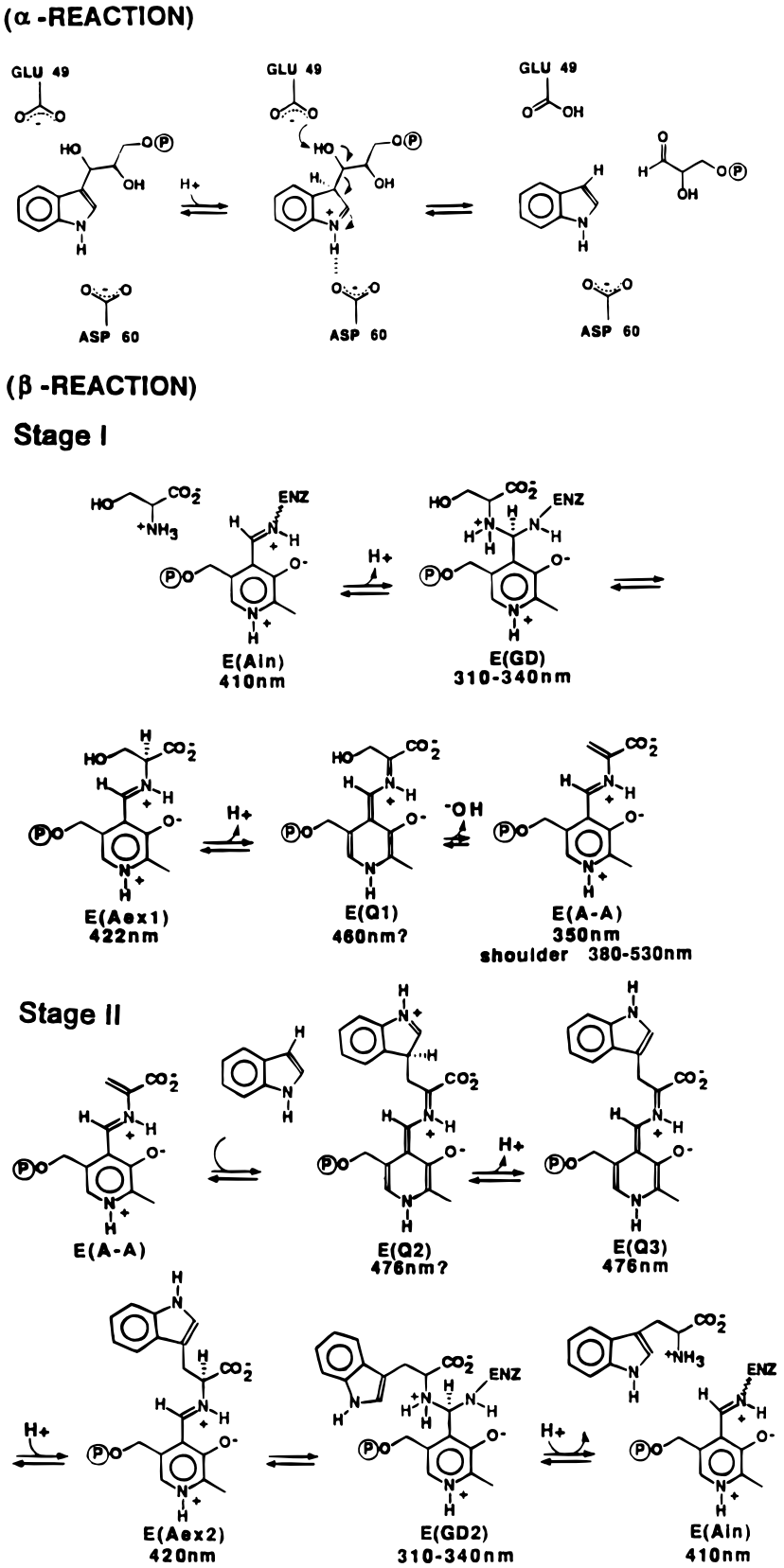
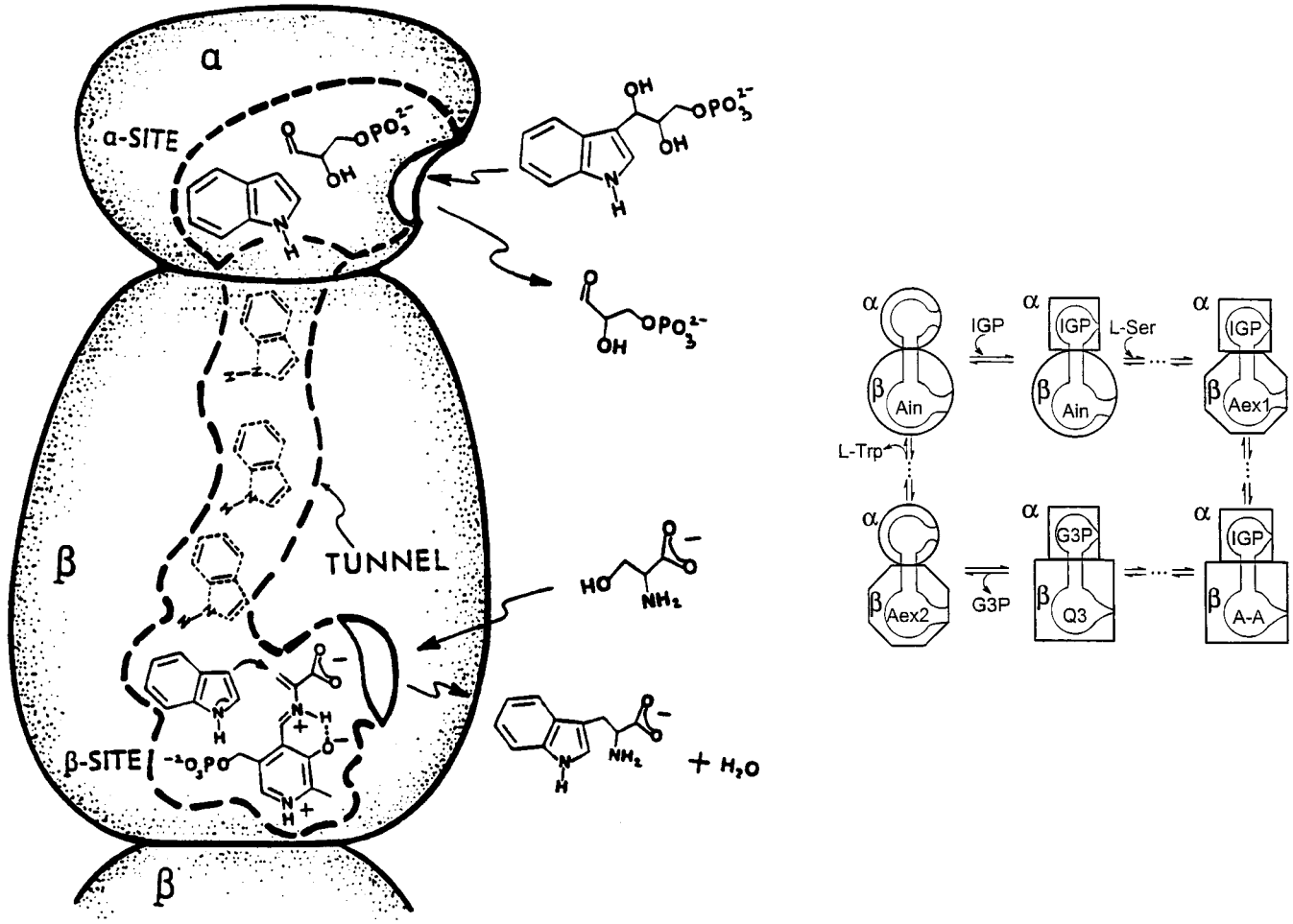
Scheme 1. Organic Structures for Reactants, Intermediates, and Products in (A) the α-Reaction, and (B) the *β*-Reaction Catalyzed by the Tryptophan Synthase Bienzyme Complex[a]



[a] The *β*-reaction takes place in two stages. In stage I, L-Ser reacts with enzyme-bound PLP to give an equilibrating mixture of the quasistable E(Aex$_1$) and E(A-A) species and a water molecule. In stage II, E(A-A) undergoes nucleophilic attack by indole to give L-Trp and regenerate E(Ain).

1993; Yang & Miles, 1993), and the X-ray structure of the $\alpha_2\beta_2$ bienzyme complex have implicated various *β*-site residues in catalysis, the identity of the active site base involved in abstraction of the α-proton of E(Aex$_1$) has not been unambiguously established. Early work involving chemical modification and photo-inactivation studies led to

Scheme 2 [a]



[a] (A) Cartoon showing an $\alpha\beta$-dimeric unit of $\alpha_2\beta_2$, the active sites of the α- and β-sites, the entrance and exit of IGP and G3P at the α-site, the entrance and exit of Ser and L-Trp at the β-site, and the transfer of indole from the α-site to the β-site via the 25 Å-long tunnel. The dashed indole structures indicate the path of diffusion of indole between the two sites. (B) Reaction scheme showing the proposed conformational transitions between open (circles), partially open (hexagons), and closed (squares) conformations of the α- and β-subunits during the $\alpha\beta$-reaction. The binding of IGP and conversion of E(Aex_1) to E(A-A) triggers conversion to the completely closed conformation. Conversion of E(Q_3) to E(Aex_2) and dissociation of G3P trigger a return to the open conformation. Redrawn from Pan and Dunn (1996) with permission.

the proposal that this base is βHis86 (Higgins et al., 1980; Tsai et al., 1978; Miles & Kumagi, 1974). Recent site-directed mutagenesis work (Miles et al., 1989) supports assignment of this role to βLys87, the PLP Schiff base Lys.

Low-Barrier H-Bonds (LBHBs) in Enzyme Catalysis. A growing body of literature supports the hypothesis that in certain enzyme site environments low-barrier H-bonds (LBHBs) play significant roles in catalysis (Cleland, 1992; Gerlt, 1994; Gerlt & Gassman, 1993; Frey et al., 1994; Cleland & Kreevoy, 1995; Frey, 1995). LBHBs are characterized by short donor−acceptor atomic distances (approximately 2.5 Å), very broad, low-frequency IR bands, strongly deshielded ^{1}H NMR chemical shifts of the H-bonded proton, and very low ^{1}H−^{2}H isotope fractionation factors (Hibbert & Emsley, 1990; Cleland, 1992; Frey et al., 1994). The bond energies for such systems are proposed to be 12−24 kcal/mol. LBHBs have been identified in several enzymes and inferred for many others (Cleland, 1992; Gerlt, 1994; Gerlt & Gassman, 1993; Frey et al., 1994; Cleland & Kreevoy, 1994; Tong & Davis, 1995). Thus far, discoveries of LBHBs in proteins are associated primarily with the catalytic site apparatus and/or coenzyme interactions with the site (Gerlt, 1994; Frey et al., 1994; Tong & Davis, 1995). However, the roles played by LBHBs in catalysis are not clear. Gerlt (1994), Gerlt and Gassman (1993), and Cleland and Kreevoy (1994) have proposed that the expected bond strength (12−24 kcal/mol) may provide a driving force to stabilize intermediates and transition states which otherwise would not be kinetically compentent, and this involvement is particularly important in solvent-excluded active sites. Frey et al. (1994) have proposed that a LBHB in serine proteases stabilizes the transition state for nucleophilic attack of the active site Ser-OH on the substrate. On the basis of ^{1}H NMR chemical shifts and NOE-based assignments of resonances, Tong and Davis (1995) propose that LBHBs between site residues, the pyridinium ring N−H, the Schiff base proton, and a third group function to "anchor" PLP at the active site of 2-amino-3-ketobutyrate-CoA ligase (a PLP enzyme). ^{1}H NMR data suggest that several other PLP enzymes exhibit similar LBHBs (Metzler et al., 1991a, 1994; Kintanar et al., 1991). While not contesting the evidence of LBHBs in enzyme active sites, Warshel et al. (1995) argue that the evidence for the short bond lengths of LBHBs in enzymes does not imply an unusually strong bond nor does the presence of an LBHB mean that it has an important role in catalysis, and they point out that there are no reported measurements of the energetics of LBHBs in transition states. Schwartz et al. (1995) and Usher et al. (1994) report that

carboxylate and amide analogues of acetyl-CoA at the active site of citrate synthase give very short H-bonds to Asp375 which may qualify as LBHBs. However, there does not seem to be any correlation between the affinities of these inhibitors, their pK_a values, and the length of the H-bond.

In this study we will show that during the interconversion of E(Aex_1) and E(A-A) in stage I (Scheme 1), the abstracted C-α proton exchanges only very slowly with external solvent, indicating a solvent-excluded active site. The distribution of species obtained with α-^{1}H vs α-^{2}H Ser gives an equilibrium isotope effect with an abnormally low isotope fractionation factor, indicating that the abstracted $^1H^+$ or $^2H^+$ resides in a LBHB. We propose this H-bond stabilizes the closed conformation of the E(A-A) species (Scheme 2B), and we speculate that it might play a role in lowering the energy of one or more of the transition states for the conversion of E(Aex_1) to E(A-A) (Scheme 1).

MATERIALS AND METHODS

Materials. Purification of *S. typhimurium* tryptophan synthase, determination of protein concentrations, and measurement of enzyme activity have been previously described (Kawasaki et al., 1987; Miles et al., 1987, 1989; Brzovic' et al., 1992a,b). L-Ser, morin, and indole were purchased from Sigma. α-^{2}H-D,L-Ser was synthesized by the method described previously (Miles & McPhie, 1974). Using ^1H_2O as the solvent rather than ^2H_2O, α-^{1}H-D,L-Ser was synthesized by the same method as that of α-^{2}H-D,L-Ser so that the samples of α-^{1}H- and α-^{2}H-D,L-Ser were prepared under identical conditions except for the isotopic composition of the solvent. All reactions and spectra were measured in 0.05 M bicine buffer, pH 7.8, containing 1 mM EDTA and 0.1 M NaCl at 25 °C. The concentrations reported refer to conditions after mixing.

Characterization of α-*^{2}H*-D,L-*Ser*. The ^{1}H-NMR spectra taken in ^2H_2O for commercially purchased α-^{1}H-Ser from Sigma show two multiplets, one at 3.81 ppm and the other at 3.69 ppm, with an integral ratio 2:1, respectively. On the basis of chemical shift, *J* coupling, and this integral ratio, the multiplet at 3.81 ppm is assigned to the two β-protons and the multiplet at 3.69 ppm is assigned to the α-proton (spectrum no. 13157M; Sadtler, 1969). The 3.81 ppm multiplet arises from the two diastereotropic β-protons which are split by each other and by the adjacent α-proton. The 3.69 ppm multiplet for the α-proton is a doublet of a doublet due to splitting by the two adjacent diastereotropic β-protons. In comparison, the ^{1}H-NMR spectrum taken in ^2H_2O for the α-^{2}H-Ser sample shows only a singlet at 3.81 ppm (data not shown). The disappearance of the multiplet at 3.69 ppm in the ^{1}H-NMR spectrum shows that the deuteration step is complete.

Characterization of α-*^{1}H*-D,L-*Ser*. Wild-type $\alpha_2\beta_2$ tryptophan synthase stereospecifically and quantitatively reacts with L-Ser to yield L-Trp. The yield of L-Trp was determined by measuring the change in absorbance at 290 nm (Weischet & Kirschner, 1976; Brzovic' et al., 1992b). A solution containing 0.030 mM α-^{1}H-D,L-Ser synthesized by the above-described procedure and 0.11 mM of indole was prepared, and a control solution made with exactly the same concentrations of indole and a commercial sample of α-^{1}H-L-Ser (Sigma). Addition of 15 μM wild-type tryptophan synthase to the sample containing D,L-Ser yields 0.015 ± 0.003 mM L-Trp, whereas the L-Ser sample yields 0.030 ± 0.005 mM of L-Trp. This result shows that racemization in the D,L sample is complete.

Qualitative Determination of Aluminum Ion and Its Effect on Enzymatic Activities. The synthetic procedure used to synthesize samples of α-^{2}H-D,L-Ser and α-^{1}H-D,L-Ser utilizes Al^{3+} ion and pyridoxal as catalysts. After isolation and purification by crystallization, the amounts of aluminum ion present in the samples of α-^{2}H-D,L-Ser and α-^{1}H-D,L-Ser were determined by a fluorescence assay involving the Al^{3+}−morin complex in acidic condition (Will, 1961). This fluorescence study showed that the 50 mM stock solutions of α-^{2}H-D,L-Ser and α-^{1}H-D,L-Ser used in these studies contained 12 ± 2 μM Al^{3+} and 11 ± 2 μM Al^{3+}, respectively. Assays to determine the effects of Al^{3+} on the β-reaction for wild-type $\alpha_2\beta_2$ tryptophan synthase showed that the enzyme is unaffected by Al^{3+} concentrations in this range (data not shown).

UV/Visible Spectroscopy. All static spectral measurements were performed with a Hewlett-Packard 8452A diode-array spectrometer. Decomposition of UV/vis absorption spectra via lognormal fitting was carried out as described by Houben and Dunn (1990), Metzler et al. (1985, 1991b). Siano and Metzler (1969), and Woehl and Dunn (1995).

Stopped-Flow Kinetic and Spectral Measurements. Single-wavelength stopped-flow (SWSF) absorbance kinetic studies and stopped-flow fluorescence studies were carried out as previously described (Dunn et al., 1979; Drewe & Dunn, 1985). All SWSF time courses were fitted to eq 3 by nonlinear least-squares regression analysis.

$$R_t = R_\infty \pm \sum_{i=1}^{n} R_i \exp(-t/\tau_i) \qquad (3)$$

In eq 3, R_t and R_∞ are the absorbance of fluorescence values at time *t* and time infinity, respectively; R_i and τ_i are the *i*th amplitude and the *i*th relaxation time, respectively. All time courses were collected under pseudo-first-order conditions. Rapid-scanning, stopped-flow (RSSF) kinetic studies were performed using the instrumentation and methodology described by Koerber et al. (1979), Drewe and Dunn (1985), Woehl and Dunn (1995), and Brzovic' and Dunn (1993, 1995).

The RSSF instrumentation and methodology have been described previously (Houben et al., 1989; Drewe & Dunn 1985; Koerber et al., 1983; Brzovic' & Dunn, 1993, 1995). In each RSSF experiment, a set of 25 scans is collected with delays between scans under programatic control. The repetitive scan rate is 8.54 ms. In our study, the collection of the initial scan relative to flow cessation is 8.5 ms.

^{1}H NMR Spectroscopy. The ^{1}H NMR spectra were collected with a Techmag Libra upgrade on a Joel FX 200 computer.

RESULTS

Comparison of the Reactions of α-*^{1}H*-L-*Ser and* α-*^{1}H*-D,L-*Ser with* $\alpha_2\beta_2$. In stage I of the β-reaction, the E(Ain) form of the enzyme reacts rapidly with L-Ser to give E(Aex_1), and this species then decays to a quasistable equilibrium mixture of E(Aex_1) and E(A-A) (Table 1 and Scheme 1). The time course for stage I consists of three relaxations, $1/\tau_1$, $1/\tau_2$, and $1/\tau_3$, with amplitudes A_1, A_2, and A_3 (Lane & Kirschner,

Table 1: Comparison of Relaxation Rates and Amplitudes of α-Proton Removal for D,L-Ser and L-Ser Catalyzed by $\alpha_2\beta_2$[a,b]

observation time scale (s)	relaxation rates (s^{-1}) and amplitudes[c]	α-^{1}H-D,L-Ser	α-^{1}H-L-Ser
1	$1/\tau_2$	7.64 ± 0.96	7.54 ± 0.97
	A_2	0.07 ± 0.01	0.07 ± 0.01
10	$1/\tau_2$	7.00 ± 1.10	7.50 ± 0.98
	A_2	0.06 ± 0.01	0.07 ± 0.009
	$1/\tau_3$	0.37 ± 0.05	0.38 ± 0.05
	A_3	0.004 ± 0.0006	0.004 ± 0.0007

[a] Absorbance studies were carried out as described under Materials and Methods. [b] The relaxation rates ($1/\tau_1$) for the formation of E(A-A) are too fast to be quantified. [c] Amplitudes are measured as ΔAbs.

1983a; Drewe & Dunn, 1985). Values for $1/\tau_2$ and $1/\tau_3$ and the corresponding amplitudes A_2 and A_3 are given in Table 1. Due to the fast rate of appearance of E(Aex$_1$) and the modest amplitude of the accompanying absorbance changes at 420 nm, values for $1/\tau_1$ and A_1 were not determined in this experiment. On much slower time scales (data not shown), the E(A-A) species undergoes decomposition to pyruvate and NH_4^+, and, as L-Ser is depleted, the enzyme very slowly reverts to E(Ain). During $1/\tau_2$ and $1/\tau_3$, the absorbance at 420 nm decreases as E(Aex$_1$) is converted to E(A-A) through removal of the α-proton and elimination of the β-hydroxyl group (Scheme 1). Comparisons of the single-wavelength, stopped-flow (SWSF) time courses measured at 420 nm for the reactions of 14 μM wild-type $\alpha_2\beta_2$ with either α-^{1}H-D,L-Ser (50 mM) or α-^{1}H-L-Ser (25 mM) at 1 or 10 s show there is no difference in observed relaxation rates and amplitudes (Table 1). *Since* D,L-*Ser and* L-*Ser give time courses with identical rate constants and amplitudes, we conclude that the* D-*isomer does not react with the enzyme*, a finding in agreement with earlier observations (Miles & McPhie, 1974; Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985). Therefore, to facilitate the preparation of ^{2}H-substituted samples for the isotope work described below, D,L-Ser is used in place of L-Ser.

Time-Resolved Spectra for the Deprotonation Step of the β-Reaction. Figure 1 presents time-resolved spectra obtained in the reactions of α-^{1}H- and α-^{2}H-D,L-Ser with $\alpha_2\beta_2$ at pH 7.8. Consistent with the findings of Drewe and Dunn (1985), these rapid-scanning stopped-flow (RSSF) spectra show that substitution of ^{2}H for ^{1}H at C-α alters the time course for the approach to equilibrium during the conversion of the E(Aex$_1$) intermediate (λ_{max} = 425 nm) to E(A-A) (λ_{max} = 349 nm) (compare spectra 1 with spectra 9 in panels A and B of Figure 1). In the ^{2}H-substituted sample, the conversion of the E(Aex$_1$) species to E(A-A) is slowed by the primary kinetic isotopic effect, causing a greater transient accumulation of E(Aex$_1$) (Drewe & Dunn, 1985). *Upon completion of this interconversion, the distribution of species at equilibrium shows less accumulation of E(A-A) in the ^{2}H sample, indicating a significant equilibrium isotope effect.* When these reactions are repeated in the presence of the α-site ligand α-glycerolphosphate (GP), which is an analogue of G3P and a potent allosteric effector which stabilizes E(A-A) (Houben & Dunn, 1990; Dunn et al., 1990), the large difference in distribution of species observed in Figure 1 is no longer seen (data not shown).

Reactions of α-^{2}*H-*D,L-*Ser and* α-^{1}*H-*D,L-*Ser with* $\alpha_2\beta_2$. As previously stated, stopped-flow time courses measuring either the absorbance changes at 420 nm (data not shown) or the

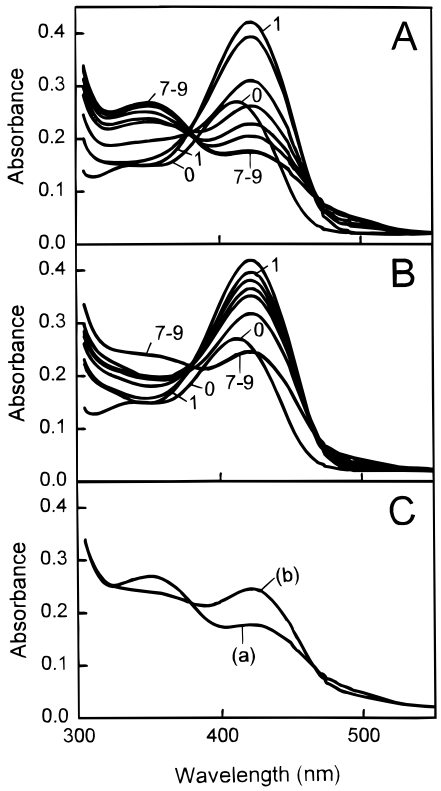


FIGURE 1: Time-resolved RSSF UV/vis absorption spectra showing the time courses for the reactions of 40 mM α-^{1}H-Ser (A) or α-^{2}H-D,L-Ser (B) with 10 μM $\alpha_2\beta_2$. Panel C compares spectrum 9 from panel A with spectrum 9 from panel B. In both A and B, the sequences of time-resolved spectra show the rapid conversion of the internal aldimine, E(Ain) (spectrum 0), to the Ser external aldimine, E(Aex$_1$) (spectrum 1), followed by the decay of this intermediate to an equilibrium distribution of E(Aex$_1$) and the α-aminoacrylate Schiff base, E(A-A) (spectrum 9) (Drewe & Dunn, 1985). The different patterns in A and B are due to the primary KIE on the rate of conversion of E(Aex$_1$) to E(A-A). The different distribution of species obtained at quasiequilibrium (viz., C) is due to the equilibrium isotope effect. The repetitive scan rate was 8.54 ms/scan. The scanning sequence used is (1) 8.54, (2) 42.7, (3) 102.5, (4) 162.3, (5) 222.1, (6) 341.7, (7) 6877.9, (8) 7775.0, and (9) 8672.2 ms after flow had stopped. Scan 0 is the spectrum of E(Ain) prior to reaction.

fluorescence changes due to formation and decay of E(Aex$_1$) (Figure 2) for reaction of α-^{1}H-D,L-Ser with $\alpha_2\beta_2$ in stage I are composed of three kinetic phases with relaxation rates $1/\tau_1^H > 1/\tau_2^H > 1/\tau_3^H$ (Figure 2; Table 2). A very slow process corresponding to the decomposition of E(A-A) is also observed. The E(Aex$_1$) intermediate is the only species in the tryptophan synthase pathway that gives a strong fluorescence signal (Miles & McPhie, 1974; Lane & Kirschner, 1983a; Drewe & Dunn, 1985). Hence, when excited at 420 nm, the system gives a fluorescence emission signal that unambiguously measures the formation and decay of E(Aex$_1$).

When ^{2}H is substituted for ^{1}H at the C-α of D,L-Ser (Figure 2), in addition to the three phases (τ_1^D, τ_2^D, and τ_3^D) associated with conversion of E(Ain) to the equilibrium mixture of E(Aex1) and E(A-A), there is a somewhat slower fourth phase, τ_4^D, not observed in the ^{1}H sample (Table 2). As implied by the RSSF data (Figure 1), the first and third relaxations are unaffected by substitution of ^{2}H for ^{1}H. As a consequence of the primary KIE on $1/\tau_2$ with $(1/\tau_2^H)/(1/\tau_2^D) = 3.0$ (Table 2), there is a greater transient accumulation of E(Aex$_1$) in the ^{2}H sample. This finding is in agreement

Table 2: Comparison of Relaxation Rates, Amplitudes, and KIE for the Reactions of α-^{1}H-D,L-Ser or α-^{2}H-D,L-Ser with $\alpha_2\beta_2$[a]

observation time scale	relaxation rates (s^{-1}) and amplitudes[b]	α-^{1}H-D,L-Ser	α-^{2}H-D,L-Ser	$(1/\tau_n)^H/(1/\tau_n)^D$ [c]
1	$1/\tau_1$	422 ± 50	450 ± 58	0.94
	A_1	1.00 ± 0.16	1.30 ± 0.18	
	$1/\tau_2$	6.50 ± 0.81	2.18 ± 0.34	2.98
	A_2	1.95 ± 0.24	1.28 ± 0.21	
10	$1/\tau_2$	7.00 ± 0.98	2.37 ± 0.36	2.95
	A_2	1.91 ± 0.25	1.17 ± 0.25	
	$1/\tau_3$	0.42 ± 0.06	0.34 ± 0.05	1.23
	A_3	0.18 ± 0.02	0.19 ± 0.03	
600	$1/\tau_4$[d]		0.002 ± 0.0003	
	A_4		0.53 ± 0.07	

[a] Fluorescence studies were carried out as described under Materials and Methods and in the legend to Figure 2. [b] Amplitudes are measured as relative fluorescence (with A_1 increasing, and A_2, A_3, and A_4 decreasing). [c] The KIE in each phase is defined as $(1/\tau_n)^H/(1/\tau_n)^D$. [d] The slow kinetic phase ($1/\tau_4$) for solvent exchange is not observed in the α-^{1}H-D,L-Ser sample.

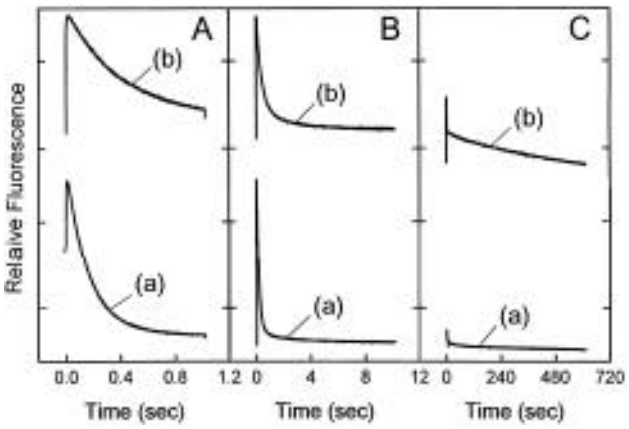


FIGURE 2: Stopped-flow fluorescence time courses for the reactions of 13 μM $\alpha_2\beta_2$ with 50 mM of α-^{1}H-D,L-Ser (a) or α-^{2}H-D,L-Ser (b) for 1 s (A), 10 s (B), and 600 s (C). For clarity in presentation, traces a and b in each panel are offset by the same amount. Each trace is plotted on the same relative fluorescence scale. The difference in the rate of decay ($1/\tau_2$) seen in panel A is a consequence of the primary KIE on the conversion of E(Aex_1) to E(A-A). The additional relaxation (τ_4) apparent in panel C, trace (b), is due to exchange of enzyme-bound ^{2}H for the ^{1}H of solvent.

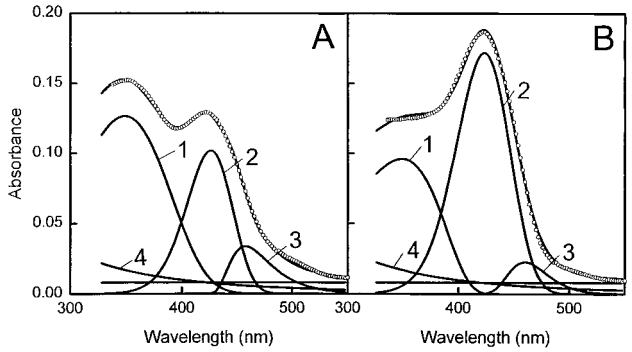


FIGURE 3: UV/vis static absorbance spectra (○) for the products of the reactions of 14 μM $\alpha_2\beta_2$ with 50 mM of ^{1}H-Ser (A) or ^{2}H-Ser (B) in quasiequilibrium measured approximately 5 s after mixing. The spectra are fitted with lognormal and scattering curves (solid lines). In A and B, lognormal curves 1 and 3 are assigned to the α-aminoacrylate Schiff base, E(A-A), curve 2 is assigned to the Ser external aldimine, E(Aex_1), and curve 4 is assigned as the scattering curve. The band position, bandwidth, and band skewness of bands 1, 2, and 3 were held constant, while the amplitudes were treated as unknowns. The solid line running through the individual data points is the summation of contributions from curves 1−4. Due to the complexity of the spectra at wavelengths below 325 nm, no attempt was made to fit this region.

with earlier literature which shows that the abstraction of the C-α proton is partially rate limiting for the formation of E(A-A) (Miles & McPhie, 1974; Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985). Detailed spectroscopic studies (data not shown) establish that the additional relaxation detected in the ^{2}H sample, τ_4^D, is due to the slow exchange of enzyme-bound ^{2}H for solvent ^{1}H.

The time courses presented in Figure 2 establish that stage I reaches a quasiequilibrium between E(Aex_1) and E(A-A) within seconds after mixing. Due to the primary kinetic isotope effect for the α-^{2}H-Ser sample, the quasiequilibrium is reached in ∼5 s, whereas for α-^{1}H-Ser it is reached in ∼2.5 s. Moreover, in this quasiequilibrium, more of the E(Aex_1) species is present in the α-^{2}H-Ser system than in the α-^{1}H-Ser system (viz., Figures 1 and 2), demonstrating the existence of a relatively large equilibrium isotope effect on the interconversion of E(Aex_1) and E(A-A).

When examined on longer time scales (600 s), the fluorescence of both the α-^{2}H-Ser and α-^{1}H-Ser samples undergo further changes (Figure 2C). The α-^{2}H-Ser sample undergoes a slow conversion of E(Aex_1) to E(A-A) in τ_4, approaching the distribution of intermediates found in the reaction of the α-^{1}H-Ser sample, whereas the α-^{1}H-Ser sample does not undergo a comparable spectral change. The slow decay of both samples is due to the conversion of E(A-A) to pyruvate and NH_4^+.

Evaluation of the Equilibrium Isotope Effect via UV/Vis Spectral Band Analysis. UV/vis spectra (Figure 3) were obtained ∼5 s after mixing either α-^{1}H-Ser or α-^{2}H-Ser with $\alpha_2\beta_2$, a time at which these systems have reached quasiequilibria for the interconversion of E(Aex_1) with E(A-A) (Figure 2). Earlier, Drewe and Dunn (1985) established that the λ_{max} of E(Aex_1) is ∼425 nm, while the λ_{max} of E(A-A) is ∼349 nm with a shoulder ∼460 nm (Woehl & Dunn, 1995). When the three λ_{max} and the parameters for band width and band skewness are all held constant (Table 3), the UV/vis spectra between 320 and 500 nm for both the α-^{2}H-Ser and α-^{1}H-Ser samples obtained at quasiequilibrium could be decomposed into three components using lognormal distribution curve fits (Figure 3) (Siano et al., 1969; Metzler et al., 1985, 1991b; Houben & Dunn, 1990; Woehl & Dunn, 1995). It can be shown that, provided the spectra are dominated by contributions from only these species, the ratio of band amplitudes at 349 nm and at 424 nm for α-^{1}H-Ser ($K_H = A_{349}/A_{425})_H$ divided by the ratio of amplitudes at 349 nm and at 424 nm for α-^{2}H-Ser ($K_D = A_{349}/A_{425})_D$ gives the magnitude of the isotope effect on the equilibrium constant (K_H/K_D) for interconversion of E(Aex_1) and E(A-A). From

Table 3: Lognormal Peak Fit Analyses of UV/Vis Static Absorbance Spectra for the Distribution of Species Formed in the Reactions of α-^{1}H-D,L-Ser and α-^{2}H-D,L-Ser with $\alpha_2\beta_2$[a]

samples	peak	amplitude	skewness	λ_{max} (nm)	bandwidth
α-^{1}H-D,L-Ser	1	0.127 ± 0.004	0.80	349	100
	2	0.102 ± 0.004	0.85	426	49.6
	3	0.034 ± 0.001	1.7	458	45
α-^{2}H-D,L-Ser	1	0.096 ± 0.002	0.6	349	105
	2	0.172 ± 0.005	0.87	424	55.6
	3	0.023 ± 0.001	1.5	460	42

[a] UV/vis static absorbance spectra were determined as described under Materials and Methods and in Figure 3.

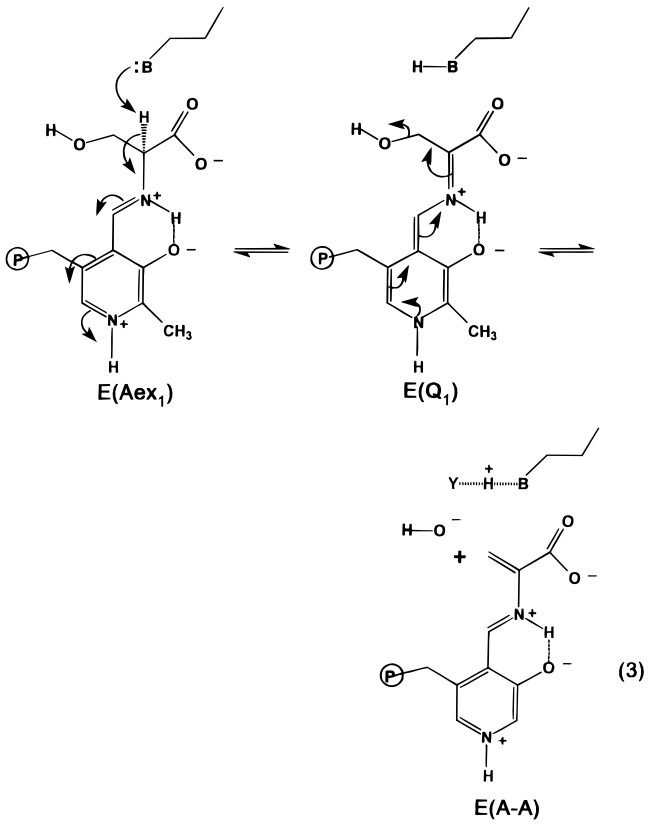
this analysis, the magnitude of the equilibrium isotope effect was determined to be 1.80 ± 0.18 (Table 3). Since the fractionation factor for the α proton of an amino acid is 1.13 (Cleland, 1980), the fractionation factor for the residue which functions as the base to deprotonate and protonate the C-α proton in E(Aex_1) is estimated to be 0.62 (Cleland, 1980).

DISCUSSION

While investigating kinetic and equilibrium isotope effects on the reaction of L-Ser with $\alpha_2\beta_2$, we noticed that reaction of α-^{1}H-Ser and α-^{2}H-D,L-Ser with $\alpha_2\beta_2$ gives different quasiequilibrium distributions of E(Aex_1) and E(A-A) (Figures 1−3). On a slower time scale (600 s) the quasiequilibrium initially attained by the deuterated sample is converted to a distribution of intermediates approaching that of the isotopically normal system (Figure 2C). *This slow equilibrium is due to a slow exchange of enzyme-bound ^{2}H for the ^{1}H of solvent water*. Analysis of the effects of ^{2}H substitution for ^{1}H at the C-α of D,L-Ser on the distribution of intermediates in the quasiequilibrium of stage I (Figure 3) gives an equilibrium isotope effect of 1.80 ± 0.18, implying a fractionation factor of 0.62 for the base group involved in the abstraction of the C-α proton of serine. When reaction is carried out in the presence of 50 mM GP (data not shown), the apparent difference in quasiequilibrium distribution becomes small due to the allosteric effect of GP. It is known that GP binding to the α-site strongly shifts the distribution of species formed in the reaction of L-Ser with E(Ain) in favor of E(A-A) (Dunn et al., 1987, 1990; Brzovic' et al., 1992a,b, 1993; Woehl & Dunn, 1995), thus making the equilibrium isotope effect difficult to discern.

The unusual equilibrium isotope effect requires an unusual isotope fractionation factor. Thiol groups can give rise to small fractionation factors (Cleland, 1992); however, there is no Cys thiol group at the β-site of tryptophan synthase (Hyde et al., 1988; Miles et al., 1989). Functional group candidates for acid−base transformations at the site consist of O or N from Glu, Asp, Lys, and His side chains, and a water or hydroxide ion; low fractionation factors could arise from LBHB formation within a pair derived from these donor−acceptor atoms. *In the absence of alternative, reasonable explanations, the low fractionation factor qualifies as evidence for a low-barrier H-bond (LBHB).* Slow exchange of the deuterated complex with solvent water is indicative of a special environment where access of solvent water to the catalytic site is highly restricted, *i.e., the E(A-A) species behaves as if the β-site has a solvent-excluded environment*.

These findings indicate that the abstracted $^1H^+$ or $^2H^+$ resides in a LBHB which stabilizes E(A-A), perhaps as depicted below (eq 3), where −B: is the active site base and −B···H^+···Y represents the LBHB:



Atoms B and Y have not been identified. If the base which abstracts the α-proton is βK87, as proposed by Miles et al. (1989), then −B: should be the ϵ-N of βK87. Atom Y may be the oxygen of the sequestered OH^-.

From the experimental findings presented herein, we are unable to determine the strength of the LBHB nor are we able to estimate the effects of this LBHB on the activation energies of steps involving its formation. If LBHBs in enzyme sites are unusually strong, as has been argued (Cleland, 1992; Gerlt, 1994; Gerlt & Gassman, 1993; Frey et al., 1994; Cleland & Kreevoy, 1994; Tong & Davis, 1995), then the effects on stage I of the β-reaction could be large. While Warshel et al. (1995) argue against an important role of LBHBs in enzyme catalysis, it seems significant to us that all of the examples of LBHBs reported for enzymes involve bonding interactions between site residues either involved directly in catalysis (Cleland, 1992; Gerlt, 1994; Gerlt & Gassman, 1993; Frey et al., 1994; Cleland & Kreevoy, 1994, 1995; Schwartz et al., 1995; Frey, 1995 or in coenzyme binding (Tong & Davis, 1995).

The (stabilizing) energy of a strong LBHB could reduce the rate of exchange with solvent ^{1}H (Eigen & Hammes, 1963; Hibbert & Emsley, 1990). If −B: is the ϵ-N of βK87 (eq 3), then the slow exchange of the ^{2}H-substituted LBHB in E(A-A) with solvent ^{1}H requires that the ϵ-N group be tied down such that its protons are unable to rapidly exchange positions with the abstracted deuterion. Alternatively, the slow exchange rate is consistent either with a His imidazolyl group, or a monoprotic base such as a Glu or Asp carboxylate. While the β-site contains all three groups (E109, E305, H86), none of these residues seems ideally placed to serve this base catalyst role. However, since the conformation of

E(A-A) (Brzovic' et al., 1992a,b, 1993; Leja et al., 1995; Pan & Dunn, 1996) is almost certainly different from the conformations of the structures solved (viz., Scheme 2B) [E(Ain) and the (IPP)E(Ain) complex (Hyde et al., 1988)], the available X-ray structures very likely are not entirely appropriate for analysis of the roles played by site residues in the $E(Aex_1)$ to E(A-A) interconversion. According to current theories for LBHBs (Hibbert & Emsley, 1990; Cleland, 1992; Frey et al., 1994; Gerlt, 1994; Gerlt & Gassman, 1993), in order for an LBHB to exist, the proton affinities of −B: and −Y: must be comparable. If true, this obligatory matching of proton affinities no doubt places restrictions on the possible identities of −B and −Y and has implications for how the proton affinities of −B and −Y are modulated by the site microenvironment. However, the findings of Schwartz et al. (1995) and Usher et al. (1994) indicate that abnormally short H-bonds can be formed in systems where the proton affinities of the donor and acceptor appear to differ by a factor $>10^8$. They conclude that in their enzyme system there is no direct correlation between H-bond length and bond strength.

In contrast to the L-Ser reaction, reaction of Gly with $\alpha_2\beta_2$ gives a mixture of equilibrating species dominated by the Gly external aldimine, the Gly *gem*-diamine, and a trace of the Gly quinonoid (Houben et al., 1989). The binding of GP to the α-site shifts this distribution slightly toward the quinonoid species. Comparison of the spectra obtained for isotopically normal and α,α-$^2H_2$-Gly (data not shown) gave no indication that the equilibrium between the quinonoid species, the external aldimine and the *gem*-diamine species is significantly altered by the isotope substitution.

In studies of the β_2-form of tryptophan synthase from *Escherichia coli*, Miles and McPhie (1974) were the first to report unusual equilibrium isotope effects. In the absence of high concentrations of monovalent cations and at pH 7.8, Ser reacts with β_2 to give $E(Aex_1)$ almost exclusively (Goldberg & Baldwin, 1967; Goldberg et al., 1968). Miles and McPhie (1974) found that in the presence of 1.5 M NH_4-Cl, the relative stabilities of species in stage I are altered such that comparable amounts of $E(Aex_1)$ and E(A-A) are formed. Just as we have found with the $\alpha_2\beta_2$-form of the enzyme (Figures 1−3), substitution of ^2H for ^1H at the α-C of D,L-Ser in the β_2-system shifts this equilibrium in favor of $E(Aex_1)$, and the equilibrium isotope effect estimated by Miles and McPhie ($K^H/K^D \approx 3$) is unusually large. This result also clearly implies an unusually low fractionation factor, and the existence of a LBHB in the β_2 reaction.

LBHB Alteration of Ground-States and Transition-States in β-Site Catalysis. The presence of an LBHB in stage I of the β-reaction raises important questions about its role in tryptophan synthase catalysis. As argued by Gerlt (1994), Gerlt and Gassman (1993), and Frey et al. (1994), LBHBs may play important roles both in the stabilization of transition-states and the stabilization or destabilization of ground-states of enzyme-catalyzed reactions. With energies in the range of 12−24 kcal/mol or more, the introduction of an LBHB into the structure of a ground-state or a transition-state (or both) could cause a significant lowering of the activation energy of a step either via stabilization of the activated complex, stabilization of a high-energy intermediate, or via destabilization of the ground-state. In the tryptophan synthase system, we do not understand either the chemical role or the strength of the LBHB. The LBHB involves the proton abstracted from the α-C of the external aldimine species $E(Aex_1)$ and presumably the base catalytic group to which it is transferred upon abstraction (eq 3). Since the quinonoid formed in this step is a fleeting transient (Drewe & Dunn, 1985), the species with an LBHB that accumulates in stage I is the α-aminoacrylate Schiff base, E(A-A) (eq 3 and Scheme 1). The primary KIE on the rate of removal of the α-proton from $E(Aex_1)$ (Lane & Kirschner, 1983a,b; Drewe & Dunn, 1985) establishes that C−H bond scission is an important component of the transition state for the rate-determining step in the conversion of $E(Aex_1)$ to E(A-A). If an LBHB occurs in the transition-state for abstraction of the α-proton of $E(Aex_1)$, then the energy of this transition-state will be lowered by an amount equivalent to the strength of the LBHB. If LBHB formation occurs following proton abstraction, but before elimination of the β-hydroxyl group, then $E(Q_1)$ would be stabilized and this stabilization could result in lowered activation energies for its formation and decay. Since the Ser β-hydroxyl group is eliminated as E(A-A) is formed, it may be that OH^- is the other electronegative species participating in the LBHB (as depicted in eq 3) and that an LBHB occurs only at this stage in the reaction. The formation of an LBHB to the hydroxyl oxygen in the transition-state of the elimination reaction would provide a significant lowering of the activation energy for scission of the $O-C_\beta$ bond. The LBHB in E(A-A) must stabilize the α-aminoacrylate structure. Finally, the LBHB present in E(A-A) may play a role in the reaction of indole with E(A-A).

Effects of the LBHB on the Conformation of Tryptophan Synthase. Our work (Dunn et al., 1990, 1994; Brzovic' et al., 1992a,b, 1993; Leja et al., 1995; Pan & Dunn, 1996) and the work of others (Ruminov et al., 1995; Lane & Kirschner et al., 1991; Kirchner et al., 1991) have provided compelling evidence that during the catalytic cycle of the αβ-reaction, αβ-subunit pairs cycle between open and closed conformations that switch the complex between low- and high-activity states (Scheme 2B). One function of these conformational transitions is to provide a mechanical/chemical mechanism for phasing the catalytic cycles of the α- and β-sites; a second function is to prevent the escape of indole from the environs of the α- and β-sites and the interconnecting tunnel so that reaction with E(A-A) is assured (Brzovic' et al., 1992a; Leja et al., 1995; Pan & Dunn, 1996).

During the αβ-reaction, the conversion to a closed, catalytically-activated structure is triggered by the combination of IGP binding to the α-site and the chemical transformation of $E(Aex_1)$ to E(A-A) at the β-site; conversion back to the open, catalytically inactivated conformation occurs when $E(Q_3)$ is converted to $E(Aex_2)$ (Brzovic' et al., 1992a; Leja et al., 1995; Pan & Dunn, 1996) (Scheme 2B). Since the conversion of $E(Aex_1)$ to E(A-A) coincides with formation of the LBHB in E(A-A), the (exothermic) energy associated with this bonding interaction almost certainly must contribute stability to the closed conformation of the E(A-A) species. The Gly reaction gives species predominantly in the open (perhaps solvent-exposed) conformation (Brzovic' et al., 1992b) and may explain the absence of evidence for an LBHB in this reaction.

Conclusions. The discovery of the slow exchange of ^2H for solvent ^1H of α-^2H-substituted Ser, together with a large equilibrium isotope effect on the interconversion of $E(Aex_1)$ and E(A-A), provides new insights into the catalytic mechanism and allosteric regulation of the tryptophan synthase

bienzyme complex. The slow exchange supports the view that the closed conformation of the β-subunit generates a solvent excluded active site. The equilibrium isotope effect implies an unusual isotope fractionation factor and indicates that the abstracted α-proton is sequestered at the site in a LBHB. We speculate that this LBHB may play important roles in modulating the relative stabilities of ground-states, lowering the activation energies of covalent steps in catalysis, or stabilizing the closed conformation of the E(A-A) species, a process which helps prevent the escape of the common metabolite, indole, thereby contributing to the efficiency with which indole is channeled between the two active sites.

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