Interactions of Tryptophan Synthase, Tryptophanase, and Pyridoxal Phosphate with Oxindolyl-L-alanine and 2,3-Dihydro-L-tryptophan: Support for an Indolenine Intermediate in Tryptophan Metabolism†

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ABSTRACT: We have examined the interaction of tryptophan synthase and tryptophanase with the tryptophan analogues oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan. Since these analogues have tetrahedral geometry at carbon 3 of the heterocyclic ring, they are structurally similar to the indolenine tautomer of L-tryptophan, a proposed intermediate in reactions of L-tryptophan. Oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan are potent competitive inhibitors of both tryptophan synthase and tryptophanase, with K_I values (3–17 μM) 10–100-fold lower than the corresponding K_m or K_I values for L-tryptophan. Addition of oxindolyl-L-alanine or 2,3-dihydro-L-tryptophan to solutions of the $\alpha_2\beta_2$ complex of tryptophan synthase results in new absorption bands at 480 or 494 nm, respectively, which are ascribed to a quinonoid or α-carbanion intermediate. Spectrophotometric titration data give half-saturation values of 5 and 25 μM, which are comparable to the K_I values obtained in kinetic experiments. Our finding that both enzymes catalyze incorporation of tritium from ^3H_2O into oxindolyl-L-alanine is evidence that both enzymes form α-carbanion intermediates with oxindolyl-L-alanine. These results support the proposal that the indolenine tautomer of L-tryptophan is an intermediate in reactions catalyzed by both tryptophanase and tryptophan synthase. In addition, we have found that oxindolyl-L-alanine reacts irreversibly with free pyridoxal phosphate to form a covalent adduct.

The $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli* (EC 4.1.2.20) catalyzes reactions 1–3. Pyridoxal phosphate,

indole-3-glycerol phosphate ⇄
D-glyceraldehyde 3-phosphate + indole (1)

indole + L-serine $\xrightarrow[\text{PLP}]{}$ L-tryptophan + H_2O (2)

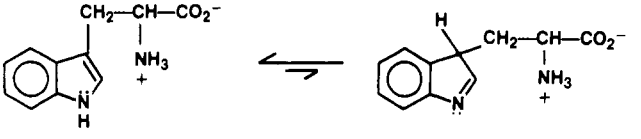
indole-3-glycerol phosphate + L-serine $\xrightarrow[\text{PLP}]{}$
L-tryptophan + D-glyceraldehyde 3-phosphate (3)

the cofactor of the β_2 subunit, is required for reactions 2 and 3. [See Miles (1979) for a review.] Kumagai & Miles (1971) noted that although the β_2 subunit alone catalyzes several pyridoxal phosphate dependent β-elimination reactions it does not catalyze the degradation of L-tryptophan to pyruvate, indole, and ammonia (reaction 4). The degradation of L-

L-tryptophan ⇄ indole + pyruvic acid + NH_3 (4)

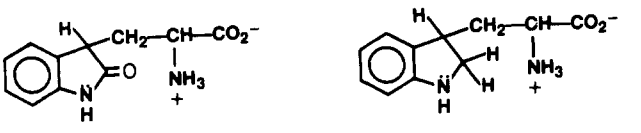
tryptophan is the primary reaction of the closely related enzyme tryptophanase (EC 4.1.99.1). The $\alpha_2\beta_2$ complex of tryptophan synthase does, however, bind L-tryptophan and forms a pyridoxal phosphate intermediate with L-tryptophan with maximum absorbance at 476 nm (Miles, 1980; Tschopp & Kirschner, 1980; Lane & Kirschner, 1981). This intermediate probably has a quinonoid structure and results from removal of the α proton from the Schiff base formed between L-tryptophan and pyridoxal phosphate. Tryptophan synthase catalyzes α-hydrogen exchange of L-tryptophan in D_2O (Tsai et al., 1978; Miles, 1980). Tryptophanase also forms an intermediate with substrates or analogues which exhibits λ_{max} at about 500 nm (Morino & Snell, 1967, 1970; Watanabe & Snell, 1977) and catalyzes α-hydrogen exchange of amino acids (Morino & Snell, 1967, 1970), even those such as alanine, which are not substrates.

The β-elimination reaction involving indole differs from most other pyridoxal phosphate dependent β-elimination reactions in that a carbon–carbon bond is cleaved. Indole would be expected to be an exceedingly poor leaving group in a β-elimination reaction that required cleavage of an unactivated vinylic carbon–carbon bond; the vinylic carbanion so produced has an estimated pK_a of 44 (Lowry & Richardson, 1978). Furthermore, indole would be expected to be a very poor nucleophile for β-replacement reactions, since it is a very weak base with a $pK_a = -3.62$ for protonation at C-3 (Hinman & Lange, 1964). Thus, enzymatic catalysis of these β-elimination and β-replacement reactions must involve an activated intermediate. It has been proposed that a pyridoxal phosphate Schiff base of the indolenine tautomer (B) of tryptophan (A)



A B

is an intermediate in the β elimination of indole catalyzed by tryptophanase (Davis & Metzler, 1972) or in the synthesis of tryptophan catalyzed by tryptophan synthase (Lane & Kirschner, 1983) (see Discussion). If the Schiff base of B is a reaction intermediate, then compounds such as oxindolyl-L-alanine (C) and 2,3-dihydrotryptophan (D), which resemble



C D

B in having tetrahedral geometry at C-3 of the ring, might then act as inhibitors of these enzymes. The results of our experiments indicate that both C and D are strong competitive inhibitors of tryptophan synthase and tryptophanase, with K_I values 10–100-fold lower than the K_I or K_m for tryptophan. These results provide experimental support for the existence

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of the indolenine tautomer of tryptophan as a reactive intermediate in these enzymatic reactions.

Materials and Methods

Materials. Pyridoxal hydrochloride was from Sigma; pyridoxal phosphate was from Sigma or P-L Biochemicals. Indole-3-glycerol phosphate was synthesized enzymatically from indole and fructose 1,6-bisphosphate plus aldolase and the $\alpha_2\beta_2$ complex of tryptophan synthase (Hardman & Yanofsky, 1965). Oxindolyl-L-alanine was prepared by oxidation of L-tryptophan with dimethyl sulfoxide and 12 N HCl in glacial acetic acid, essentially as described by Savige & Fontana (1980). Oxindole-3-propionic acid was prepared by acid hydrolysis of 1-[3-(methylpropionyl)-2-indolyl]pyridinium bromide, obtained by reaction of *N*-bromosuccinimide with methyl indole-3-propionate (Kobayashi & Inokuchi, 1964). Oxytryptamine (2-oxo-2,3-dihydrotryptamine) was prepared by reaction of tryptamine hydrochloride with dimethyl sulfoxide and 12 N HCl (Szabo-Pustav & Szabo, 1979). 2,3-Dihydro-L-tryptophan was a generous gift of Dr. John W. Daly (Daly et al., 1967). ^3H_2O (specific activity 25 mCi/g) was obtained from New England Nuclear.

Enzymes and Enzyme Assays. The tryptophan synthase $\alpha_2\beta_2$ complex from *Escherichia coli* strain W3110 *trpRcysB*Δ*trpLD102trpB*$^+$*trpA*$^+$/*F'colVBcysB*$^+$-Δ*trpLD102trpB*$^+$*trpA*$^+$ was prepared and assayed as described by Higgins et al. (1979). The *E. coli* strain was a generous gift of Dr. C. Yanofsky and of Dr. I. P. Crawford.

Reaction 3 was measured by a spectrophotometric assay coupled with glyceraldehyde-3-phosphate dehydrogenase as described by Creighton & Yanofsky (1966). The K_m for L-serine and the K_I for L-tryptophan and analogues were determined by using the assays for reaction 3 modified to contain final concentrations of L-serine from 0.2 to 2.0 mM and final concentrations of L-tryptophan and analogues from 0.05 to 0.5 mM.

Apotryptophanase was purchased from Sigma and was reconstituted at a concentration of 5 mg/mL in 0.1 M potassium phosphate, pH 8.0, containing 0.1 mM pyridoxal phosphate, 1 mM glutathione, and 25% glycerol. The commercial enzyme has a specific activity of about 0.2 μmol min^{-1} mg^{-1} under the assay conditions described below, whereas pure tryptophanase has a specific activity of 30 μmol min^{-1} mg^{-1} with *S*-methyl-L-cysteine under these conditions (Morino & Snell, 1970). Tryptophanase was assayed by the continuous spectrophotometric assay (Morino & Snell, 1970), in which the formation of pyruvate is coupled to oxidation of NADH catalyzed by lactate dehydrogenase. *S*-Methyl-L-cysteine was used as the substrate rather than L-tryptophan since the progress curves observed with L-tryptophan are markedly nonlinear, due to the strong product inhibition exhibited by indole (Morino & Snell, 1970; Kazarinoff & Snell, 1980). Enzyme was added last to an otherwise complete assay mixture. Linear, steady-state rates were measured with *S*-methyl-L-cysteine as substrate in the presence or absence of the inhibitors oxindolyl-L-alanine or 2,3-dihydro-L-tryptophan subsequent to initial lag periods. The maximum inhibition by 2,3-dihydro-L-tryptophan is not observed until after about 5 min, suggesting that this inhibitor binds slowly. That 2,3-dihydro-L-tryptophan binds slowly and reversibly is supported by our finding that when tryptophanase is preincubated with 10 μM 2,3-dihydro-L-tryptophan for 15 min before addition of substrate, the initial rate is linear and equal to the steady-state rate obtained in the presence of 2,3-dihydro-L-tryptophan without preincubation. Because of the rapid nonenzymatic reaction of oxindolyl-L-alanine with pyridoxal phosphate (see Results), the inhibition studies using this compound were carried out by adding the inhibitor immediately prior to initiation of the reaction by addition of tryptophanase.

Table I: Steady-State Kinetic Constants for Substrates or Analogues with the $\alpha_2\beta_2$ Complex of Tryptophan Synthase

substrate or analogue	K_m or K_I[a] (μM)	$[S]_{0.5}$[b] (μM)	λ_{max}[c] (nm)	$\epsilon_{\lambda_{max}}$[c] (mM^{-1} cm^{-1})
none			412	5.2
L-serine	200			
L-tryptophan	170	80	474	1.8
oxindolyl-L-alanine	6	5	480	6.0
2,3-dihydro-L-tryptophan	17	25	494	2.0

[a] K_m or K_I in reaction 3; data from Figure 1. [b] Half-saturation values ($[S]_{0.5}$) are obtained from Hill plots where log $[R/(1-R)] = 0$; data from Figure 3. [c] From absorption spectra (Figure 2) at 1 mM analogue.

Spectrophotometric Titration of the $\alpha_2\beta_2$ 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 ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol] at the indicated wavelengths was determined by use of a Cary 118 spectrophotometer at 23 °C after each addition of amino acid analogue; the observed differences between absorbance at 474 and at 412 nm (L-tryptophan), between absorbance at 480 and at 413 nm (oxindolyl-L-alanine), or between absorbance at 494 and at 412 nm (2,3-dihydro-L-tryptophan) were 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).

Results

Kinetics of Inhibition of Tryptophan Synthase by L-Tryptophan and Analogues. The experimental results of Figure 1A show that oxindolyl-L-alanine, 2,3-dihydro-L-tryptophan, and L-tryptophan are competitive with L-serine in reaction 3 with inhibition constants of 6 μM, 17 μM, and 170 μM, respectively (Table I).

Inhibition of Tryptophanase by L-Tryptophan Analogues. Oxindolyl-L-alanine was also found to be a potent inhibitor of tryptophanase, as can be seen from Figure 1B. A replot of these data (inset, Figure 1B) is linear, indicating simple competitive inhibition (Cleland, 1962). From the replot, a K_I of 2.5 μM is obtained, which is approximately 2 orders of magnitude less than the K_m for L-tryptophan (Table II). 2,3-Dihydro-L-tryptophan exhibits the slightly higher K_I value of 4.5 μM under the same conditions (Table II). These two amino acids are the most potent inhibitors of tryptophanase reported.

We also compared the ability of the amine and acid analogues of oxindolyl-L-alanine to inhibit tryptophanase (Table II). As reported by Watanabe & Snell (1977), we find that indole-3-propionic acid exhibits a K_I (0.36 mM) comparable to the K_m (0.30 mM) for L-tryptophan. Although the K_I for oxindolyl-L-alanine is about 100-fold smaller than the K_m for L-tryptophan, oxindole-3-propionic acid is a somewhat less potent inhibitor than indole-3-propionic acid, with a K_I of 0.8 mM. In agreement with the results of Watanabe & Snell (1977), we also find that the α-carboxyl group is necessary

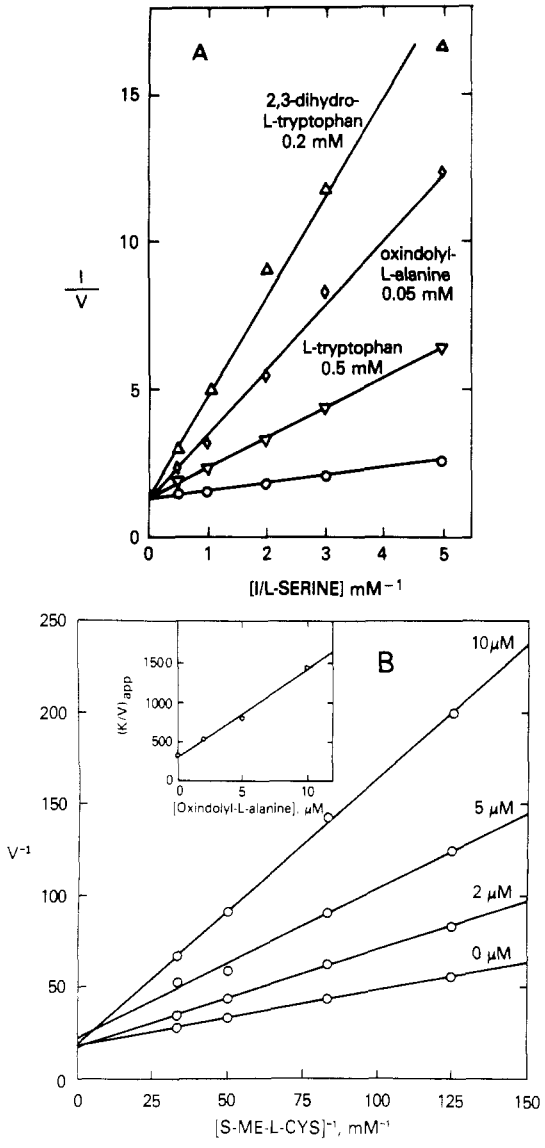


FIGURE 1: (A) Inhibition of the tryptophan synthase catalyzed synthesis of L-tryptophan from L-serine and indoleglycerol phosphate by L-tryptophan, by 2,3-dihydro-L-tryptophan, and by oxindolyl-L-alanine. Double-reciprocal plots of initial velocity vs. serine concentration at no inhibitor (○) and at the fixed concentration of L-tryptophan (0.5 mM) (▽), of oxindolyl-L-alanine (0.05 mM) (◇), or of 2,3-dihydro-L-tryptophan (0.2 mM) (△). Each assay contained 10 μg of $\alpha_2\beta_2$ complex; the velocity is the change in the absorbance at 340 nm/5 min. (B) Inhibition of tryptophanase-catalyzed elimination of *S*-methyl-L-cysteine by oxindolyl-L-alanine. Double-reciprocal plots of steady-state velocity (measured in change in absorbance at 340 nm/min) vs. concentration of *S*-methyl-L-cysteine at 0, 2, 5, and 10 μM oxindolyl-L-alanine are presented. Within experimental error, all lines intersect on the *y* axis. Inset: Replot of the slopes, $(K_m/V_{max})_{app}$, according to the equation $(K_m/V_{max})_{app} = (K_m/V_{max}) + (K_m/V_{max})[I]/K_I$. K_I determined from this analysis is 2.5×10^{-6} M.

for oriented binding, as both tryptamine and oxytryptamine are much poorer inhibitors. Thus, these data indicate that the potent inhibitory activity of oxindolyl-L-alanine is not a general characteristic of oxindoles and requires the intact amino acid side chain for expression. Furthermore, tryptophans substituted at the 2-position of the indole ring are, in general, poor inhibitors (Snell, 1975); we find no significant inhibition of either tryptophanase or tryptophan synthase by 2-chloro- or 2-bromo-L-tryptophan (Phillips & Cohen, 1983) at a concentration of 1 mM.

Detection of α-Carbanion Intermediates by Spectral Studies and by Tritium Exchange Studies. Addition of L-tryptophan, oxindolyl-L-alanine, or 2,3-dihydro-L-tryptophan to the $\alpha_2\beta_2$ complex of tryptophan synthase leads to formation of new absorption bands at 474, 480, or 494 nm, respectively (Figure 2 and Table I). Spectrophotometric titration of the enzyme with the three ligands (see Materials and Methods and Figure 3) gave binding constants that are similar in magnitude to the inhibition constants (Table I). Since absorption bands in the 470–500-nm region have been ascribed to quinonoid or α-carbanion intermediates which result from the removal of the

Table II: Steady-State Kinetic Constants for Tryptophan and Analogues with Tryptophanase

substrate or inhibitor	K_m or K_I (μM)	literature[c]
L-tryptophan	300[a]	320
indole-3-propionic acid	360[b]	420
tryptamine	2400[b]	
oxindolyl-L-alanine	2.5[b]	
oxindole-3-propionic acid	800[b]	
oxytryptamine	>1000[b]	
2,3-dihydro-L-tryptophan	4.5[b]	

[a] K_m. [b] K_I determined by using *S*-methyl-L-cysteine as substrate. [c] Watanabe & Snell (1977).

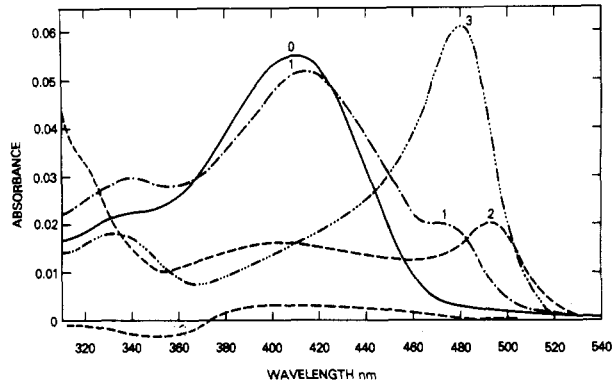


FIGURE 2: Absorption spectra of the $\alpha_2\beta_2$ complex of tryptophan synthase (0.01 mM in $\alpha\beta$ protomer in 0.1 M potassium phosphate buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol) before (curve 0) and after the addition of 1 mM L-tryptophan (curve 1), 1 mM 2,3-dihydro-L-tryptophan (curve 2), or 1 mM oxindolyl-L-alanine (curve 3). Curves 1–3 were corrected for dilution; the unnumbered curve is the base line. The absorbance of the protein at 278 nm is 0.45.

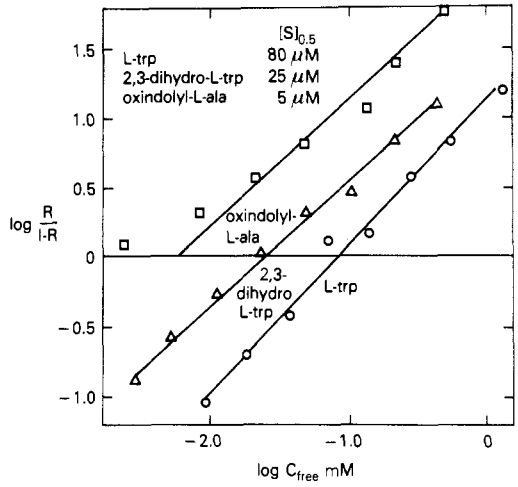


FIGURE 3: Hill plots of spectral titration data for binding of amino acids to the $\alpha_2\beta_2$ complex of tryptophan synthase (0.01 mM $\alpha\beta$) (see Materials and Methods). The data for binding of low concentrations of oxindolyl-L-alanine (below 15 μM) are poor because the concentration of enzyme used (10 μM) is higher than the dissociation constant (≃5 μM), and there is consequently very little free ligand. The slopes of the Hill plots are 1.0 for L-tryptophan, 0.86 for oxindolyl-L-alanine, and 0.88 for 2,3-dihydro-L-alanine.

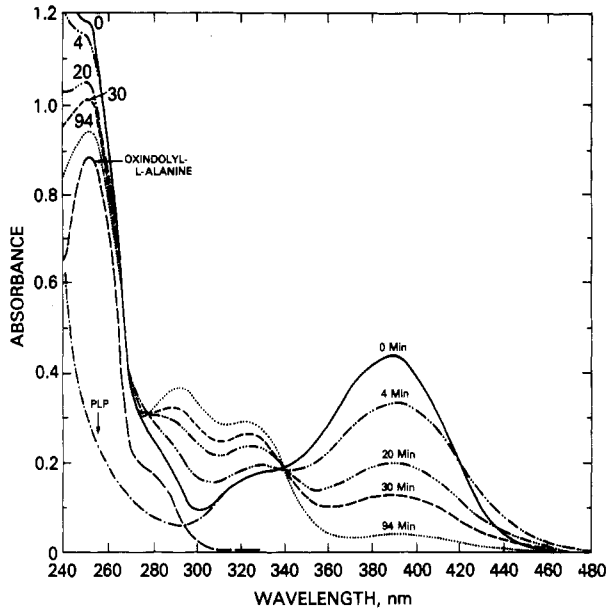


FIGURE 4: Absorption spectra of pyridoxal phosphate and of oxindolyl-L-alanine before and after mixing. A total of 1.0 mL of 0.2 mM pyridoxal phosphate in 0.1 mM potassium phosphate, pH 7.8, containing 2 mM EDTA and of 1.0 mL of 0.24 mM oxindolyl-L-alanine was placed in each of the two sides of a split cuvette (light path of each side = 0.44 cm). Absorption spectra were recorded before (curve 0) and at the indicated number of minutes after mixing the contents of the two sides. Spectra of the separate solutions of pyridoxal phosphate (···) and oxindolyl-L-alanine (—) are shown for comparison.

α-hydrogen of the substrate or quasi-substrate, our results indicate that tryptophan synthase can form these α-carbanion intermediates with L-tryptophan, oxindolyl-L-alanine, and 2,3-dihydro-L-tryptophan (see Discussion). The formation of α-carbanion intermediates should also result in incorporation of tritium into the α-position of the substrate or quasi-substrate when the reaction mixture contains ^3H_2O. Both tryptophanase (Morino & Snell, 1967, 1970) and tryptophan synthase (Tsai et al., 1978; Miles, 1980) are known to catalyze the exchange of the α-hydrogen of substrates and analogues with water. Since we were unable to use our impure preparation of tryptophanase to obtain spectral evidence for the formation of the α-carbanion of oxindolyl-L-alanine or 2,3-dihydro-L-tryptophan, we examined the ability of tryptophanase and tryptophan synthase to catalyze exchange of the α-hydrogen of oxindolyl-L-alanine with ^3H_2O (Table III). Our finding that both tryptophanase and tryptophan synthase catalyze incorporation of tritium into oxindolyl-L-alanine is evidence that both enzymes form α-carbanion intermediates with oxindolyl-L-alanine.

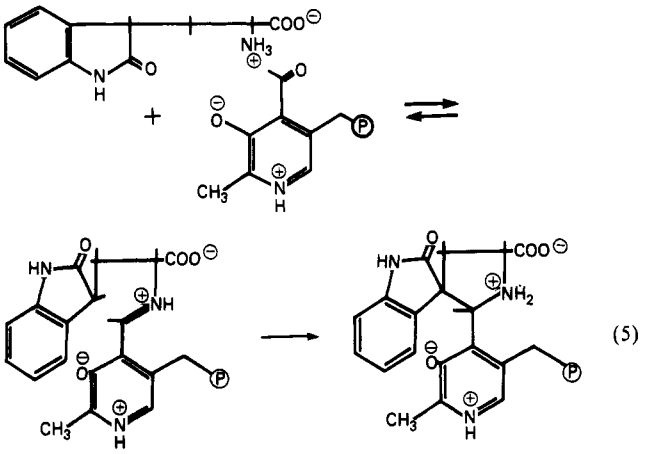
Studies of the Nonenzymatic Reactions between Oxindolyl-L-alanine and Pyridoxal Phosphate or Pyridoxal. Addition of 0.12 mM oxindolyl-L-alanine to 0.1 mM pyridoxal phosphate at 22 °C results in progressive decrease in absorbance at 388 and at 250 nm; concomitant increases in absorbance at 297 and at 320 nm occur (Figure 4). These observations suggest that a reaction occurs between pyridoxal phosphate and oxindolyl-L-alanine. Since little or no change in absorbance occurs when L-tryptophan or 2,3-dihydro-L-tryptophan is mixed with pyridoxal phosphate under the same conditions (data not shown), the reactivity of oxindolyl-L-alanine with pyridoxal phosphate probably results from the presence of the very labile proton at the 3-position of the oxindole ring, which is not present in L-tryptophan or 2,3-dihydro-L-tryptophan. Thus, the C-3 proton of oxindolyl-L-alanine readily exchanges in D_2O (Ohno et al., 1974), as has been shown by NMR

Table III: Incorporation of Tritium from ^3H_2O into Oxindolyl-L-alanine Catalyzed by Tryptophanase and Tryptophan Synthase[a]

addition	oxindolyl-L-alanine (dpm/nmol)	exchange (nmol/nmol)
none	28.2	0.14
tryptophanase	104.5	0.52
tryptophan synthase	99.4	0.50

[a] Reaction mixtures contained, in a total volume of 0.1 mL, 10 μmol of potassium phosphate, pH 8.0, 100 nmol of glutathione, 10 nmol of pyridoxal phosphate, 100 nmol of oxindolyl-L-alanine, 1 mCi of ^3H_2O, and either no addition, crude tryptophanase (800 μg), or purified $\alpha_2\beta_2$ complex of tryptophan synthase (160 μg). The reactions were carried out for 60 min at 22 °C and were stopped by boiling for 2 min. After cooling, the protein was removed by centrifugation, and a 40-μL aliquot of the supernatant was chromatographed on a Waters HPLC system by using a 4 cm × 10 cm C_{18} reverse-phase column (Rainin). Elution was carried out with 20% MeOH–80% 0.1 M ammonium acetate at 0.7 mL/min; detection was by absorbance at 254 nm. The peaks corresponding to the amino acid (retention time about 5 min) were collected, lyophilized, dissolved in 1 mL of H_2O, and relyophilized. The residue was dissolved in 1 mL of H_2O, recovery was quantified by the absorbance at 250 nm, and an aliquot (250 μL) was counted. Complete exchange under these conditions should result in a specific activity of 200 dpm/nmol.

measurements. We propose that C-3 of the enol tautomer of oxindolyl-L-alanine adds to the double bond of the aldimine formed between oxindolyl-L-alanine and pyridoxal phosphate to give a stable, cyclic derivative (reaction 5). This derivative

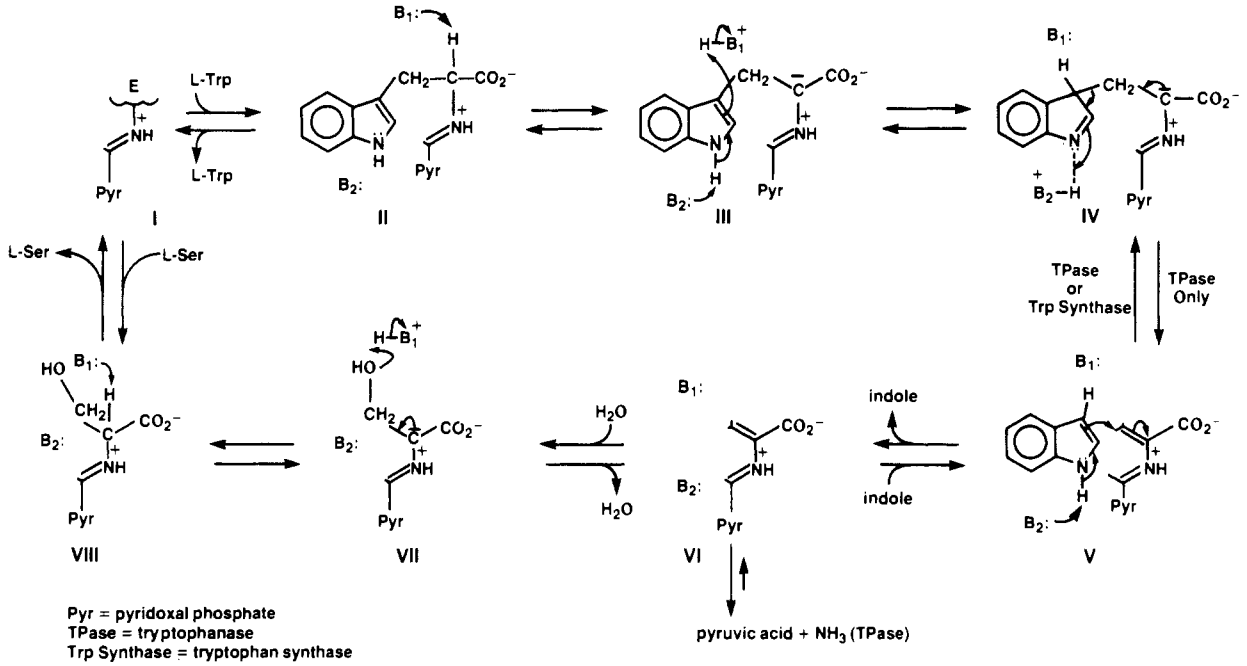


(5)

is analogous to the spirocyclic product formed from the reaction of 1-(methyloxindolyl)ethylamine and benzaldehyde (Jackson & Smith, 1968). The equilibrium between the free reactants and the aldimine shown in reaction 5 probably favors the free reactants, since there is no evidence in Figure 4 for the accumulation of an aldimine derivative (which would be expected to absorb near 410 nm) and since there is no increase in absorbance at 410 nm when pyridoxal phosphate is mixed with L-tryptophan or 2,3-dihydro-L-tryptophan under the same conditions. However, the final, irreversible cyclization reaction shown in reaction 5 would be expected to pull the overall reaction to completion.

In order to characterize the cyclic product by mass spectrometry, a similar reaction was carried out by incubating 10 μmol of pyridoxal hydrochloride with 10 μmol of oxindolyl-L-alanine at 37 °C in a final volume of 2.0 mL after adjustment of the pH to 7.0 with NaOH. The absorbance at 318 and at 252 nm measured on 1:40 dilutions was observed to decrease for 120 min. Thin-layer chromatography on silica gel G in $CHCl_3$–MeOH (3:1) after 120 min showed that pyridoxal (R_f 0.8) had been completely converted to a product which remained at the origin. For examination by californium-252 plasma desorption mass spectrometry (Macfarlane,

Scheme I: Mechanism of Reaction of Tryptophanase and Tryptophan Synthase



1983), this sample was dissolved in water and electrosprayed on an aluminized mylar film. Ions were accumulated over a 2-h period. Because of the presence of sodium chloride, multiple peaks were observed in the molecular ion region. Thus, 371, 392, 414, and 437 correspond to M + H, M + Na, M + Na − H, and M + 3 Na − 2H for the expected structure (the dephosphorylated form of the product shown in reaction 5). Although the dephosphorylated form of the aldimine intermediate shown in reaction 5 would be expected to have the same molecular ion, the accumulation of the aldimine intermediate is excluded by the absence of absorbance at 410 nm (see above). Thus, the identification of the product is based on both the mass spectrum and on the absorption spectrum.[1]

Discussion

Tryptophan synthase and tryptophanase catalyze respectively the formation of tryptophan from indole and serine (reaction 2) and the cleavage of tryptophan to indole, pyruvate, and ammonia (reaction 4). Whereas tryptophanase can also catalyze the synthesis of tryptophan from serine (or pyruvate and ammonia) and indole, tryptophan synthase does not appear to catalyze tryptophan cleavage. Nevertheless, it is reasonable to assume that both these enzymes carry out their respective reactions through a similar series of intermediates. A key activated intermediate that has been proposed in reactions catalyzed by both tryptophanase and tryptophan synthase is the pyridoxal phosphate aldimine of the indolenine tautomer of tryptophan (structure IV, Scheme I; see discussion of mechanism below). We have examined two compounds [oxindolyl-L-alanine (C) and 2,3-dihydro-L-tryptophan (D)], which have structures similar to the indolenine tautomer of tryptophan (B), to see whether these analogues mimic the proposed intermediate by being quasi-substrates or very good competitive inhibitors for tryptophanase and tryptophan synthase. We have found that the two analogues are very good competitive inhibitors of tryptophan synthase (Figure 1A) and tryptophanase (Figure 1B). Since linear competitive inhibition is observed and since preincubation of either enzyme with either of the analogues for up to 90 min resulted in no irreversible inhibition, the binding of these analogues to either enzyme must be fully reversible. The two inhibitors do appear to differ in their initial rates of binding to tryptophanase since different pre-steady-state rates are observed in the presence of each of the two inhibitors (see Materials and Methods). Oxindolyl-L-alanine must bind rapidly since the onset of inhibition is immediate. The occurrence of a delay in the onset of inhibition by 2,3-dihydro-L-tryptophan suggests that this analogue is a slow-binding inhibitor (Morrison, 1981). Since this delay is overcome by preincubation of the enzyme with 2,3-dihydro-L-tryptophan and since the same steady-state rates and the same K_I values are obtained whether or not tryptophanase is preincubated with either of the two inhibitors (see Materials and Methods), inhibition is fully reversible. Although the commercial tryptophanase used in these studies was not pure, it appears to be suitable for kinetic studies since we obtained kinetic constants for L-tryptophan and for indole-3-propionic acid (Table II) similar to values reported for homogeneous tryptophanase (Watanabe & Snell, 1977).

In the case of tryptophan synthase we obtained spectral evidence for α-carbanion formation with oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan. Interaction of oxindolyl-L-alanine or 2,3-dihydro-L-tryptophan with the $\alpha_2\beta_2$ complex of tryptophan synthase changes the absorption band at 412 nm (Figure 2) due to pyridoxal phosphate, which is bound to the enzyme as an internal aldimine, I (Scheme I), to a new band at 480 or 494 nm, respectively, characteristic of a quinonoid intermediate. A similar spectral band at 474 nm, thought to be the quinonoid, which is a resonance form of intermediate III (Scheme I), is seen with L-tryptophan bound to tryptophan synthase (Figure 2) and in the synthesis of L-tryptophan from indole and L-serine (Miles, 1980; Tschopp & Kirschner, 1980; Lane & Kirschner, 1981). There is kinetic evidence that this quinonoid intermediate is directly on the pathway of synthesis of L-tryptophan (Lane & Kirschner, 1983). The occurrence of intermediate III, which results from removal of the α proton of L-tryptophan, is also supported by the finding that tryptophan synthase catalyzes the exchange of the α proton of L-tryptophan with D_2O (Tsai et al., 1978; Miles, 1980). We did

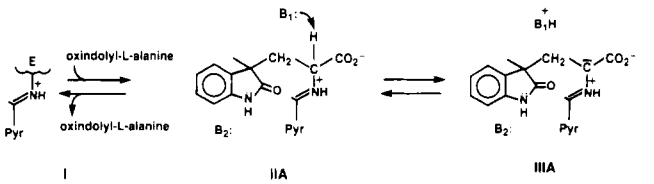
[1] This spectrum was generously determined and interpreted by Drs. Henry M. Fales and Lewis Pannell, Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health.

not examine the effects of oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan upon the absorption spectrum of tryptophanase because we do not have a preparation of homogeneous enzyme. These studies would be of interest especially since tryptophanase has been shown to form quinonoid intermediates with several substrates and analogues (Morino & Snell, 1967; Kazarinoff & Snell, 1980). However, our results demonstrating tritium incorporation into oxindolyl-L-alanine (Table III) during incubation with either tryptophan synthase or tryptophanase suggest that the quinonoid intermediate is formed in the complex with tryptophanase as well as with tryptophan synthase. Additional evidence that tryptophanase forms an intermediate with oxindolyl-L-alanine can be derived from our kinetic data in Table II. Whereas the K_I for indole-3-propionic acid is comparable to the K_m for L-tryptophan, the K_I for oxindolyl-L-alanine is about 300-fold lower than the K_I for oxindole-3-propionic acid. These results imply that the inhibition by oxindolyl-L-alanine is due not simply to its ability to bind but to its conversion to an intermediate which is bound more tightly.

The formation of quinonoid intermediates has also been observed upon addition of substrates or substrate analogues to several other pyridoxal phosphate enzymes, including tyrosine phenol-lyase (Kumagai et al., 1975), serine transhydroxymethylase (Schirch & Jenkins, 1964; Schirch, 1974; Ulevitsch & Kallen, 1977), and aspartate aminotransferase (Jenkins, 1961; Metzler et al., 1978). The absorption bands at 492–505 nm attributed to quinonoid derivatives with these enzymes are sharp, narrow, and highly skewed; in addition to the main peak there is usually a shoulder at approximately 470 nm (Davis & Metzler, 1972). Although the quinonoid derivatives formed by tryptophan synthase and either L-serine and β-mercaptoethanol or L-tryptophan (Miles, 1980; Lane & Kirschner, 1981) have absorption bands at somewhat lower wavelengths (468 and 474 nm, respectively), these bands are probably quinonoid derivatives since they have narrow band widths and have shoulders at lower wavelengths. The lower than usual wavelengths of these bands might result from interaction with protein groups (Davis & Metzler, 1972). The absorption bands observed with oxindolyl-L-alanine (480 nm) and 2,3-dihydro-L-tryptophan (494 nm) are at higher wavelengths than the bands observed with L-tryptophan (474 nm). Although the peak heights of the bands with L-tryptophan and 2,3-dihydro-L-tryptophan are low in Figure 2, the extinction coefficients cannot be estimated since the fraction of the enzyme-bound pyridoxal phosphate present in this form is not known. The absorbance of the 474-nm band with L-tryptophan is enhanced about 3-fold in the presence of indolyl-3-propanol phosphate (Miles, 1980; Lane & Kirschner, 1981).

A mechanism consistent with our present results is presented in Scheme I. Addition of indole to the aminoacrylate intermediate, V, is most likely facilitated by a basic group, B_2, which abstracts the proton on N-1 of the indole ring concomitant with attack of indole on the aminoacrylate, thereby increasing the nucleophilicity of the indole. The enhanced reactivity of indole N-1 anions to alkylation, both at C-3 and N-1, is well documented in organic chemistry (Sundberg, 1970). Furthermore, our mechanism, based on "push–pull" general acid–base catalysis of the tautomerization of the indole ring, is consistent with the observation that N^1-methyltryptophan is not a substrate for tryptophanase (Baker et al., 1946), even though N^1-methylindoles are stronger bases for protonation at C-3 (Hinman & Lang, 1964). The indolenine, IV, which is the initial product of the indole attack, is identical with the unstable tetrahedral intermediate, W′, proposed by

Scheme II: Mechanism of Inhibition of Tryptophanase and Tryptophan Synthase by Oxindolyl-L-alanine



Lane & Kirschner (1983) to be a key intermediate in the synthesis of L-tryptophan (although W′ was protonated at N-1), and to intermediate IIb, proposed by Davis & Metzler (1972) to be an intermediate in β elimination by tryptophanase. Note that, for simplicity, we have presented structures III and IV in the α-carbanion form rather than the quinonoid resonance form in Schemes I and II. Tautomerization of IV to III is readily carried out by abstraction of the C-3 proton by a basic group, B_1, concomitant with protonation of N-1 by the protonated B_2, yielding the carbanion of tryptophan (III). B_1 can then protonate the α-carbon to give the Schiff's base of L-tryptophan. The data of Schleicher et al. (1976) and Vederas et al. (1978), which show intramolecular proton transfer from the α-carbon of the side chain to C-3 of the indole ring in tryptophan cleavage by tryptophanase, support the idea that a single basic group is involved in abstraction of the α-hydrogen and protonation of C-3 of the indole. However, Lane & Kirschner (1983) suggest that two different basic groups in tryptophan synthase catalyze the abstraction of the α proton and deprotonation at C-3 of the indolenine intermediate.

The initial complex with oxindolyl-L-alanine (IIA in Scheme II) can also undergo abstraction of the α proton to form a carbanion (IIIA), which closely resembles the indolenine intermediate, IV in Scheme I, in the normal catalytic pathway. However, IIIA is not converted to pyruvate by either tryptophanase or tryptophan synthase, as we found no evidence for pyruvate formation (by the lactate dehydrogenase reaction) when oxindolyl-L-alanine was incubated with tryptophanase or tryptophane synthase. Thus, oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan act as competitive inhibitors. We note, however, that the real K_I value may be lower than our observed K_I values, since both of these compounds contain a mixture of two diastereomers at C-3 of the heterocyclic ring. Since intermediate IV contains a new chiral center at C-3, and since the enzymatic formation of this intermediate is most likely stereospecific (i.e., proton donation will occur from only one face of the 2,3-double bond), the inhibition which we observe with the analogues may be due to only one isomer. It is also interesting that oxindolyl-L-alanine is a better inhibitor of both enzymes than is 2,3-dihydro-L-tryptophan. This is in accord with our expectations, as the lactam ring of oxindolyl-L-alanine contains a planar C-2 carbon atom, as in the case of the putative indolenine intermediate, whereas 2,3-dihydro-L-tryptophan has a tetrahedral C-2 carbon.

During the course of these studies we have noted that oxindolyl-L-alanine reacts nonenzymatically with free pyridoxal phosphate (Figure 4) and have suggested that the product is the cyclic derivative formed by reaction 5. This structure is supported by the mass spectrum and absorption spectrum (see Results). Since the absorption spectrum of oxindolyl-L-alanine bound to tryptophan synthase (curve 3, Figure 2) does not change after several hours and is completely different from the spectrum of the nonenzymatic reaction product (Figure 4), we conclude that oxindolyl-L-alanine does not form a cyclic derivative with pyridoxal phosphate bound to tryptophan

synthase. The quinonoid intermediate IIIA (Scheme II) formed enzymatically would not be expected to undergo the cyclic reaction.

In conclusion, our results provide strong support for an indolenine intermediate in the reactions of tryptophanase and tryptophan synthase. The potent binding of the tryptophan analogues which we have studied may be due to their resemblance to this intermediate or to their ability to act as transition-state analogues (Pauling, 1946; Wolfenden, 1972; Lienhard, 1973) for the reactions leading to this intermediate. In this regard, we note that the Hammond postulate (Hammond, 1955) proposes that transition states will, in general, resemble unstable intermediates in structure. Furthermore, the data of Kazarinoff & Snell (1980) suggest that cleavage of tryptophan by tryptophanase is accompanied by a conformational change in the enzyme. Our observation that 2,3-dihydro-L-tryptophan is a slow-binding inhibitor (see Materials and Methods) is consistent with this suggestion, since a requirement for a protein conformational change upon binding is one factor that can lead to slow-binding inhibition (Morrison, 1981). Protein isomerizations also occur when tryptophan synthase binds L-tryptophan (Lane & Kirschner, 1981) and during the synthesis of L-tryptophan by tryptophan synthase (Lane & Kirschner, 1983). It is possible that our compounds are powerful inhibitors because they bind more strongly to the active conformations of these enzymes.

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Registry No. A, 73-22-3; B, 6536-35-2; C, 32999-55-6; D, 7536-97-2; L-serine, 56-45-1; indole-3-propionic acid, 830-96-6; tryptamine, 61-54-1; oxindole-3-propionic acid, 2971-17-7; oxytryptamine, 60716-71-4; pyridoxal phosphate, 54-47-7; tryptophan synthase, 9014-52-2; tryptophanase, 9024-00-4.

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