Substrate-Protein Interaction in Tryptophanase from Bacillus alvei

KINETIC AND SPECTRAL EVALUATIONS*

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JANE D. FENSKE and RALPH D. DEMOSS

From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801

This investigation studied the substrate protein interaction of the α,β elimination reaction in tryptophanase (EC 4.1.99.1). The results of this work are 2-fold. (a) The presence of multiple enzyme sites was found to be related to the observed kinetic patterns of inhibition. Indole analogues caused competitive inhibition in the tryptophanase reaction and noncompetitive inhibition in the dehydratase reaction. Inhibition patterns of alanine for these activities were reversed. (b) Under some conditions, compounds which bind presumably at the indole site modified the spectral and fluorescent characteristics of the enzyme. The addition of anthranilate to the enzyme resulted in a broad absorption band around 350 nm. This absorption band was distinct from that formed by alanine addition. Based on absorption data, both of these compounds could be bound simultaneously. The optical activity of tryptophanase was reported for the first time. Indole analogues caused greater conformational alterations in the circular dichroism spectra than 3-carbon analogues. The calculated anisotropy factors, as well as fluorescent quenching data, suggest a more direct interaction between indole analogues and pyridoxal-P than between 3-carbon compounds and the coenzyme. It is proposed that the indole site is the dominant recognition site. The data are consistent with the three-dimensional aspects of space-filling models of Schiff's bases evaluated in terms of multiple site binding.

Tryptophanase catalyzes the breakdown of tryptophan to indole, pyruvate, and ammonia as described by the general reaction in Scheme 1 (1). This enzyme is constitutive in Bacillus alvei and was first purified and characterized from this source by Hoch, Simpson and DeMoss (2), and was further chemically characterized by Hoch and DeMoss (3).

It is a well recognized concept that enzymes combine with their substrates at more than one site. Tryptophanase can catalyze α,β elimination reactions with a variety of substrates but the enzyme has greatly enhanced affinity for the substrate tryptophan as compared with serine, cysteine, or S-CH₇-cysteine (4). In addition, this enzyme can catalyze a β replacement reaction, which results in tryptophan synthesis when indole is in the presence of serine or cysteine, for example (5). Morino and Snell (1) have used spectral evaluations to demonstrate a combining site for alanine. They showed that a 500 nm absorption band was indicative of a semiquinoid structure formed between an amino acid and pyridoxal-P. There are other lines of evidence which also support this interpretation (6, 7). This 500 nm absorption band was also generated when substrates were added to the enzyme (1).

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† Public Health Service Predoctoral Trainee (GM 510). Present address, Department of Pathobiology SC-38, University of Washington, Seattle, Washington 98195.

| α,β ELIMINATION | α,β REACTION |
|-----------------|---------------|
| R-CH₂-C-H       | R-CH₂-C-(-)   |
| COO⁻             | COO⁻           |
| R=PLP⁺N         | R=PLP⁺N       |

SCHEME 1. Proposed mechanism of an α,β elimination reaction as suggested by Morino and Snell (1). The amino acid substrate bound through a Schiff's base to pyridoxal 5'-phosphate (PLP) loses the α-hydrogen forming a semiquinoid intermediate. In turn, the β group is removed from the amino acid resulting in the formation of the key aminoacrylic intermediate. This species then degrades to produce pyruvate and ammonia.

These and similar such observations demonstrate conclusively that a separate combining site is present for indole and the 3-carbon substrates.

Spectral evaluations of pyridoxal-P-containing enzymes have been extensively reported in the literature, as reviewed by Fasella (8) for example. These enzymes exhibit absorption bands in the visible spectrum which can be modified in the presence of substrates and inhibitors (1, 9, 10). Optical activity was first observed in a protein-pyridoxal-P system, in 1963, using aspartate aminotransferase (11). Similar extrinsically
induced Cotton effects caused by the coenzyme, which serves as a very special probe in the active site, have been demonstrated using Escherichia coli glutamate decarboxylase (12), Alcaligenes faecalis aspartate β-decarboxylase (13), E. coli threonine deaminase (14), and phosphorylase b from rabbit muscle (15).

The present study investigates the kinetics of the tryptophanase and the dehydratase reaction in light of the multiple enzyme combining sites, and evaluates the capability of indole and 3-carbon analogues to modify the spectral characteristics of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Bacteria and Growth**

*Bacillus alvei* F was isolated and grown in medium as described by Hoch et al. (16). The cells were grown in an American Sterilizer Company fermentor at 35°C with forced aeration, or in a 250-ml New Brunswick fermentor at 35°C with agitation at 175 rpm and aeration of 2 ft³/min. Silicone antifoam A (Dow Corning) was added in either case. The cells were harvested after 24 to 36 hours of growth. Yields were approximately 10 g of wet cell weight/liter with specific activities from 0.014 to 0.035. Cells were stored in 500 g lots at 20°C.

**Enzyme Preparation and Criteria of Homogeneity**

*B. alvei* tryptophanase was purified by the method of Hoch et al. (2) except that the heat extract was not frozen since this resulted in a 30 to 40% loss of activity. Instead, this step was followed directly with the DEAE-step and then continued as described. The final pellet obtained from the 25% AmSO₄-fractionation was routinely analyzed for contamination by analytical polyacrylamide gel electrophoresis (3). The gels were prerun for 30 min before the protein was applied. Destaining was performed using a Canalco Quick Gel Destainer. Sedimentation velocity has also been used for homogeneity analyses.

**Spectral Analyses**

**Absorption**—Absorption spectra were recorded on a Cary model 14 recording spectrophotometer. The buffer system used was 0.05 M potassium, pH 7.5, 0.01 mM pyridoxal-P, and 0.1 mM 2-mercaptoethanol. For the anionic acid studies, the protein was dialyzed against this compound for 48 hours and the dialyze was used as the buffer system in the pH studies utilizing other analogues. Inhibitors were added to the protein solution and spectra were recorded after a 5-min incubation period. All spectra have been corrected for volume dilution resulting from substrate and analogue additions and were recorded at 25°C.

**Fluorescence**—Fluorescent spectra were measured on an Aminco-Bowman spectrophotofluorometer in a square quartz cuvette (1.0 x 1.0 cm) at 25°C. The same buffer system used in the absorption studies was employed. The relative fluorescence was recorded directly from the photomultiplier microphotometer. When fluorescence quenching experiments were performed, the ligand was added in 5- to 10-μl samples to minimize volume changes. The fluorescence emission was read 5 to 10 min after ligand addition at 350 and 510 nm using an excitation wavelength of 280 nm. Corrections for volume alteration and fluorescence attenuation were made in calculating the final relative fluorescence (17). The protein used was 0.1 to 0.2 mg/ml and was illuminated by the light source only while the fluorescence reading was made.

**Circular Dichroism**—Optical activity was measured with a Jasco ORD/CD/VUV spectropolarimeter which was equipped with an interface to an IBM model 1800 containing an analog-to-digital converter for data collection. The program to collect and process the data was written by B. J. Carey prior to addition in the assay mixture. The pH of the substrates and inhibitors used was adjusted to near neutrality. Duplicates were always run. The Kᵣ, Vₑ, and Vₑ parameters were calculated on an IBM 360/75 computer using an iterative program to fit the experimental data to a hyperbola. The inhibitor constants for competitive inhibition were calculated using Equation 2.

**RESULTS**

**Kinetic Evaluation of Substrate Binding Sites**—Compounds which resemble the alanine portion of the substrate were analyzed for their capacity to inhibit the tryptophanase reaction (Table I). All of the analogues demonstrated noncompetitive inhibition as defined by Cleland (26). Since rather high concentrations of inhibitors which could form Schiff's bases with pyridoxal-P were used, it was conceivable that the inhibitory action was caused by reducing the available concentration of pyridoxal-P. However, when a 2-fold increase of pyridoxal-P was included in the assay using glycine, for example, there was essentially no difference.

Inhibition of the tryptophanase reaction exhibited by indole and some indole derivatives was analyzed (Table II). Com-
pared with the indole derivatives, exceedingly low concentrations of indole caused inhibition. This suggests that the indole binding site is the dominant recognition site in substrate binding. An investigation of these compounds also serves to further assess the 3-carbon side chain portion of the substrate molecule. For example, the carboxylate ion of indole propionic acid appears to be a more effective inhibitory group than the amino group of α-CH₂-tryptamine. This is consistent with Dunathan's proposals concerning the mechanism of amino acid binding in pyridoxal-P-dependent enzymes (27).

Further assessment of the modes of inhibition as a function of the substrate binding sites was achieved by capitalizing on the protein's ability to catalyze the a,b elimination reaction using either tryptophan or serine. These kinetic experiments are summarized in Tables III and IV. The most striking observation is that the indole analogues serve as competitive inhibitors in the tryptophanase reaction and as noncompetitive inhibitors in the dehydratase reaction. The reverse is true for alanine. Different recognition sites for the substrates appear to be related to the observed variance in inhibition patterns.

Spectral Analysis of Different Recognition Sites—Spectral alterations observed for preparations of Bacillus alvei tryptophanase in the presence of substrates were similar to those previously reported in Escherichia coli (1). The addition of d-alanine did not elicit any spectral change in the E. coli tryptophanase system (1). When d-alanine is added to B. alvei

| Inhibitor | Conc (mM) | Kᵢ (mM) | % Inh. init. vel | % Inh. Vₘₙ | Inhibition pattern | Replots | Intercept | Slope |
|-----------|-----------|---------|-----------------|-------------|------------------|---------|-----------|-------|
| Indole    | 0         | 0.047   | 12              | 18          | Noncompetitive    | Linear  | Linear    |       |
| α-CH₂-    | Tryptamine| 0.02    | 0.044           | 32          |                   |         |           |       |
| Tryptamine| 5         | 0.66    | 5               | 7           | Noncompetitive    | Linear  | Linear    |       |
| Acetic    | 10        | 0.87    | 8               | 18          |                   |         |           |       |
| Indole    | 0         | 0       | 0               | 0           | Noncompetitive    | Hyperbolic | Linear   |       |
| Propionic | Acid      | 5       | 0.34            | 21          | Noncompetitive    | Parabolic | Parabolic |       |
| Acid      | 2         | 0.42    | 2               | 43          |                   |         |           |       |
| Indole    | 0         | 0       | 0               | 0           | Noncompetitive    | Linear  | Linear    |       |
| Acetic    | 1         | 1.13    | 9               | 19          |                   |         |           |       |
| Acetic    | 2         | 3.00    | 19              | 38          |                   |         |           |       |
| Acetic    | Acid      | 4       | 5.62            | 34          | Noncompetitive    | Hyperbolic | Hyperbolic |       |
| Acid      | 4         | 7.1     | 44              | 57          |                   |         |           |       |

The reaction mixture contained (in a final volume of 0.5 ml): potassium phosphate buffer, pH 8.0, 2.5 × 10⁻³ M pyridoxal-P, 1 to 2 μg of protein, and L-tryptophan using concentrations from 0.2 to 3.0 mM. After addition of substrate, the tryptophanase reaction was allowed to proceed for 5 min at 37°C and was assayed as indicated under "Experimental Procedures."

The replots were determined from a family of inhibitor curves by plotting both the intercepts and the slopes against inhibitor concentration (20). The approximate inhibitor constant determined for alanine is similar to the inhibition constant determined indirectly from the spectrophotometric titration of tryptophanase with alanine.
Table III

Kinetic parameters determined for four benzenoid derivatives and alanine in tryptophanase reaction

| Inhibitor         | Conc (mM) | K_i (mM) | % Inhib Init Vel | % Inhib max | Inhibition Pattern | Intercept | Replots Slope |
|-------------------|-----------|----------|------------------|-------------|-------------------|-----------|---------------|
| L-phenylalanine   | 0         | 6.1      | 10               | 0           | Competitive       | Linear    | Linear        |
|                   | 10        | 6.6      | 17               | 0           |                   |           |               |
| Anthranilic acid  | 0.1       | 0.67     | 7                | 0           | Competitive       | Linear    | Linear        |
|                   | 0.5       | 0.44     | 9                | 0           |                   |           |               |
|                   | 1.0       | 0.39     | 28               | 0           |                   |           |               |
| 2-NH_2-3-CH_3 benzoic acid | 0 | 1.8 | 15 | 0 | Competitive | Linear | Hyperbolic |
| 4                  | 2.6       | 20       | 0                |             |                   |           |               |
| 2-NH_2-4-CH_3 benzoic acid | 0 | 2.4 | 8 | 0 | Competitive | Linear | Parabolic |
| 4                  | 2.2       | 26       | 0                |             |                   |           |               |
| L-alanine         | 0         | 50       | 24               | 77          | Noncompetitive    | Hyperbolic| Hyperbolic    |
|                   | 100       | 28       | 17               |             |                   |           |               |

Table IV

Kinetic parameters determined for some benzenoid derivatives and alanine in dehydratase reaction using L-serine as substrate

| Inhibitor            | Conc (mM) | K_i (mM) | % Inhib Init Vel | % Inhib max | Inhibition Pattern | Intercept | Replots Slope |
|----------------------|-----------|----------|------------------|-------------|-------------------|-----------|---------------|
| L-phenylalanine      | 0         | 5        | 24               | 12          | Noncompetitive    | Hyperbolic| Hyperbolic    |
|                      | 10        | 32       | 19               |             |                   |           |               |
| Anthranilic acid     | 0         | 1        | 33               | 50          | Noncompetitive    | Parabolic | Parabolic     |
|                      | 2         | 71       | 73               |             |                   |           |               |
| 2-NH_2-3-CH_3 benzoic acid | 0 | 42 | 58 | 0 | Noncompetitive | | |
| 4                  | 43        | 58       | 58               |             |                   |           |               |
| 2-NH_2-4-CH_3 benzoic acid | 0 | 43 | 18 | 0 | Noncompetitive | Hyperbolic| Hyperbolic |
| 4                  | 43        | 18       | 18               |             |                   |           |               |
| L-alanine            | 0         | 50       | 38               | 0           | Competitive       | Linear    | Hyperbolic    |
|                      | 100       | 34       | 44               | 0           |                   |           |               |

\(^a\)The reaction mixture contained in 0.5 ml: 0.2 M potassium phosphate buffer, pH 7.8, 2.5 x 10^{-4} M pyridoxal-P, 5 to 10 \mu g of protein, and L-serine concentrations ranging from 80 to 400 mM. The serine dehydratase reaction was allowed to proceed for 10 min at 37°C and pyruvate formation was monitored as described under "Experimental Procedures."
tryptophanase, alterations occur in the 425 and 333 nm bands but no 500 nm band is observed. Glycine, threonine, valine, isoleucine, leucine, and pyruvate caused spectral alterations similar to those observed for α-alanine. The addition of 17 mM β-alanine does not alter the enzyme absorption spectrum. This is consistent with a separate kinetic experiment which showed that β-alanine was not an inhibitor of the tryptophanase reaction.

In this study it is shown that compounds binding presumably at the indole portion of the substrate site can modify the spectral characteristics of the enzyme. The 350 nm band generated when anthranilic acid is in the presence of the enzyme (Fig. 1) has been interpreted to result from an intramolecular complex between anthranilate and pyridoxal-P. Charge transferlike interactions have been reported in numerous physiological systems (28-30). Such complexes commonly result in broad absorption bands and in bathochromic shifts of 10 to 20 nm (31). As discussed by Shifrin (32), tryptophan has been shown to be the most effective electron donor among the amino acids because of the electron distribution of the indole ring, and pyridinium derivatives are classic electron acceptors. Assuming that the amino group of anthranilic acid functions in a capacity similar to the pyrrole nitrogen of indole, the potential exists for an intramolecular complex to form between anthranilic acid and pyridoxal-P. The addition of the same concentration of anthranilic acid to the pyridoxal-P bovine plasma albumin system described by Dempsey and Christensen (33) caused no significant spectral alterations.

Fig. 1 also demonstrates that the anthranilate spectral alterations are distinct from those caused by alanine site compounds such as L α,β-diaminopropionic acid. Both analogues can modify the spectrum simultaneously. When anthranilic acid and L alanine are used in a similar experiment, the results are essentially the same as shown in Fig. 1.

Circular Dichroism—It was found that both absorption bands of holotryptophanase are optically active (Fig. 2), exhibiting positive extrinsic Cotton effects in the 425 and 335 nm regions. The addition of substrates to the enzyme altered the CD spectra as shown in Figs. 2 and 3. Serine and S-CH$_3$-cysteine, which are presumed to bind at the alanine recognition site, elicit CD spectral modifications distinct from those caused by tryptophan. These differences are further substantiated by separate experiments in which the anisotropy factors for tryptophanase in the presence of substrates were determined (Table V). In general, the higher the anisotropy factor the greater the degree of asymmetry (34). The data in Table V give a direct indication of the degree of association between pyridoxal-P and the protein environment of the active center under the conditions given. The substrates listed...
generate three patterns of CD modifications: (a) decreases in both optically active bands as with tryptophan; (b) a decrease in one band and an increase in the other shown for serine and S-CH$_3$-cysteine; and (c) decreases in both bands accompanied by the appearance of a new optically active band at 484 nm demonstrated by L-$\alpha$,$\beta$-diaminopropionic acid. L-tryptophan did not elicit any CD modifications nor did it inhibit the enzyme activity when tested at concentrations up to 4.0 mM.

CD alterations which occur in the presence of an alanine site analogue as compared with those of an indole site analogue are shown in Figs. 4 and 5, respectively. The differences in the CD modifications are further supported by the calculated anisotropy factors for the inhibitors shown in Table VI. The addition of alanine elicits moderate decreases in both optically active bands of the enzyme and generates a new optically active maximum at 504 nm. The addition of indole site analogues greatly decreases the optical activity, almost abolishing the CD effect in the 425 nm region, and no new optically active band near 500 nm is generated.

Fluorescence—When holotryptophanase is excited at 280 nm, the emission spectrum exhibits a prominent band at 350 nm and another peak at 510 nm in a ratio of approximately 4:1. The emission peak at 510 nm is probably due to resonance energy transfer between tryptophan emission, at 350 nm and pyridoxal-P absorption bands as suggested by Jones and Cowgill (35) to explain results in the phosphorylase b system which are similar to those seen here. The possibility of energy transfer is further supported by the fact that apotryptophanase only gives a 350 nm emission. Free pyridoxal-P is not activated at 290 nm (35). In our investigations, there was no attempt made to determine the number of tryptophan residue sites close enough in proximity to pyridoxal-P to be responsible for this postulated energy transfer mechanism.

Tryptophanase was titrated with alanine and anthranilic acid in separate experiments. Neither analogue, even at high concentrations, associates with the protein in a manner which serves to decrease the tryptophan residue emission at 350 nm (Fig. 6). However, both served as a quenching agent of the 510 nm emission. Therefore, each was bound in such a manner as to interfere with the postulated resonance energy transfer from tryptophan to pyridoxal-P. Alanine caused a 50% quenching effect, indicating that the transfer mechanism was only partially hindered. Anthranilic acid totally quenched this emission which suggests that the transfer is completely blocked in its presence.

**DISCUSSION**

The prominent findings from this work show that (a) different modes of inhibition may be explained in terms of multiple enzyme recognition sites; and (b) compounds which presumably bind at either alanine or indole recognition sites cause different modifications in the absorption, CD, and fluorescent properties of the enzyme.

In general, the alanine analogues shown in Table I are required at higher concentrations than the indole analogues shown in Table II to cause similar levels of inhibition. Although the modes of inhibition are all noncompetitive, the variety of replot functions indicates that the actual mechanisms of inhibitor action are quite complex and distinct from one another (26). Using the indole analogues listed in Table III, which are smaller than those listed in Table II, binding at the indole site alone may be more closely approximated. The data suggest that compounds which occupy the indole binding site result in competitive inhibition when tryptophan is the substrate but result in noncompetitive inhibition with serine as the substrate. The inhibition patterns are reversed for compounds which occupy the alanine binding site. However, the replot data still serve to indicate the complex nature of these interactions.

This is the first report of optical activity for tryptophanase. When observed, the optical activity of the 800 nm species in other pyridoxal-P enzyme systems has a negative Cotton effect (36, 37). That the 500 nm band in tryptophanase is positive indicates that the electronic transition generating this band is opposite in direction as compared to the other systems (38). From the preparations of holoenzyme examined, the average $\Delta A_{350:335}$ was $1.5 \times 10^{-3}$ and the average $\Delta A_{350:335}$ was $0.7 \times 10^{-4}$. These values are in the same order of magnitude as compared with published anisotropy values for threonine deaminase, aspartate $\beta$-decarboxylase, glutamate decarboxylase, phosphorylase, and aspartate aminotransferase (14). The trend in our data indicates that variation in anisotropy factors determined for unaltered tryptophanase is correlated with the specific activities of the preparations evaluated. However, a study of the optical activity over a wide range of enzyme specific activities was not undertaken.

As discussed by Hayaishi and Shizuta (14), CD and optical rotatory dispersion data can provide a basis for the classifica-
TABLE V

Anisotropy factors for tryptophanase in presence of substrates

| Sample          | $\Delta A_{335}/A_{335}$ | $\Delta A_{425}/A_{425}$ | $\Delta A_{484}/A_{484}$ | $\frac{(\Delta A/A) + \text{Substrate}}{335 \text{ nm}}$ | $\frac{(\Delta A/A) + \text{Substrate}}{425 \text{ nm}}$ |
|-----------------|-------------------------|-------------------------|-------------------------|--------------------------------------------------|--------------------------------------------------|
| TPase           | 0.89 x 10^{-3}          | 1.25 x 10^{-3}          | 0.52                    | 0.74                                             |
| TPase + TRP     | 0.46 x 10^{-3}          | 0.93 x 10^{-3}          | 0.08                    | 1.12                                             |
| TPase           | 0.85 x 10^{-3}          | 1.65 x 10^{-3}          | 0.64 x 10^{-3}          | 0.13 x 10^{-3}                                   |
| TPase + L-SER   | 0.07 x 10^{-3}          | 1.88 x 10^{-3}          | 0.54 x 10^{-3}          | 1.32 x 10^{-3}                                   |
| TPase + S-CH$_3$-L-CYS at 160 min | 0.64 x 10^{-3} | 1.27 x 10^{-3} | 0.41 x 10^{-3} | 1.13 x 10^{-3} |
| TPase + L-DAP$^b$ | 0.54 x 10^{-3} | 1.32 x 10^{-3} | 0.38 x 10^{-3} | 0.76 |
| TPase + L-DAP$^b$ | 0.41 x 10^{-3} | 1.13 x 10^{-3} | 0.85 |

$^a$Conditions for the CD analyses and the method of calculation are given under "Experimental Procedures." $\Delta A$ values (except as noted) were recorded as the optical density values obtained after the CD scans were completed.

$^b$The anisotropy factors were determined by using an average $A$ value between a 2- and a 160-min spectrum recording. The $K_d$ determined from the two sets of data is 15.9 ± 1.9 mM and 13.0 ± 1.7 mM. The velocity is approximately 90% of that obtained with serine.

Fig. 4. Optical activity (top) and absorption spectrum (bottom) of holotryptophanase in the presence of L-alanine. L-alanine at a final concentration of 136 mM was added to an enzyme solution (specific activity = 8.3) containing 2.56 mg/ml. In the circular dichroic spectra, each curve is an average of four scans. Other conditions are described under "Experimental Procedures."

Fig. 5. Optical activity (top) and absorption spectrum (bottom) of holotryptophanase in the presence of phenylalanine. L- and D-phenylalanine were added at a final concentration of 14.2 mM to separate protein samples containing 4.2 mg/ml (specific activity = 7.8). The CD spectra represent data averaged from four scans. The D isomer caused no CD alteration as compared with the control. Both isomers altered the absorption spectrum as indicated. Phenylalanine has been reported to cause no absorption changes in Escherichia coli tryptophanase (1).
Table VI

Anisotropy factors for tryptophanase in the presence of inhibitors

| Sample      | \( \Delta A_{335}/A_{335} \) | \( \Delta A_{425}/A_{425} \) | \( \Delta A_{504}/A_{504} \) | \((\Delta A/A) + \text{Inhibitor}\) | \((\Delta A/A) - \text{Inhibitor}\) |
|-------------|-------------------------------|-------------------------------|-------------------------------|----------------------------------|----------------------------------|
| TPase       | \(0.55 \times 10^{-3}\)       | \(1.21 \times 10^{-3}\)       | \(0.43 \times 10^{-3}\)       | 0.31                             | 0.74                             |
| TPase + L-ALA| \(0.17 \times 10^{-3}\)       | \(0.90 \times 10^{-3}\)       |                               | 0.15                             | 0.27                             |
| TPase       | \(0.61 \times 10^{-3}\)       | \(1.29 \times 10^{-3}\)       |                               | 0.25                             | 0.32                             |
| TPase + L-PHE| \(0.20 \times 10^{-3}\)       | \(0.35 \times 10^{-3}\)       |                               |                                  |                                  |

*An anisotropy factor can be calculated at this wavelength but there is no distinct absorption maximum at 425 nm after alanine addition (see Fig. 5).

*This was determined from the \(\Delta A\) and A values at 350 nm where the new absorption band appears after anthranilate addition.

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**Fig. 6.** Fluorescence quenching of holotryptophanase. The relative fluorescence intensity of holotryptophanase was recorded as a solution of protein, approximately 0.1 mg/ml, and was titrated with either alanine or anthranilic acid. The fluorescence emission was monitored at 360 and 510 nm and recorded as relative per cent transmission. These curves are an average of four separate titration experiments done with each inhibitor. Other conditions are described under “Experimental Procedures.”

**Scheme 2.** Proposed modes of binding of pyridoxal phosphate for a type III pyridoxal phosphate enzyme (10).

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the 330 and 410 nm species being the active pyridoxal-P form, respectively (14). The nature of the reactive form(s) of pyridoxal-P in tryptophanase is presently unresolved and deserves further attention.

Although the \(\alpha\)-isomers analyzed in this study caused absorption spectral alterations other than the appearance of a

**Fig. 7.** Pyridoxal 5'-phosphate in Schiff’s base formation with the substrates tryptophan and serine. The pyridoxal-P-typtophan Schiff’s base is shown from the pyridoxal-P side of the structure (e), from the tryptophan side (b), and from the side so the ring interactions are visible with pyridoxal-P in the lower position and tryptophan located in the upper position (c). The atom designations for the model shown in Panels a to c are: I'CO, oxygen atom of the amino acid substrate carboxyl group; aN, imine nitrogen originating from the amino group of the substrate; C'4, carbonyl carbon at the 4-carbon position of pyridoxal-P; C'5P, phosphate group at the 5-carbon position of pyridoxal-P; N1, pyridine nitrogen in the pyridoxal-P ring; \(\alpha\)-hydrogen bond shown to be perpendicular to the pyridoxal-P ring. The atom designations for this model are: 3’OH, hydroxyl group on the side chain of serine; and \(\alpha\)H, \(\alpha\)-hydrogen on the amino acid. The other designations are as previously explained. See Scheme 3 for clarification of these designations.
510 nm band or the generation of a 350 nm band, only the small alanine site compound n-serine also caused a conformational change (Fig. 3b). It is conceivable that n-serine could form a Schiff's base with pyridoxal-P, thus specifically modifying the CD spectrum, but since the α-hydrogen would be positioned inadequately for an elimination, no further changes would occur. This is supported by the fact that n-serine could not elicite the formation of a 510 nm band in the presence of the enzyme. α-Phenylalanine caused absorption changes similar to those caused by n-serine but no CD conformational change occurred. The CD spectra and anisotropy factors indicate that the conformational changes resulting from the interaction of L-phenylalanine and anthranilate with pyridoxal-P are similar to each other yet distinct from those modifications caused in L-phenylalanine and anthranilate with pyridoxal-P are similar to each other yet distinct from those modifications caused in L-phenylalanine and anthranilate with pyridoxal-P are similar to each other yet distinct from those modifications caused.

However, it is clear that the interactions of these compounds near the indole site are not identical since the absorption data suggests (a) the formation of an intramolecular complex for anthranilate with no similar evidence for phenylalanine, which is however, consistent with the weak electron donating characteristics of phenylalanine (32); and (b) that phenylalanine can also interact with the enzyme to form a 510 nm band (Fig. 5).

Space-filling models of the Schiff’s bases formed between pyridoxal-P and tryptophan (Fig. 7, a to c), and between the coenzyme and serine (Fig. 7d), were constructed as a means of summarizing and visualizing the experimental data from this study. The notation that was adopted to designate the various atoms and functional groups is shown in Scheme 3. In these models, the required orientation of the α-hydrogen (αH) perpendicular to the pyridoxal-P ring system is satisfied (27) so that this atom may be removed from the substrate during the first step in the reaction as proposed by Morino and Snell (1). The orientation of the αH in relation to the serine-pyridoxal-P Schiff’s base product is shown in Fig. 7d. Fig. 7c provides for a dramatic visualization of the ring interactions that could occur between pyridoxal-P and the indole ring of tryptophan, thus supporting the interpretation of an intramolecular complex forming between the two. The absorption data in Fig. 1 experimentally support this interpretation. These models also indicate that an analogue which binds in the indole position of tryptophan would have a larger interaction with pyridoxal-P than those analogues binding in the alanine position. The CD spectra, calculated anisotropy factors, and fluorescent data directly support this interpretation. When Schiff’s base structures formed between pyridoxal-P and serine, S-CH₂-cysteine or α,β-diaminopropionic acid are constructed (not shown here), these substrates do not extend over the pyridine ring as does the indole portion of tryptophan. This decreased amount of interaction with pyridoxal-P could be a primary reason for the reduced rates of the αβ elimination reaction when these substrates are utilized. The alanine-pyridoxal-P Schiff’s base model has a geometry similar to the substrate complexes. However, compared with the substrates, this inhibitor has no electronnegative component in its side chain structure, a quality which apparently is required for the β elimination step of the reaction to occur.

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