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# Isomerization of (3*S*)-2,3-Dihydro-5-fluoro-L-tryptophan and of 5-Fluoro-L-tryptophan Catalyzed by Tryptophan Synthase: Studies Using Fluorine-19 Nuclear Magnetic Resonance and Difference Spectroscopy

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ABSTRACT: We are exploring the active site and the mechanism of the pyridoxal phosphate dependent reactions of the bacterial tryptophan synthase $\alpha_2\beta_2$ complex by use of substrate analogues and of reaction intermediate analogues. Fluorine-19 nuclear magnetic resonance studies and absorption spectroscopy are used to study the binding and reactions of the D and L isomers of 5-fluorotryptophan, of tryptophan, and of (3*S*)- and (3*R*)-2,3-dihydro-5-fluorotryptophan. Tryptophan synthase specifically and tightly binds the 3*S* diastereoisomer of both 2,3-dihydro-5-fluoro-D-tryptophan and 2,3-dihydro-5-fluoro-L-tryptophan, whereas it binds 5-fluoro-D-tryptophan more tightly than 5-fluoro-L-tryptophan. Unexpectedly, we find that the D and L isomers of 5-fluorotryptophan, of tryptophan, and of (3*S*)-2,3-dihydro-5-fluorotryptophan are slowly interconverted by isomerization reactions. Since these isomerization reactions are $10^3$–$10^5$ times slower than the $\beta$-replacement and $\beta$-elimination reactions catalyzed by tryptophan synthase, they have no biochemical significance in vivo. However, the occurrence of these slow reactions does throw some light on the nature of the active site of tryptophan synthase and its requirements for substrate binding. Our results raise the interesting question of whether tryptophan synthase itself serves a catalytic role in these slow reactions or whether the enzyme simply binds the substrate and pyridoxal phosphate stereospecifically and thus promotes the intrinsic catalytic activity of pyridoxal phosphate.

We are investigating the interaction of tryptophan synthase of *Escherichia coli* and of *Salmonella typhimurium* with substrates, products, and analogues of reaction intermediates to increase our understanding of the specificity, mechanism, and stereochemistry of the pyridoxal phosphate dependent reactions of this enzyme. The $\alpha_2\beta_2$ complex of tryptophan synthase (EC 4.1.2.20) catalyzes the synthesis of L-tryptophan from L-serine and indole (eq 1) as well as a number of other

$$\text{L-serine} + \text{indole} \rightarrow \text{L-tryptophan} \quad (1)$$

pyridoxal phosphate dependent $\beta$-addition and $\beta$-elimination reactions that occur at the active site of the $\beta_2$ subunit. The cleavage of indole-3-glycerol phosphate occurs at the active site of the $\alpha$ subunit [for review, see Miles (1979 and 1986)]. The pyridoxal phosphate Schiff base of the indolenine tautomer

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of L-tryptophan, a proposed intermediate in eq 1 (Davis & Metzler, 1972; Lane & Kirschner, 1983), has tetrahedral geometry at C-3 of the heterocyclic ring. Our finding that tryptophan synthase is strongly inhibited by one diastereoisomer of 2,3-dihydro-L-tryptophan (2,3-$H_2$-L-Trp),[1] an analogue of this proposed intermediate, is evidence that tryptophan synthase catalyzes eq 1 via a chiral indolenine intermediate (Phillips et al., 1984, 1985a,b). Addition of either L-tryptophan or (3*S*)-2,3-$H_2$-L-Trp to tryptophan synthase leads to formation of new absorption bands at 474 or 494 nm, respectively, ascribed to quinonoid or $\alpha$-carbanion intermediates that result from the removal of the $\alpha$-hydrogen (Miles, 1980; Phillips et al., 1984, 1985a). Although tryptophan synthase binds D-tryptophan more tightly than L-tryptophan, the enzyme does not exchange the $\alpha$-hydrogen of D-tryptophan at a detectable rate and thus does not appear to form a carbanion intermediate with D-tryptophan (Miles, 1980).

In this work, we have separated L and D isomers of 5-fluorotryptophan and of (3*S*)- and (3*R*)-2,3-dihydro-5-fluorotryptophan for fluorine-19 nuclear magnetic resonance ($^{19}$F NMR) studies and absorption spectra. Fluorine-19 is a very useful spectroscopic reporter group for nuclear magnetic resonance studies since it permits studies of binding of specifically labeled compounds by a very sensitive technique (Gerig, 1978). Fluorine substitution frequently has little effect on biological activity since the carbon–fluorine bond is not much longer than a carbon–hydrogen bond (Gerig, 1978). $^{19}$F NMR studies and spectrophotometric titrations show that 5-fluoro-D-tryptophan and specific diastereoisomers, (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan, are tightly bound by tryptophan synthase, whereas 5-fluoro-L-tryptophan is less tightly bound. Unexpectedly, some of these compounds undergo slow, enzyme-catalyzed reactions at the high enzyme concentrations used for the $^{19}$F NMR experiments. The interconversion of 5-fluoro-L-tryptophan and 5-fluoro-D-tryptophan and of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan is demonstrated by changes in the absorption spectra of enzyme–substrate complexes. These reactions do not depend on the presence of the 5-fluoro group since the isomerization of (3*S*)-2,3-dihydro-L-tryptophan and of L-tryptophan occurs under the same conditions. A slow cleavage of 5-fluoro-L-tryptophan to 5-fluoroindole is also observed and is the subject of the accompanying paper (Ahmed et al., 1986b).

Our results contribute to understanding the stereochemistry of the substrate binding site of tryptophan synthase and the relative roles of the enzyme and enzyme-bound pyridoxal phosphate in catalyzing these reactions.

## Materials and Methods

*Enzymes.* The $\alpha_2\beta_2$ complex of tryptophan synthase was prepared from *Escherichia coli* strain *W3110 trpR cysB Δtrp LD102 trpB*$^+$ *trpA*$^+$/*F' colVB cysB*$^+$ *Δtrp LD102 trpB*$^+$ *trpA*$^+$ as described by Higgins et al. (1979). Concentrated solutions of $\alpha_2\beta_2$ complex (1.5 mM in $\alpha\beta$ promoter = 110 mg/mL) for $^{19}$F NMR experiments were prepared in 0.05 M sodium *N*,*N*-bis(2-hydroxyethyl)glycine, pH 7.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and about 20% $D_2O$ by concentration in Amicon Centriflo membrane cones (CF25) with three additions of buffer to obtain equilibration. Tryptophanase was prepared from *E. coli* B/1t7-A by a modification of the method of Suelter et al. (1976).[2]

*Resolution of 5-Fluoro-*DL*-tryptophan.* 5-Fluoro-DL-tryptophan (Sigma) was converted to the *N*-(trifluoroacetyl) derivative with ethyl trifluoroacetate and triethylamine in methanol as described by Curphey (1979) for tryptophan. The *N*-(trifluoroacetyl)-5-fluoro-DL-tryptophan was digested with carboxypeptidase A, as described by Coy et al. (1974) for the corresponding *N*-(chloroacetyl) derivative. The remaining *N*-(trifluoroacetyl)-5-fluoro-D-tryptophan was converted to the free amino acid by treatment with 1 M aqueous piperidine (Cohen, 1970). Both 5-fluoro-L-tryptophan and 5-fluoro-D-tryptophan were recrystallized from $CHCl_3$–methanol (1:1). The optical purity of the L and D isomers was established by digestion with L-amino acid oxidase (Greenstein & Winitz, 1961). The resultant 5-fluoroindole-3-pyruvic acid was determined spectrophotometrically at 310 nm; these results indicated that the 5-fluoro-L-tryptophan used in our experiments is >97% L, while the 5-fluoro-D-tryptophan is >99% D.

*Preparation of 2,3-Dihydro-5-fluorotryptophans and Separation of Diastereoisomers.* 5-Fluoro-L-tryptophan and 5-fluoro-D-tryptophan were reduced with pyridine–borane complex in trifluoroacetic acid, as described for L-tryptophan (Phillips et al., 1985a; Kikugawa, 1978). The diastereoisomers were separated by HPLC as described for the diastereoisomers of 2,3-$H_2$-L-Trp (Phillips et al., 1985a).

Since the diastereoisomers of 2,3-$H_2$-L-Trp and 2,3-dihydro-5-fluoro-L-tryptophan show similar elution patterns on HPLC (see below) and similar relative inhibition of tryptophan synthase (Table I), we assume that the stereochemical assignments in our previous paper (Phillips et al., 1985a) also hold for the fluorinated derivatives. Thus, the first diastereoisomer eluted (previously designated isomer A) is (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan, and the second isomer (previously referred to as isomer B) is (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan. Similarly, for the D-amino acids, the first compound to elute is (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan, and the second is (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan.

*Spectrophotometric Titration of $\alpha_2\beta_2$ Complex with Amino Acids.* The absorbance of solutions of unmodified or modified holo-$\alpha_2\beta_2$ complex (0.01 mM in $\alpha\beta$ protomer in 0.1 M potassium phosphate, pH 7.8, containing 1 mM EDTA) at the indicated wavelengths was determined by use of a Cary 118 spectrophotometer at 23 °C within 5 min after each addition of amino acid analogue; the observed differences between absorbance at 494 and at 412 nm [(3*S*)-2,3-dihydro-5-fluoro-L-tryptophan], between absorbance at 470 and at 412 nm (5-fluoro-D-tryptophan), and between absorbance at 476 and at 412 nm (5-fluoro-L-tryptophan) or the decrease in absorbance at 412 nm [(3*R*)-2,3-dihydro-5-fluoro-L-tryptophan, (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan, and (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan] was corrected for changes in volume and for the initial difference absorbance of the solution before addition of amino acid. The maximum absorbance change ($\Delta\epsilon_{max}$) was estimated from a plot of $1/\Delta\epsilon$ vs. $1/S_T$, where $S_T$ is the total ligand concentration; the straight line through the data points at high $S_T$ intersects the $y$ axis at $1/\Delta\epsilon_{max}$. The experimental data are then plotted as log $[R/(1 - R)]$ vs. log $C_{free}$, where $R = \Delta\epsilon/\Delta\epsilon_{max}$ and $C_{free} = S_T - RE_T$, where $E_T$ is the enzyme monomer concentration (0.01 mM) (Tanizawa & Miles, 1983).

*Analysis of Diastereoisomers by HPLC.* Protein-free filtrates (2–10 μL containing 2–10 nmol of tryptophan deriva-

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; 2,3-$H_2$-L-Trp, 2,3-dihydro-L-tryptophan; HPLC, high-performance liquid chromatography; $^{19}$F NMR, fluorine-19 nuclear magnetic resonance.

[2] We thank Dr. Esmond E. Snell for a gift of *E. coli* B/1t7-A and Dr. Syed Ashrafuddin Ahmed for help in preparing the tryptophanase (R. S. Phillips and S. A. Ahmed, unpublished results).

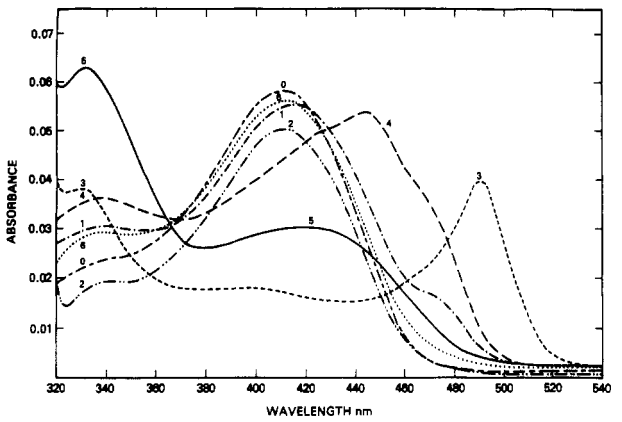



FIGURE 1: Effects of 5-fluorotryptophan compounds on absorption spectra of $\alpha_2\beta_2$ complex of tryptophan synthase. Spectra of the $\alpha_2\beta_2$ complex (0.01 mM $\alpha\beta$ protomer in 0.1 M potassium phosphate, pH 7.8, containing 2 mM EDTA) were recorded before (curve 0) and after addition of 1 mM 5-fluoro-L-tryptophan (curve 1), (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan (curve 2), (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (curve 3), 5-fluoro-D-tryptophan (curve 4), (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan (curve 5), and (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan (curve 6). Corrected spectrophotometric titration data are given in Table I.

tives) were applied to a 4.6 mm × 150 mm $C_{18}$ column and eluted at 0.7 mL/min with 0.05 M sodium dihydrogen phosphate, pH 6.0, containing 10% methanol. Peaks were located by absorbance at 254 nm. The relative concentrations of diastereoisomers were determined from the peak heights. Isomers A and B of 2,3-dihydro-5-fluorotryptophan eluted at 10 and 12 min, respectively. Isomers A and B of 2,3-$H_2$-Trp eluted at 8.5 and 9.5 min, respectively. For stereochemical assignments, see above.

*Analysis of* L-*Tryptophan by Difference Spectroscopy after Conversion to Indole by Tryptophanase.* Protein-free filtrates (0.2 mL containing 0–200 nmol of L-tryptophan) were diluted were 2.0 mL of 0.1 M potassium phosphate buffer, pH 7.8, containing 2 mM EDTA. A solution of tryptophanase (0.12 mg/mL) was prepared in the same buffer. Difference spectra were measured between two split cuvets (Hellma) with a total path length of 0.88 cm, each containing 1.0 mL of L-tryptophan solution and 1.0 mL of tryptophanase. Spectra were recorded at 37 °C before and 60 min after mixing the sample cuvet. The concentration of L-tryptophan was determined from the difference absorbance at 290 nm between L-tryptophan and indole ($\Delta\epsilon_{290nm}$ = 1.85 $mM^{-1}$ $cm^{-1}$) (Higgins et al., 1979). No change in difference absorbance was obtained with a control solution containing D-tryptophan.

*$^{19}F$ NMR Spectra.* $^{19}F$ NMR spectra were recorded at 282.4 MHz on a Varian XL-300 spectrometer with a 5-mm broad-band switchable probe. All spectra were recorded without proton decoupling. In general, a 6000-Hz spectral width was acquired in 4K data points with 45° pulse angle and 1.5-s repetition time. A 5-Hz exponential line broading was used prior to Fourier transformation for enzyme samples. Buffer for $^{19}F$ NMR measurements contains 20% $D_2O$ for field/frequency lock. Sample temperatures were controlled at 22 ± 1 °C. $^{19}F$ chemical shift was reported in ppm with respect to 2% trifluoroacetic acid in $D_2O$ contained in a capillary.

## RESULTS

*Absorption Spectra and Spectrophotometric Titrations of $\alpha_2\beta_2$ Complex of Tryptophan Synthase with 5-Fluorotryptophan Compounds.* Figure 1 shows that addition of 5-fluoro-L-tryptophan (curve 1), 5-fluoro-D-tryptophan (curve 4), or (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (curve 3) to the $\alpha_2\beta_2$ complex (curve 0) results in spectral changes with formation of new bands at 476, 445 or 494 nm, similar to those previously reported for the corresponding compounds lacking the 5-fluoro group (Miles, 1980; Lane & Kirschner, 1981; Phillips et al., 1984, 1985a,b). Addition of (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan (curve 5) results in a large decrease in absorbance at 412 nm and formation of a shoulder at 460 nm and a peak at 330 nm. Addition of (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan (curve 2) or (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan (curve 6) results in small decreases in absorbance at 412 nm. These spectral changes can be used for spectrophotometric titrations to obtain equilibrium constants for binding of these compounds (Table I) as described previously (Tanizawa & Miles, 1983; Phillips et al., 1984, 1985a). 5-Fluoro-D-tryptophan, (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan, and (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan bind strongly and have very low dissociation constants near 3 μM; the dissociation constant for 5-fluoro-L-tryptophan (30 μM) is 10-fold higher. Since the dissociation constants for (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan (600 μM) and for (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan (500 μM) are 160–200-fold higher, the analogues are bound much more weakly. Since addition of these two weak binding diastereoisomers (compounds **2** and **6**) produces different spectral changes (Figure 1) and different maximum changes in millimolar extinction coefficients at 412 nm than does addition of the two tight binding diastereoisomers (compounds 3 and 5), compounds **2** and **6** must actually bind rather than contain small amounts of contaminating diastereoisomers.

Table I: Binding Constants of Tryptophan Compounds for $\alpha_2\beta_2$ Complex of Tryptophan Synthase[a]

| compd | $[S]_{0.5}$ (μM) | change measured[b] | $\Delta\epsilon_{412}$ (mM)[c] |
|---|---|---|---|
| 5-fluoro-L-tryptophan (**1**) | 30 | $A_{476} - A_{412}$ | 1.2 |
| (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan (**2**)[e] | 600 | $A_{412}$ | 1.0 |
| (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (**3**)[e] | 3 | $A_{494} - A_{412}$ | 5 |
| 5-fluoro-D-tryptophan (**4**) | 3 | $A_{470} - A_{412}$ | 2.2 |
| (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan (**5**)[e] | 3 | $A_{412}$ | 4 |
| (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan (**6**)[e] | 500 | $A_{412}$ | <1.0 |
| (3*R*)-2,3-dihydro-L-tryptophan (**7**)[e] | 600[d] | $A_{412}$ | |
| (3*S*)-2,3-dihydro-L-tryptophan (**8**)[e] | 5[d] | $A_{494} - A_{412}$ | |
| L-tryptophan | 80[d] | $A_{474} - A_{412}$ | |
| D-tryptophan | 30[f] | $A_{460}$ | |

[a] Half-saturation ($[S]_{0.5}$) was determined by spectrophotometric titrations indicated from Hill plots as described under Materials and Methods. [b] The indicated change in absorbance at 412 nm or the difference between absorbance at an absorbance peak and at 412 nm was measured for each compound. [c] The maximum change in millimolar extinction coefficient at 412 nm at saturating compound was determined from the spectrophotometric titration data as described under Materials and Methods. [d] From Phillips et al. (1985a). [e] For stereochemical assignment, see Materials and Methods and Phillips et al. (1985a). [f] Tanizawa & Miles (1983).

*$^{19}F$ NMR Spectra of (3S)-2,3-Dihydro-5-fluoro-*L-*tryptophan in the Presence and Absence of $\alpha_2\beta_2$ Complex.* The $^{19}F$ NMR spectrum of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan exhibits a resonance centered at −48.74 ppm upfield from the trifluoroacetic acid (Figure 2, trace A). When the $\alpha_2\beta_2$ complex is present at a molar concentration ratio of 1:1.1, two spectral changes are noted: (1) a new peak with a line width of 180 Hz appears at −52.00 ppm (Figure 2, trace B), and (2) the intensity of the low-field peak (−48.74 ppm) gradually

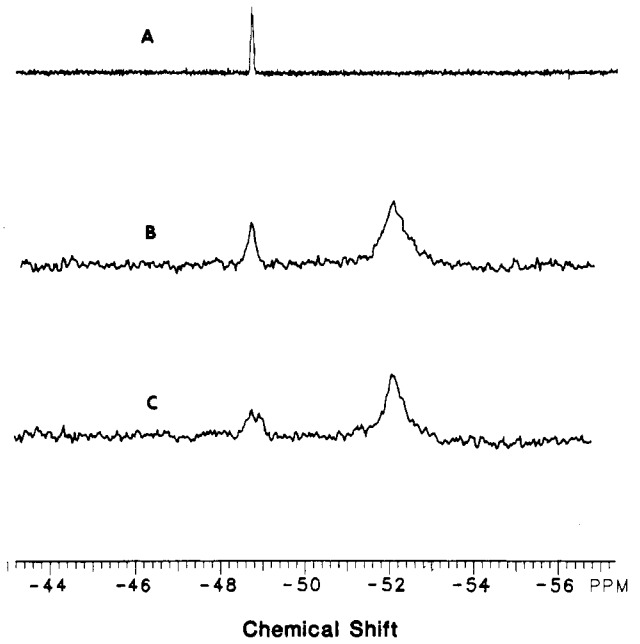



FIGURE 2: $^{19}F$ NMR spectra of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan in the presence and absence of the $\alpha_2\beta_2$ complex: (trace A) 2 mM (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan in buffer ($^3J_{FH}$ = 7.2 Hz and $^4J_{FH}$ = 2.0 Hz); (trace B) 1.55 mM (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and1.38 mM $\alpha_2\beta_2$ complex (total acquisition time = 2 h); (trace C) same sample as trace B but spectrum was taken 24 h later.

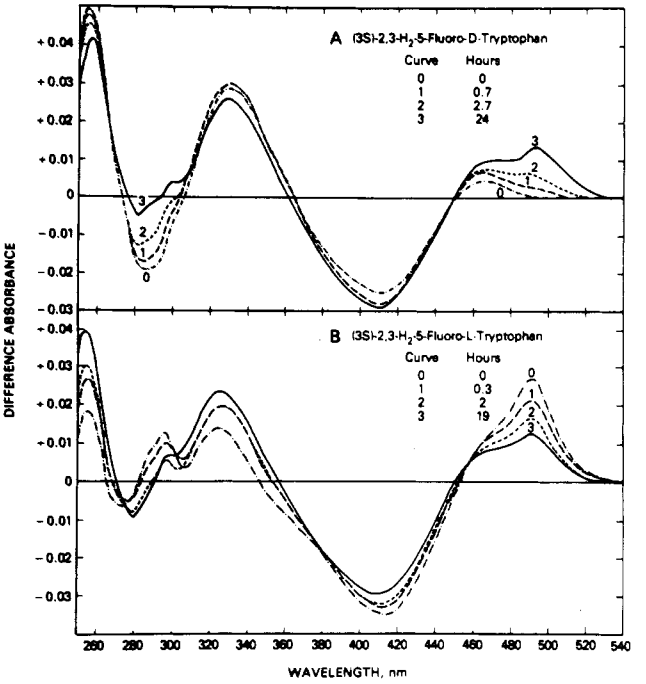



FIGURE 3: Evidence from difference absorption spectra for epimerization of (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan (A) and of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (B) by $\alpha_2\beta_2$ complex. Difference spectra were measured in split cuvets (Hellma) with a total path length of 0.88 cm. The initial concentrations of $\alpha\beta$ promoter was 0.02 mM before mixing 1.0 mL of enzyme with 1.0 mL of dihydrotryptophan compound. Concentratioyns of dihydotryptophan compounds were 0.2 mM before mixing. All solutions contained 0.1 M potassium phosphate, pH 7.8, and 2 mM EDTA. Additional spectra were made at the indicated hours after mixing at 21 °C.

decreases with concomitant appearance of an adjacent new peak at −48.90 ppm. The resonances at −48.74 and −48.90 ppm have approximately the same intensity after 24 h (Figure 2, trace C). We assign the broad peak at −52.00 ppm to enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan, which is in slow exchange with free (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (−48.74 ppm) on the NMR time scale. We estimate the maximum off-rate for the enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan as 920 $s^{-1}$.

The assignment of the resonance at −52.00 ppm to enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan is supported by the following two experiments. In the first experiment, a $^{19}F$ NMR spectrum of a mixture of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and $\alpha_2\beta_2$ complex (5:1 molar ratio) was taken to ensure the existence of the broad peak at −52.00 ppm. The mixture was then centrifuged in an Amicon ultrafiltration membrane (CF25) to yield a protein-free filtrate and a residual solution containing concentrated protein. As expected, the broad peak at −52.00 ppm ascribed to enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan appears in the concentrated protein solution but not in the protein-free filtrate. The peaks at −48.74 and −48.90 ppm ascribed to free (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and free (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan (see below for assignment), respectively, were present in both solutions. In a second experiment, excess D-tryptophan (18 mM), a strong inhibitor with $[S]_{0.5}$ = 30 $\mu$M (see below), was added to the sample used in trace C of Figure 2. The relative intensity of the peak at −48.74 ppm due to free (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan increased from 23 to 80%, while the peak at −52.00 ppm decreased from 77 to 20%, indicating that enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan had been displaced by the excess D-tryptophan.

The decrease in intensity of the resonance at −48.74 ppm and the increase in the intensity of the peak at −48.90 ppm suggest a slow conversion of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan to a new product, which is identified as (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan on the basis of its $^{19}F$ chemical shift (48.88 ppm, data not shown). This conversion by epimerization is confirmed by changes in difference absorption spectra of the enzyme–inhibitor complexes and by HPLC of the free ligands (see below).

*Difference Absorption Spectra after Mixing $\alpha_2\beta_2$ Complex with (3S)-2,3-Dihydro-5-fluoro-D-tryptophan or with (3S)-2,3-Dihydro-5-fluoro-L-tryptophan.* The initial difference absorption spectra (curves 0) obtained at time zero in Figure 3 are quite different, since the D- and L-amino acids form distinct spectral intermediates with the $\alpha_2\beta_2$ complex (see also curves 3 and 5, Figure 1). However, slow increases in difference absorbance at 494 and at 285 nm and decreases at 325 nm are observed in Figure 3A, whereas somewhat faster decreases in difference absorbance at 494 and at 285 nm and increases at 325 nm are observed in Figure 3B. The final spectra observed after 19–24 h (curves 3, Figure 3) are very similar to each other, suggesting that approximately equal amounts of D and L ligands are bound after 24 h, that is, that the two amino acids are being interconverted by epimerization.

*Time Course of Epimerization of (3S)-2,3-Dihydro-5-fluoro-L-tryptophan and (3S)-2,3-Dihydro-5-fluoro-D-tryptophan.* When the $\alpha_2\beta_2$ complex is incubated with (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan, the changes in absorbance at 490 nm and in the amount of L-amino acid in the protein-free filtrate show the same time course and approach 50% after 46 h (Figure 4, bottom). In contrast, incubation of the $\alpha_2\beta_2$ complex with (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan results in a more rapid decrease in absorbance at 490 nm than in the disappearance of L-amino acid; both values converge and reach 50% after 46 h (Figure 4, top) (see Discussion).

Control experiments done under the same conditions (Table II) show that the epimerization reaction is enzyme-dependent

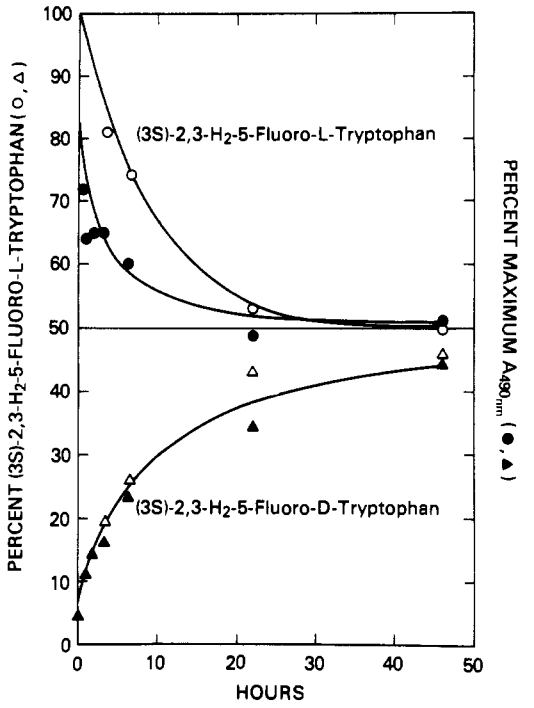



FIGURE 4: Time course of spectral changes and of epimerization on incubation of $\alpha_2\beta_2$ complex with (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and with (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan. (3*S*)-2,3-Dihydro-5-fluoro-L-tryptophan or (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan (1.0 mM in 0.1 M potassium phosphate, pH 7.8, containing 2 mM EDTA) was incubated at 21 °C with $\alpha_2\beta_2$ complex (0.1 mM) in a final volume of 0.5 mL. The absorbance of each solution at 490 nm was determined at intervals. The percent of the maximum absorbance at 490 nm (0.34) observed immediately after addition of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan is plotted with open symbols. Aliquots (25 μL) were filtered at intervals through Amicon YMT filters in MPS-1 micropartition systems to remove protein. The filtrate was assayed for the diastereoisomers by HPLC.

Table II: Epimerization of Dihydrotryptophan Compounds by $\alpha_2\beta_2$ Complex of Tryptophan Synthase[a]

| compd added | $\alpha_2\beta_2$ complex | HPLC peaks found | % L | % D |
|---|---|---|---|---|
| (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan | + | A + B | 50 | 50 |
| (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan | − | B | 100 | 0 |
| (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan | + | A + B | 50 | 50 |
| (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan | − | A | 0 | 100 |
| (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan | + | A | 100 | 0 |
| (3*R*)-2,3-dihydro-5-fluoro-D-tryptophan | + | B | 0 | 100 |
| (3*S*)-2,3-dihydro-L-tryptophan | + | A + B | 50 | 50 |

[a] The indicated dihydrotryptophan compound (1.0 mM in 0.1 M potassium phosphate, pH 7.8, containing 2 mM EDTA) was incubated in the presence or absence of $\alpha_2\beta_2$ complex (0.1 mM) at 20 °C in a total volume of 0.1 mL. After 48 h the solutions were filtered through Amicon YMT filters in MPS-1 micropartition systems to remove protein. The filtrate was assayed for the diastereoisomers by HPLC.

and does not occur with the two diastereoisomers which bind weakly. Since (3*S*)-2,3-$H_2$-L-Trp is epimerized under the same conditions, the presence of the 5-fluoro group is not necessary for the reaction.

*$^{19}$F NMR Spectra of 5-Fluoro-D-tryptophan and of 5-Fluoro-L-tryptophan in the Presence and Absence of $\alpha_2\beta_2$ Complex.* The $^{19}$F NMR spectrum of 5-fluoro-D-tryptophan or of 5-fluoro-L-tryptophan displays a resonance centered at

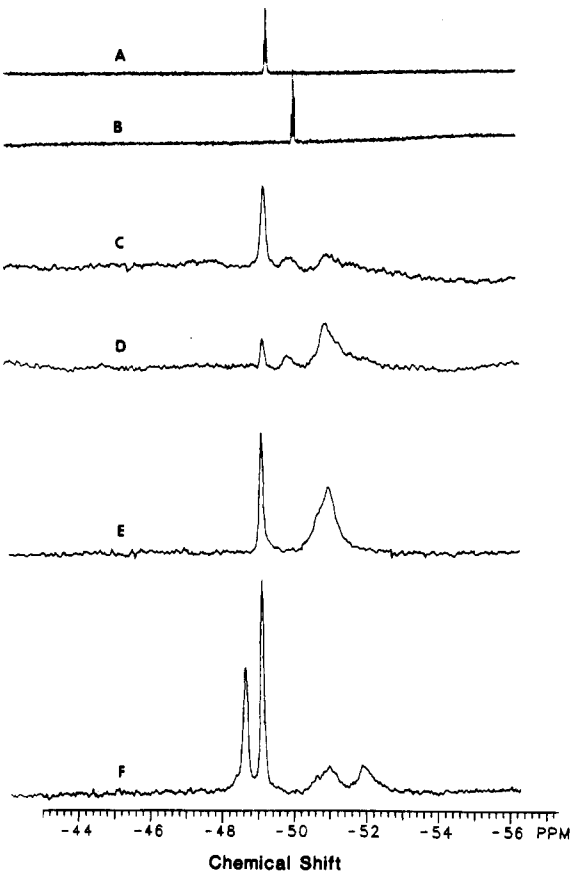



FIGURE 5: $^{19}$F NMR spectra of 5-fluoro-D-tryptophan and of 5-fluoro-L-tryptophan in the presence or absence of the $\alpha_2\beta_2$ complex: (trace A) 2 mM 5-fluoro-L-tryptophan (5-fluoro-D-tryptophan gives the same spectrum); (trace B) 2 mM 5-fluoroindole; (trace C) 1.1 mM 5-fluoro-L-tryptophan and 1.0 mM $\alpha_2\beta_2$ complex (spectrum shown about 12 h after sample preparation); (trace D) same as trace C but spectrum was taken about 3 days later; (trace E) 1.48 mM 5-fluoro-D-tryptophan and 1.39 mM $\alpha_2\beta_2$ complex; (trace F) same as trace E after addition of 1.48 mM (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan.

−49.24 ppm (Figure 5, trace A). When the $\alpha_2\beta_2$ complex and 5-fluoro-D-tryptophan are mixed in a molar ratio of 1:1.1, a second resonance consisting of about 70% of the total fluorine signal appears at −51.00 ppm with a line width of 170 Hz (Figure 5, trace E). This signal is ascribed to the enzyme-bound 5-fluoro-D-tryptophan. No further spectral change was noticed after incubation over a period of 6 days. Unlike 5-fluoro-D-tryptophan, 5-fluoro-L-tryptophan shows a time-dependent $^{19}$F NMR spectrum when the $\alpha_2\beta_2$ complex is present (Figure 5, traces C and D). The $^{19}$F NMR spectrum of 5-fluoro-L-tryptophan initially shows a single resonance at −49.24 ppm with no indication of the enzyme-bound substrate (data not shown). The intensity of this signal then slowly decreases with concomitant appearance of two new signals at −49.95 and −51.00 ppm (Figure 5, traces C and D), suggesting the formation of two new products. The peak at −49.95 ppm is probably due to a weakly bound 5-fluoroindole product, since the $^{19}$F NMR spectrum of 5-fluoroindole shows a resonance peak at the same position, both in the absence (Figure 5, trace B) and in the presence (data not shown) of the $\alpha_2\beta_2$ complex. The peak at −51.00 ppm is probably due to strongly enzyme-bound 5-fluoro-D-tryptophan, which has a signal at this position (see Figure 5, trace E). After incubation for 3 days, about 20% of 5-fluoro-L-tryptophan is converted to the product presumed to be 5-fluoroindole and 60% to the product presumed to be enzyme-bound 5-fluoro-D-tryptophan. The for-

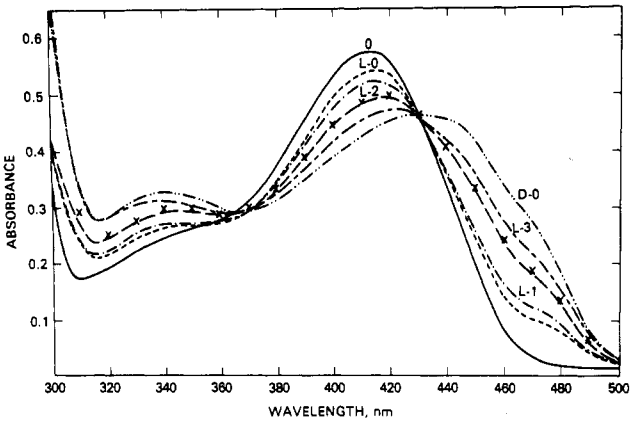



FIGURE 6: Spectroscpic evidence for racemization of 5-fluoro-L-tryptophan. Effects of 5-fluoro-L-tryptophan and of 5-fluoro-D-tryptophan on the absorption spectra of the $\alpha_2\beta_2$ complex (0.1 mM $\alpha\beta$ protomer in 0.1 M potassium phosphate buffer, pH 7.8, containing 2 mM EDTA) were recorded at 21 °C before (curve 0) and after addition of 5-fluoro-L-tryptophan (curve L-0) or 5-fluoro-D-tryptophan (curve D-0) to a final concentration of 0.1 mM. Additional spectra were recorded at the following times after addition of 5-fluoro-L-tryptophan: 2 (curve L-1), 21 (curve L-2), and 53 h (curve L-3). The calculated average absorbance of curve L-0 and curve D-0 (the absorbance expected for a 50:50 mixture of enzyme-bound D and L compounds) is shown by (×) at 10-nm intervals. The absorption spectrum in the presence of 5-fluoro-D-tryptophan did not change after 24, 48, or 72 h.

mation of these two products is supported by spectroscopic evidence [see below and Ahmed et al. (1986b)].

The induced upfield shift for enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan ($\Delta\delta$ = 3.26 ppm) (Figure 2, trace B) is quite different from that for enzyme-bound 5-fluoro-D-tryptophan ($\Delta\delta$ = 1.76 ppm) (Figure 5, trace E). This difference indicates that (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and 5-fluoro-D-tryptophan bind somewhat differently to the $\alpha_2\beta_2$ complex, either to different sites or to the same site in different orientations or conformations. The occurrence of two different sites can be ruled out by the following experiment. When (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (1.48 mM) was added into the mixture of 5-fluoro-D-tryptophan (1.48 mM) and $\alpha_2\beta_2$ complex (1.39 mM) (in Figure 5, trace E), about half of the peak intensity at −51.00 ppm (enzyme-bound 5-fluoro-D-tryptophan) was displaced by a peak at −52.00 ppm [enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan] (Figure 5, trace F). This result indicates that (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and 5-fluoro-D-tryptophan bind competitively at the same site and is consistent with kinetic results (see Discussion). We therefore conclude that the large difference in the upfield shift between these two substrates must be due to local differences in conformation or orientation in the common binding site for these two substrates.

*Spectroscopic Evidence for Conversion of Enzyme-Bound 5-Fluoro-L-tryptophan to Enzyme-Bound 5-Fluoro-D-tryptophan.* Incubation of the $\alpha_2\beta_2$ complex with approximately equimolar 5-fluoro-L-tryptophan resulted in a gradual decrease in absorbance at 412 nm and an increase centered at 460 nm (curves L-0 to L-3, Figure 6). Incubation with 5-fluoro-D-tryptophan under the same conditions gave a characteristic spectrum (D-0, Figure 6), which did not change with time. The average absorbance of the enzyme in the presence of the L-amino acid and in the presence of the D-amino acid calculated from curves L-0 and D-0 at 10-nm intervals falls close to curve L-2, which was measured after 21 h. A subsequent curve, L-3, measured after 53 h showed further changes, suggesting that about 70% of the enzyme-bound amino acid was 5-fluoro-D-tryptophan in agreement with the $^{19}F$ NMR

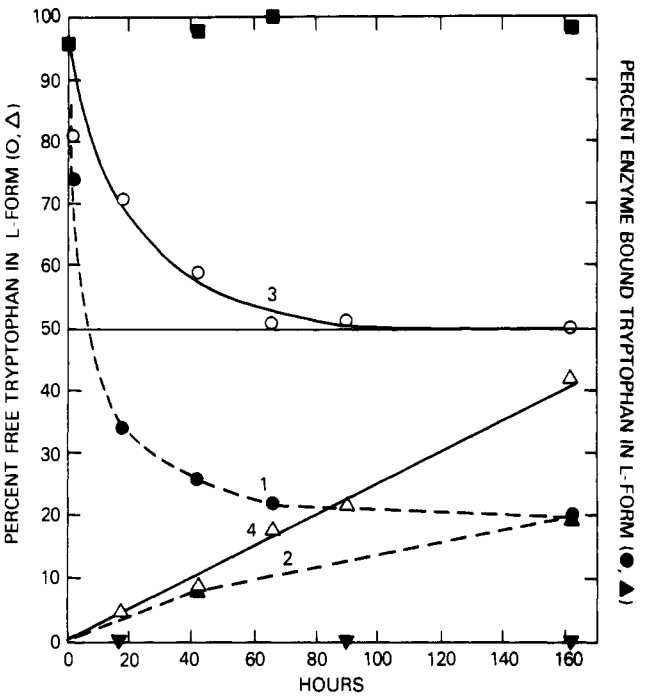



FIGURE 7: Time course of spectral changes and of racemization on incubation of $\alpha_2\beta_2$ complex with L-tryptophan or D-tryptophan. L-Tryptophan or D-tryptophan (1.0 mM in 0.1 M potassium phosphate, pH 7.8, containin 2 mM EDTA) was incubated at 37 °C with $\alpha_2\beta_2$ complex (0.1 mM) in a final volume of 3.0 mL. The visible absorption spectra and the absorbance of each solution at 460 nm was determined at intervals. The percent of the enzyme-bound tryptophan in the D form at time $X$ starting with L-tryptophan (curve 1) (●) or with D-tryptophan (curve 2) (▲) is calculated by the following relationship: (% D-Trp at $t = X$) = [$A_{460nm}$ at $t = X - A_{460nm}$ + L-Trp at $t = 0$ (0.27)]/[$A_{460nm}$ + D-Trp at $t = 0$ (0.52) − $A_{460nm}$ + L-Trp at $t = 0$ (0.27)] × 100. For example, the absorbance at 460 nm of both solutions at $t$ = 162 h = 0.47; the percent enzyme-bound D-tryptophan is 80% and the percent enzyme-bound L-tryptophan is 20%. Aliquots (0.3 mL) were filtered at intervals by centrifugation through Amicon YMT filters in MPS-1 micropartition systems to remove protein. Aliquots of the filtrate were assayed for the amount of L-tryptophan as described under Materials and Methods. The percent of free tryptophan in the L form starting from L-tryptophan (curve 3) (○) or from D-tryptophan (curve 4) (△) was calculated from this amount and from the initial concentration of free tryptophan (1.0 mM added minus 0.1 mM enzyme bound = 0.9 mM). Control solutions of 1.0 mM L-tryptophan (■) or D-tryptophan (▼) in the same buffer minus enzyme were incubated and assayed identically.

data (see above); no further changes occurred after incubation for 3 more days.

*Time Course of Racemization of L-Tryptophan and D-Tryptophan.* The effects of prolonged incubation at 37 °C of $\alpha_2\beta_2$ complex with a 10-fold molar excess of L-tryptophan or D-tryptophan upon the amount of enzyme-bound L-tryptophan (Figure 7, curves 1 and 2) and the amount of free L-tryptophan (Figure 7, curves 3 and 4) have been determined. In the experiment starting with L-tryptophan, the amount of free L-tryptophan leveled off at 50% of the initial concentration after 66 h (Figure 7, curve 3), whereas the amount of enzyme-bound L-tryptophan leveled off at 20% of bound tryptophan after 66 h (Figure 7, curve 1). Less than 5% of the initial L-tryptophan was converted to indole after 162 h (data not shown). This indole results from a very slow cleavage reaction (Ahmed et al., 1986b).

In the experiment starting with D-tryptophan, the amount of free L-tryptophan increased very slowly to 41% after 162 h (Figure 7, curve 4). The changes in the absorption spectra of the enzyme in the presence of D-tryptophan were very small and slow, since only 20% of the enzyme-bound D-tryptophan

was converted to enzyme-bound L-tryptophan after 162 h. The visible absorption spectra of the two enzyme solutions initially treated with L-tryptophan or D-tryptophan were identical after 162 h (see Discussion). Control experiments show that L-tryptophan and D-tryptophan undergo no interconversion after 162 h in the absence of enzyme.

## Discussion

*Binding of (3S)-2,3-Dihydro-5-fluoro-L-tryptophan and (3S)-2,3-Dihydro-5-fluoro-D-tryptophan.* 2,3-$H_2$-L-Trp, a structural analogue of a proposed intermediate in the synthesis of L-tryptophan, binds tightly to tryptophan synthase and forms a quinonoid intermediate with enzyme-bound pyridoxal phosphate (Phillips et al., 1984). More recent studies have demonstrated that the tryptophan synthase $\alpha_2\beta_2$ complex binds (3*S*)-2,3-$H_2$-L-Trp with high stereoselectivity (Phillips et al., 1985). This diastereoisomer was initially designated isomer B because it elutes from an HPLC column after isomer A [(3*R*)-2,3-$H_2$-L-Trp] (Phillips et al., 1985a,b). In this work we have separated the two diastereoisomers of 2,3-dihydro-5-fluoro-L-tryptophan by HPLC and find that the $\alpha_2\beta_2$ complex binds isomer B [(3*S*)-2,3-dihydro-5-fluoro-L-tryptophan] with high stereoselectivity, as expected (Table I). In addition, we have now prepared and separated the diastereoisomers of 2,3-dihydro-5-fluoro-D-tryptophan and have examined their interactions with the $\alpha_2\beta_2$ complex of tryptophan synthase, as well as the interaction of 5-fluoro-L-tryptophan and 5-fluoro-D-tryptophan.

Spectra and difference spectra of pyridoxal phosphate enzymes with substrates and analogues can be used to identify Schiff base intermediates and intermediates that result from a catalytic step, such as the removal of an $\alpha$-proton to form a quinonoid intermediate. Addition of 5-fluoro-L-tryptophan and (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan to the $\alpha_2\beta_2$ complex of tryptophan synthase results in absorption bands at 476 and 494 nm (Figure 1) identical with those ascribed to quinonoid intermediates for the analogous compounds without fluorine (Phillips et al., 1984). Addition of 5-fluoro-D-tryptophan results in an intermediate absorbing at 446 nm with a shoulder near 470 nm (Figure 1). The identical spectrum observed with D-tryptophan was previously ascribed to the Schiff base since D-tryptophan, unlike L-tryptophan, was not observed to undergo $\alpha$-proton exchange (Miles, 1980). However, the large upfield shift in this spectrum suggests that it may be due to a quinonoid intermediate. Such an intermediate might be formed in the absence of measurable $\alpha$-proton exchange if the $\alpha$-proton were removed and tightly bound to the enzyme.

Addition of (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan results in an unusual spectrum with a peak at 332 nm and a broad peak from 400 to 480 nm. The second broad peak may reflect the presence of some quinonoid intermediate. The spectra of the weakly bound (3*R*)-2,3-dihydro-5-fluoro-L- and -D-tryptophans differ only slightly from the spectrum of the enzyme alone and probably result from the formation of some Schiff base intermediate. A comparison of the spectra (Figure 1) with the binding constants suggests that the tryptophan derivatives that form a quinonoid intermediate bind tightly, whereas derivatives that only form a Schiff base bind weakly.

Tryptophan synthase $\alpha_2\beta_2$ complex has been previously shown to bind D-tryptophan more tightly than L-tryptophan (Miles, 1980; Lane & Kirschner, 1981; Tanizawa & Miles, 1983); it was thus not surprising that tryptophan synthase should also bind one of the diastereoisomers of 2,3-dihydro-5-fluoro-D-tryptophan (Table I and Figure 1). It is somewhat surprising that the $\alpha_2\beta_2$ complex should bind (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan very tightly and with high stereoselectivity, since the enzyme had not been expected to form a quinonoid intermediate with this D-amino acid.

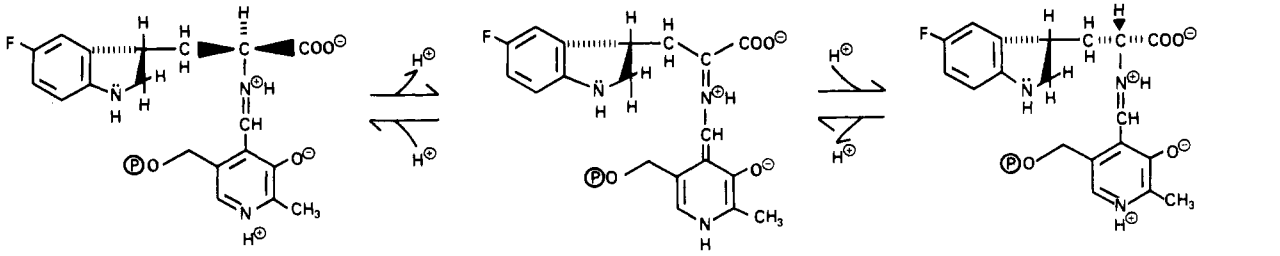
Conversion of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan by inversion at C-$\alpha$ would yield (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan. This compound is a diastereoisomer with different solubility and would be expected to elute from an HPLC column in the same position as its enantiomer, (3*R*)-2,3-dihydro-5-fluoro-L-tryptophan (isomer A). Thus, we find that the stereochemistry at C-3 of the indoline ring is critical for binding; the (3*S*)-L- and (3*S*)-D isomers bind strongly with $K_I$ values of about 3 $\mu$M (Table I), whereas the (3*R*)-L-and (3*R*)-D isomers bind about 200-fold less strongly. Our finding indicates that the $\alpha_2\beta_2$ complex binds the indoline ring of these L- and D-amino acids at the same site. This result is consistent with our previous suggestion that the indole rings of D- and L-tryptophan share the same binding site in tryptophan synthase (Miles, 1980; Tanizawa & Miles, 1983).

*Interconversion of (3S)-2,3-Dihydro-5-fluoro-L-tryptophan and (3S)-2,3-Dihydro-5-fluoro-D-tryptophan by Epimerization.* Our first evidence for an epimerization reaction came from the observation of two adjacent $^{19}F$ NMR peaks after prolonged incubation of a high concentration of $\alpha_2\beta_2$ complex with (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (Figure 2, trace C). Although L- and D-amino acids with a single chiral site cannot be distinguished by $^{19}F$ NMR or separated by HPLC, diastereoisomers of amino acids with two chiral centers (see above) have distinctive $^{19}F$ NMR spectra and solubility properties. The epimerization reaction was confirmed by changes in the absorption spectra of the enzyme (Figures 3 and 4) and by HPLC of the free ligand. The experiment in Figure 4 demonstrates that both (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan and (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan are converted to a 50:50 mixture of these two compounds when a 10:1 molar ratio of amino acid to enzyme is incubated for 46 h. Control experiments (Table II) show that the epimerization is enzyme-dependent and does not occur with the weakly bound isomers.

*Conversion of Enzyme-Bound 5-Fluoro-L-tryptophan to Enzyme-Bound 5-Fluoro-D-tryptophan and Interconversion of L-Tryptophan and D-Tryptophan by Racemization.* Since the $^{19}F$ NMR studies indicated that the $\alpha_2\beta_2$ complex converted 5-fluoro-L-tryptophan to enzyme-bound 5-fluoro-D-tryptophan, we have followed the absorption spectra of the $\alpha_2\beta_2$ complex with 5-fluoro-D-tryptophan and with 5-fluoro-L-tryptophan (Figure 6). The results are consistent with conversion of enzyme-bound 5-fluoro-L-tryptophan to enzyme-bound 5-fluoro-D-tryptophan but fail to detect the reverse reaction. However, when the $\alpha_2\beta_2$ complex is incubated with a 10-fold molar excess of L-tryptophan or D-tryptophan, each of these amino acids is converted to close to a 50:50 mixture of these two isomers after 162 h at 37 °C. The visible absorption spectra of the two enzyme incubation mixtures are identical after 162 h and indicate that the ratio of enzyme-bound D-tryptophan to enzyme-bound L-tryptophan is 4:1 under these conditions. This result is the consequence of tighter and therefore preferential binding of D-tryptophan [see Table I and Tanizawa & Miles (1983)]. In contrast, the L and D isomers of (3*S*)-2,3-dihydro-5-fluorotryptophan have identical binding constants (Table I) and are equally bound to tryptophan synthase after the epimerization reaction reaches completion (Figure 4).

The initial rate of epimerization of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan [estimated from the data in Figure 4 to be 0.032 nmol $min^{-1}$ (nmol of $\alpha\beta)^{-1}$ at 37 °C][3] is about 13-fold

Scheme I



I
SCHIFF BASE OF
(3 S)-2,3-$H_2$-5-FLUORO-L-TRYPTOPHAN

II
QUINONOID INTERMEDIATE
$A_{494\ nm}$

III
SCHIFF BASE OF
(3 S)-2,3-$H_2$-5-FLUORO-D-TRYPTOPHAN

faster than the initial rate of racemization of L-tryptophan [estimated from the data in Figure 7 to be 0.0025 nmol $min^{-1}$ (nmol of $\alpha\beta)^{-1}$ at 37 °C]. The faster rate is close to the very slow rate of L-tryptophan cleavage [0.03 nmol $min^{-1}$ (nmol of $\alpha\beta)^{-1}$] at 37 °C but about $10^4$ times slower than the rate of synthesis of L-tryptophan from L-serine and indole, a physiologically significant reaction of tryptophan synthase (Ahmed et al., 1986b). The rates of various reactions catalyzed by the $\beta_2$ subunit and $\alpha_2\beta_2$ complex of tryptophan synthase have been tabulated and discussed in the accompanying paper (Ahmed et al., 1986b). The observation that the isomerization reaction is faster with the unnatural substrate [(3*S*)-2,3-dihydro-5-fluoro-L-tryptophan] than with the natural substrate (L-tryptophan) is consistent with the statement of Snell (1985): "Such abortive reactions become more prominent when analogue substrates or coenzymes, which produce minor distortions of enzyme structure and thereby alter slightly the spatial alignment of catalytic groups, are used." [See also Miles (1986)].

*Mechanism of Epimerization.* A mechanism for the epimerization of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (Scheme I) is based on knowledge of the mechanism and stereochemistry of other pyridoxal phosphate dependent racemases (Floss & Vederas, 1982). The L-amino acid forms a Schiff base with pyridoxal phosphate (I). Removal of the $\alpha$-proton produces a resonance-stabilized carbanion at C-2 (quinonoid intermediate II). Protonation of C-$\alpha$ at the opposite face yields a Schiff base of the D-amino acid with pyridoxal phosphate (III). The absorption spectra of the $\alpha_2\beta_2$ complex recorded immediately after addition of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (Figure 1 and 3B) show a peak at 494 nm, which is attributed to the quinonoid intermediate II (Phillips et al., 1984).

Since tryptophan synthase catalyzes incorporation of tritium into a related compound, oxindolyl-L-alanine, the enzyme can form quinonoid intermediates (Phillips et al., 1984). Since the quinonoid species II with absorbance at 494 nm is observed in steady-state spectra with (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan but not with (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan, we conclude that this intermediate is formed by a rapid removal of the $\alpha$-proton from the L-amino acid I and that this intermediate II accumulates before a slow reprotonation step to form the Schiff base of (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan III. In the epimerization of (3*S*)-2,3-$H_2$-D-Trp in the reverse direction, the removal of the $\alpha$-proton is slow; the quinonoid intermediate II is then rapidly protonated to form I. It seems logical that the step that is "unnatural", i.e., protonation or deprotonation from the "wrong side", should be a very slow step. These conclusions are consistent with our previous finding (Miles, 1980) that tryptophan synthase catalyzes incorporation of deuterium into L-tryptophan but not (detectably) into D-tryptophan. The removal of the $\alpha$-proton of D-tryptophan or the reprotonation of the quinonoid formed from 5-fluoro-L-tryptophan (see Figure 6) must be a very slow step.

*Enzyme-Bound vs. Free Ligands.* In the epimerization of a 10-fold molar excess of (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (Figure 4), the spectrum of the enzyme ($A_{490nm}$) indicates that the enzyme-bound L-amino acid is converted to enzyme-bound D-amino acid at a much more rapid rate than the overall rate of conversion of the L-amino acid to a 50:50 mixture of D- and L-amino acids. This would be the expected result if the rate of release of the D-amino acid product is slower than the interconversion of the enzyme-bound D- and L-amino acids.

The proposal that (3*S*)-2,3-dihydro-5-fluoro-D-tryptophan is slowly released is consistent with our finding that this D-amino acid is a slow binding inhibitor of tryptophan synthase (unpublished results). Lane and Kirschner (1981) have found that D-tryptophan is bound more slowly than is L-tryptophan and that the isomerization of tryptophan synthase following binding D-tryptophan is slower than after binding L-tryptophan. D-Tryptophan also protects tryptophan synthase against reduction by sodium borohydride (Miles et al., 1982). These several effects of D-amino acids upon tryptophan synthase are evidence that tryptophan synthase undergoes a conformational change after binding this D-amino acid. Such a conformational change upon binding the D-amino acid might facilitate protonation from the wrong side (see below). A conformational change after binding the D-amino acid could explain our finding of quite different induced upfield shifts in the $^{19}F$ NMR spectra for enzyme-bound (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan ($\Delta\delta$ = 3.26 ppm) and for enzyme-bound 5-fluoro-D-tryptophan ($\Delta\delta$ = 1.76 ppm). Evidence that D-tryptophan and its analogues and L-tryptophan and its analogues bind at the same site in tryptophan synthase comes from the $^{19}F$ NMR studies showing competition between 5-fluoro-D-tryptophan and (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan for binding (Figure 5F) and also from previous kinetic studies. Phillips et al. (1984) demonstrated that 2,3-$H_2$-L-Trp and L-tryptophan are competitive with L-serine in the overall reaction of tryptophan synthase. Miles (1980) demonstrated that D-tryptophan competes with L-tryptophan for binding to the $\alpha_2\beta_2$ complex as determined by spectrophotometric titration studies. A model for the binding of L-tryptophan and D-tryptophan at the same site of tryptophan synthase has been proposed by Miles (1980) and by Tanizawa and Miles (1983) and is consistent with studies of modification of an active site arginine-148 of the $\beta_2$ subunit (Tanizawa & Miles, 1983).

*Mechanism, Stereochemistry, and Significance of Slow Side Reactions.* Model studies have shown that a wide variety of reactions catalyzed by pyridoxal phosphate dependent enzymes

[3] Calculated from data at 21 °C assuming $Q_{10}$ = 2.

(e.g., transamination, racemization, β-elimination, β-replacement, and aldol cleavage reactions) can be duplicated by the coenzyme alone without an enzyme (Metzler et al., 1954). The enzyme accelerates the rates of pyridoxal phosphate dependent reactions and makes them more reaction specific and stereospecific by binding the substrates and coenzyme and providing catalytic groups (Snell, 1985; Breslow, 1982). Although pyridoxal phosphate enzymes are usually reaction specific and will catalyze only a single reaction or closely related reactions with a single amino acid substrate, several pyridoxal phosphate enzymes catalyze slow side reactions, including transamination reactions (Novogrodsky & Meister, 1964; Miles et al., 1968; Ahmed et al., 1985a; Miles, 1986), racemization reactions (Kumagai et al., 1970; this work), and the slow cleavage of L-tryptophan (Ahmed et al., 1986b).

Although it has been assumed that these slow reactions are enzyme-catalyzed, it is possible that the enzyme just binds the coenzyme and substrates in a specific manner and that the bond-breaking event results from the intrinsic catalytic activity of the coenzyme itself rather than from the participation of catalytic groups on the enzyme.[4] If so, this type of reaction might be classified somewhere between a true enzyme-catalyzed reaction and an "artificial enzyme" in which the coenzyme and substrate bind to a cyclodextrin (Breslow, 1982).

It should be possible to distinguish between coenzyme-catalyzed reactions and enzyme-catalyzed reactions by determining the route of proton transfer. If the reaction is catalyzed by the coenzyme alone, the proton transferred should come from the solvent. If the reaction is catalyzed by a single base on the enzyme, some direct transfer of a proton from the substrate to the product should occur (see below). In the slow transamination reaction catalyzed by the $\beta_2$ subunit of tryptophan synthase (Miles et al., 1968), the transfer of the α-proton of L-serine substrate to C-4′ of the pyridoxamine phosphate product has been demonstrated (Tsai et al., 1978). Thus, this slow transamination reaction is enzyme-catalyzed.

The removal of the α-proton in β-replacement, β-elimination, and transamination reactions catalyzed by tryptophan synthase occurs on the *si* face of the pyridoxal phosphate Schiff base (Floss & Vederas, 1982; Tsai et al., 1978; Miles et al., 1982). Although the racemization of an L-amino acid also involves initial removal of the α-proton of the L-amino acid from the *si* face to form the quinonoid II, the distinguishing feature of this reaction is protonation of this intermediate II on the *re* face to form III (Scheme I) (Floss & Vederas, 1982).

Two mechanisms have been proposed for racemization, one involving two acid/base groups on opposite sides of the coenzyme–substrate complex and one involving a single base that first abstracts a proton on one face and then adds it back either on the same or on the opposite face (Floss & Vederas, 1982). In the latter case, the base and substrate must move in relation to each other during the catalytic process (Ahmed et al., 1986a). This might involve a conformational change, as discussed above.

If the mechanism of racemization involves a single base, it may be possible to demonstrate internal return of the α-proton (see above). This mechanism of racemization has been established for three enzymes (Shen et al., 1983; Ahmed et al., 1986a). If the mechanism of racemization involves two bases or is catalyzed by the coenzyme and solvent, no internal return would occur, and it would not be possible to distinguish between these two mechanisms. This problem is currently under investigation in our laboratories.

[4] Dr. Esmond E. Snell, personal communication.

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# Role of Head Group Structure in the Phase Behavior of Amino Phospholipids. 1. Hydrated and Dehydrated Lamellar Phases of Saturated Phosphatidylethanolamine Analogues†

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ABSTRACT: Analogues of dimyristoylphosphatidylethanolamine (DMPE) have been prepared with head groups modified by N-alkylation, alkylation of carbon 2 of the ethanolamine group, or interposition of extra methylene segments between the phosphoryl and amino groups. The phases formed by these lipids in aqueous dispersions have been examined by high-sensitivity differential scanning calorimetry and Raman spectroscopy. All of the DMPE analogues examined, excepting *N*-methyl-DMPE but including *N*-ethyl-DMPE, form hydrated gel phases that are metastable with respect to a dehydrated "high-melting" solid phase that has been observed previously for DMPE itself. The properties and the conditions of formation of this high-melting phase are qualitatively distinct from those of the "subgel" phase, which is observed for dipalmitoylphosphatidylcholine and for some of the DMPE analogues examined in this study. The high-melting phases of different DMPE analogues all exhibit similarly tight packing of the acyl chains, which however do not pack according to a single type of subcell that can be universally and specifically associated with this phase. Increasing the size of the PE head group invariably decreases the melting temperature of the hydrated gel phase, even when the normal hydrogen-bonding capability of the head group is preserved. By contrast, addition of larger alkyl substituents to either the amino group or carbon 2 of the ethanolamine moiety substantially increases the transition temperature of the high-melting solid phase, indicating that the contributions of the head group to the energies of the hydrated gel and the high-melting phases are fundamentally different. Our results suggest that the head group structural requirements for a neutral phospholipid to form stable hydrated bilayers are rather stringent, a fact that may explain the overwhelming predominance of only a few such head group structures in most natural membranes.

Phosphatidylcholine (PC)[1] and phosphatidylethanolamine (PE) together constitute the majority of the total phospholipids in most animal cell membranes. These two lipids differ markedly in several key physical properties that may be important in determining the stability and the flexibility of the bilayer organization of lipids in natural membranes. Most notably, lamellar phases of PE are less strongly hydrated than are bilayers of PC (Lis et al., 1981), and aqueous dispersions of PE's can adopt nonlamellar configurations much more readily than can PC's with the same acyl composition (Cullis & de Kruijff, 1979; Dekker et al., 1983; Verkleij, 1984). These differing properties of PE and PC can have a profound influence on the stability and the interactions of bilayer membranes containing these lipids (Düzgünes et al., 1981; Sundler et al., 1981; Silvius & Gagné, 1984a,b).

The differences observed in the physical properties of PE and PC can be attributed to two basic differences in the structural characteristics of the head groups of these species. First, of course, the sheer volume of the PC head group is greater than that of PE, and moreover, the non-hydrogen

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[1] Abbreviations: *t*-Boc, *tert*-butoxycarbonyl; $C_2$-dimethyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-2′-amino-2′-methyl-1′-propanol; $C_2$-ethyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*dl*-2′-amino-1′-butanol; *dl*-$C_2$-methyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*dl*-2′-amino-1′-propanol; *l*-$C_2$-methyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*l*-2′-amino-1′-propanol; DLPE, dilauroylphosphatidylethanolamine; DM, dimyristoyl; DPPC, dipalmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid disodium salt; *N*-ethyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(ethylamino)-1′-ethanol; *N*-methyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(methylamino)-1′-ethanol; NMR, nuclear magnetic resonance; PB, 1,2-diacyl-*sn*-glycero-3-phospho-4′-amino-1′-butanol; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PP, 1,2-diacyl-*sn*-glycero-3-phospho-3′-amino-1′-propanol; $T_c$ and $T_c$(hydrated), hydrated gel to liquid-crystalline transition temperature; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; TLC, thin-layer chromatography; $T_m$(high melting), temperature of transition of a high-melting solid phase to a liquid-crystalline phase.

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