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Pyridoxal 5′-Phosphate and Analogs as Probes of Coenzyme–Protein Interaction in *Bacillus alvei* Tryptophanase†

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ABSTRACT: Tryptophanase from *Bacillus alvei* was resolved from its coenzyme, pyridoxal phosphate, by treatment with cysteine followed by column chromatography. Spectrophotometric titration of apoenzyme with pyridoxal-P showed 1 mol of pyridoxal-P bound per 52,000 g of enzyme. Kinetic analysis of coenzyme binding showed hyperbolic activation curves with a K_m of 1.6 μM. Pyridoxal-P was used as a natural active site probe in spectrophotometric studies to distinguish differences in the active center of holotryptophanase and reconstituted enzyme that were not apparent by other techniques. The pK_a for holotryptophanase is 7.9 while the pK_a for reconstituted apoenzyme is 8.4. Apotryptophanase binds 2-nor, 2′-methyl, 2′-hydroxy, 6-methyl, and *N*-oxide pyridoxal-P to form analog enzymes distinguishable on the basis of absorption spectra and relative activity in catalyzing both the α,β-elimination and β-replacement reactions of tryptophanase. Apoenzyme also binds pyridoxal but pyridoxal analog enzyme is not active.

Tryptophanase from *Bacillus alvei* was previously reported (Hoch and DeMoss, 1966) to bind 1 mol of pyridoxal-P[1] per 125,000 g of enzyme when apotryptophanase was prepared by extensive dialysis against Tris–EDTA buffer. Techniques for successfully resolving pyridoxal-P enzymes to form apoprotein vary considerably (Wada and Snell, 1962; Newton et al., 1965; Shaltiel et al., 1966; Matsuzawa and Segal, 1968; Dowhan and Snell, 1970; etc.). Many apoprotein forms of pyridoxal-P enzymes which by kinetic and spectrophotometric criteria are resolved of coenzyme show a characteristic ability to bind 1 mol of pyridoxal-P per 50,000–60,000 g of enzyme (Wilson, 1963; Wilson and Meister, 1966; Novogrodsky and Meister, 1964; Tate and Meister, 1969; Kakimoto et al., 1969; Cowell, 1972) instead of the value of 1 mol per 125,000 g seen for *B. alvei* tryptophanase.

In this investigation *B. alvei* holotryptophanase was resolved of pyridoxal-P by treatment with cysteine followed by column chromatography. Our aim was to characterize

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[1] Abbreviations used are: pyridoxal-P, pyridoxal 5′-phosphate; Q_{max}, maximal fluorescence quenching; bicine, *N,N′*-bis(2-hydroxyethyl)glycine.

the binding and interaction of the natural coenzyme pyridoxal-P with apotryptophanase prepared in this manner.

In nature, both apotryptophanase and holotryptophanase exist and are interconverted by the addition and removal of pyridoxal-P (Gopinathan and DeMoss, 1966). Tryptophanase is purified from *B. alvei* as holotryptophanase. We will use the term reconstituted enzyme when referring to apotryptophanase to which pyridoxal-P has been added. The term analog enzyme will be used to refer to apotryptophanase plus a pyridoxal-P analog. Because we chose to carry out these studies by first removing the coenzyme from purified native holoenzyme and then asking how the coenzyme interacts when used in reconstituting the enzyme, it was also relevant to compare the properties of the reconstituted enzyme with native holoenzyme.

In order to establish the role various chemical substituents of pyridoxal-P play in the coenzyme–protein interaction, we have looked at the binding of various chemical analogs to the apoenzyme and also investigated some properties of the analog enzymes.

Materials and Methods

Compounds. Pyridoxal-P and pyridoxamine-P hydrochloride were purchased from Sigma. Pyridoxamine dihydrochloride and pyridoxal hydrochloride were obtained from Mann Research Laboratories. 4-Deoxypyridoxine-P and 4-deoxypyridoxine hydrochloride were purchased from California Corporation for Biochemical Research, and pyridoxine hydrochloride was obtained from Nutritional Biochemicals Corporation. 2-Norpyridoxal-P and 2′-methylpyridoxal-P were supplied by Dr. E. E. Snell; 2′-hydroxypyridoxal-P and *N*-oxide of pyridoxal-P by Dr. S. Fukui; isopyridoxal-4′-P by Dr. A. Pocker; and 6-methylpyridoxal-P by Dr. H. Dunathan and M. Karpeisky.

Tryptophanase was purified as holoenzyme from *B. alvei* by the method of Hoch et al. (1966). Holotryptophanase was tested for purity by ultracentrifugation and disc gel electrophoresis in a glycine–bicine buffer system (Hoch and DeMoss, 1972).

Apotryptophanase was prepared by the method of Hoch and DeMoss (1972) with the following modifications. The holotryptophanase sample was incubated with a final concentration of 0.01 *M* cysteine hydrochloride for 105 min. The Bio-Gel P-10 column was equilibrated at pH 7.0, but the pooled fractions were concentrated, centrifuged, and resuspended in 0.01 *M* potassium phosphate (pH 7.8). Final dialysis was also against pH 7.8 buffer. Increasing pH from 7.0 to 7.8 reduced the tendency for apoprotein to precipitate.

The extinction coefficients for holotryptophanase and apotryptophanase $E_{278\ nm}(1\%)$ were determined experimentally to be 12.9 and 12.4, respectively, with the protein concentration based on amino acid content (Hoch and DeMoss, 1972). Lowry protein determinations (Lowry et al., 1951) standardized with serum albumin gave protein values 14–20% higher than those obtained by extinction coefficient.

Sodium Borohydride Reduction. Reduction of enzyme for tryptic digestion and fingerprinting was according to the technique of Morino and Snell (1967b). Prior to reduction, a sample of holoenzyme, reconstituted apoenzyme, or pyridoxal analog enzyme (1.5 mg/ml) was dialyzed 72 hr against 0.5 *M* potassium phosphate buffer supplemented with 2×10^{-5} *M* coenzyme—a concentration which provides only 4 mol of coenzyme/mol of enzyme, thereby reducing the probability of nonspecific reduction of coenzyme onto the protein. Finely powdered tritiated sodium borohydride was added at a ratio of 0.6 mg of reducing agent/1.5 mg of protein in 0.05 *M* potassium phosphate (pH 6.5) at 4°. The yellow color of the enzyme disappeared almost immediately. Any foaming due to reduction was removed by centrifugation. After 10 min at 25°, the reduced enzyme was dialyzed against 0.05 *M* potassium phosphate (pH 6.8) until the radioactivity in the dialysate was no greater than background.

Activity Measurements. Tryptophanase activity of purified enzyme was measured by a modification of the method of Pardee and Prestidge (1961). The appearance of indole was measured.

The tryptophanase-catalyzed formation of tryptophan (tryptophan synthetase activity) from indole and L-serine was measured by following the disappearance of indole. Indole was determined colorimetrically (Yanofsky, 1955).

The tryptophanase-catalyzed breakdown of L-serine (serine dehydratase activity) was measured by following the formation of pyruvate (Friedemann and Haugen, 1943).

Modification of the assay techniques have been previously described (Hoch and DeMoss, 1973), and were further altered to exclude bovine serum albumin from all three reaction systems.

One unit of enzyme activity is defined as the production of 1 μmol of product or the disappearance of 1 μmol of substrate/min. Specific activity is expressed as units of activity per milligram of protein.

Apoenzyme was incubated with varying coenzyme concentrations at 4° for 20 min, and then at 37° for 10 min before the addition of substrate when used for determining activation kinetics. Time dependency curves indicated that varying the preincubation time at 37° from 5 to 120 min resulted in no significant alteration in activity.

Analyses of Kinetic Parameters. K_m and V_{max} values for all reactions were calculated on an IBM 360 computer using (1) an iterative program for fitting the data points to a hyperbola, and (2) a program that computed and plotted V vs. V/S (Eadie plot). The program eliminated all points varying by more than two standard deviations, and replotted the corrected V vs. V/S. For more than 80% of the data, no points were eliminated. For the remaining 20%, only one point was eliminated out of seven to ten samples.

Spectra. Absorption spectra were measured with a Cary Model 15 spectrophotometer equipped with a Cary-Datex Digital System (Datex Corporation) and an IBM 29 card punch. Absorbances were punched at 2-nm intervals. The data obtained were analyzed (Johnson and Metzler, 1970) using program PKA (Thomson and Metzler, 1971), adapted to the University of Illinois computer system. Modification of the program to allow plotting of the final data by a CalComp digital incremental plotter was done with the help of Dr. Thomson. Temperatures of sample and blank solutions were maintained constant.

Absorption spectra of holoenzyme samples were recorded after dialysis against pyridoxal-P for 48 hr at 4°; absorption spectra of analog enyzymes were recorded after dialysis of apoenzyme against analog for 48 hr at 4° in 3-ml chambers. Turbidity was minimized in both holoenzyme and analog samples (0.5–1.5 mg/ml) when studies were carried out at 9–10° in 0.05 *M* potassium phosphate buffer. Spectra from samples in which turbidity was observed visually or detected by changes in base-line measurement were not used.

Fluorescence measurements were made on an Aminco

Bowman spectrophotofluorometer equipped with a Honeywell X-Y recorder. Temperatures of test solutions were maintained constant. Data from fluorescence quenching experiments were corrected for dilution and fluorescence attenuation (Eisen, 1964) and analyzed on a digital computer by a program developed by Danimkoehler and Gallagher (Eisen and McGuigan, 1971).

Results

Resolution of Holotryptophanase. B. alvei apotryptophanase, prepared as described in Materials and Methods, had no pyridoxal-P absorption in the 400–450-nm region and less than 0.2% activity of the holoenzyme. Supplementing apoenzyme with excess pyridoxal-P restored 90–100% of the original activity.

Pyridoxal-P Binding. In the absence of bovine serum albumin in the reaction mixture, hyperbolic activation kinetics of apotryptophanase with pyridoxal-P were found for five independently prepared samples of apoenzyme. The average K_m was 1.6 μM pyridoxal-P. The activation curves plateau with no indication of secondary binding constants. In the presence of 200 μg of bovine serum albumin, a sigmoidal shaped pyridoxal-P saturation curve was generated indicating that the sigmoidal kinetics previously reported by Hoch and DeMoss (1966) and by O'Neil (1969) were an artifact caused by pyridoxal-P binding to serum albumin (Dempsey and Christensen, 1962).

The binding of pyridoxal-P was also investigated using fluorescence quenching. Computer analyses of data obtained from titrating apotryptophanase with increasing amounts of pyridoxal-P gave a binding constant of 1.8 μM. Kinetic and fluorimetric assays confirmed a K_m of 1.6–1.8 μM pyridoxal-P which is greater than the 0.7 μM value obtained for *Escherichia coli* tryptophanase (Morino and Snell, 1967a) and lower than the 6.3 μM value obtained for the *Sphaerophorus funduliformis* enzyme (Cecchini, 1972).

The values for maximal quenching (Q_{max}) of tryptophan fluorescence ranged from 40 to 50% with an average of 44% indicating that approximately half of the tryptophan residues are more available for energy transfer from protein to coenzyme either because of their distance from or orientation with respect to, the pyridoxal-P binding site. The 44% Q_{max} for the *B. alvei* enzyme was higher than the 23% value for *S. funduliformis* tryptophanase (Cecchini, 1972) but less than the 60–70% seen for asparate β-decarboxylase (Tate and Meister, 1971). Lack of a characteristic Q_{max} value for pyridoxal-P binding is not unexpected since both the tryptophan content per subunit and chemical composition of the active centers vary among pyridoxal-P dependent enzymes.

Since addition of pyridoxal-P to apotryptophanase restores absorption to the visible region of the spectrum, it is possible to titrate apoprotein with coenzyme. The end points for two spectrophotometric titrations corresponded to the binding of 0.91 and 0.95 mol of pyridoxal-P per 52,000 g of enzyme. These values confirm the equilibrium dialysis data (Hoch and DeMoss, 1972) that 1 mol of pyridoxal-P binds per 52,000 g of enzyme and argue against previous data that 1 mol of pyridoxal-P binds for every 125,000–130,000 g of enzyme (Hoch and DeMoss, 1966).

Similarities of Holoenzyme and Reconstituted Enzyme. Reconstituted enzyme was compared to holoenzyme. Reconstituted enzyme was 90–100% as active as holoenzyme; the K_m for tryptophan for reconstituted enzyme was 0.33 mM while the K_m for holoenzyme was 0.30 mM tryptophan. Both forms of the enzyme sedimented at 9.4 S in sucrose gradients. Tryptic peptide maps of sodium borohydride reduced reconstituted apoenzyme and holoenzyme compared well, both showing 32–34 ninhydrin positive spots. Both forms of the enzyme were reduced with tritiated sodium borohydride; the reduced protein was digested and tryptic peptide maps were prepared. The heavily tritiated peptide from either reduced reconstituted enzyme or from reduced holoenzyme migrated to the same position when subjected to electrophoresis and paper chromatography. The location of the labeled peptide was the same as had been seen previously for reduced holoenzyme (D. D. Whitt, personal communication). The V_{max} values, K_m values, and the structural analyses indicated no major differences between reconstituted enzyme and holoenzyme.

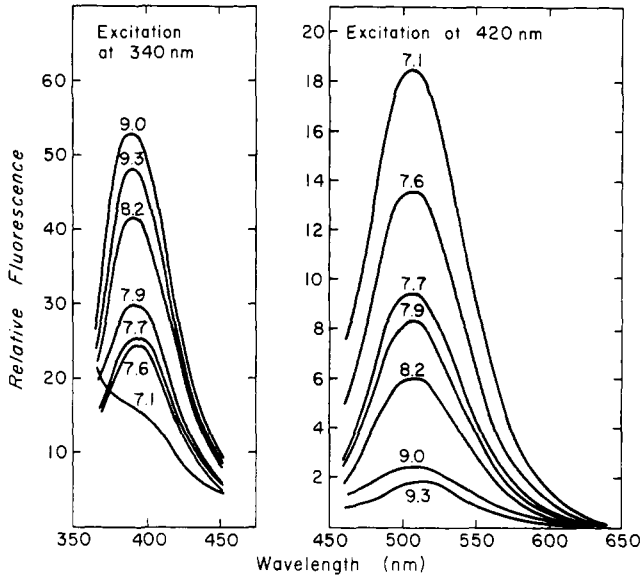


FIGURE 1: Effect of change in pH on the fluorescence emission spectrum of holotryptophanase. Holotryptophanase (0.75 mg/ml) was dialyzed extensively against 0.05 M potassium phosphate (pH 7.7) supplemented with 10^{-4} M pyridoxal-P. At this pH, the OD_{340} = 0.110 and OD_{420} = 0.067. The pH was adjusted from pH 7.1 to 9.3 at 29°. The pH was measured after each spectrum was recorded.

Differences between Holoenzyme and Reconstituted Enzyme. Spectral studies, however, revealed differences in the two enzyme forms. Samples prepared for use in spectral experiments were assayed for tryptophanase activity to establish that the observed spectra represented active protein. Reconstituted enzyme had 91–94% as much tryptophanase activity as holoenzyme.

Native holotryptophanase has two pH dependent bands at 337 and 420 nm. The relative heights of the two peaks reflect how much of the total enzyme is protonated (420 nm) and how much is not protonated (337 nm). Protonated enzyme is protein in which a hydrogen bond links the hydroxyl group of pyridoxal-P to a nitrogen from a lysine in the protein. A dissociation constant for this hydrogen ion (pK_a) can be calculated from the change in the two bands with pH. As the pH is increased, the height of the 420-nm peak decreases while that of 337-nm peak increases. This observation was made both for the fluorescence and absorption spectra but is more easily visualized in the fluorescent spectra because of the increased sensitivity of this technique (Figure 1).

Since the changes in the absorption pattern were small,

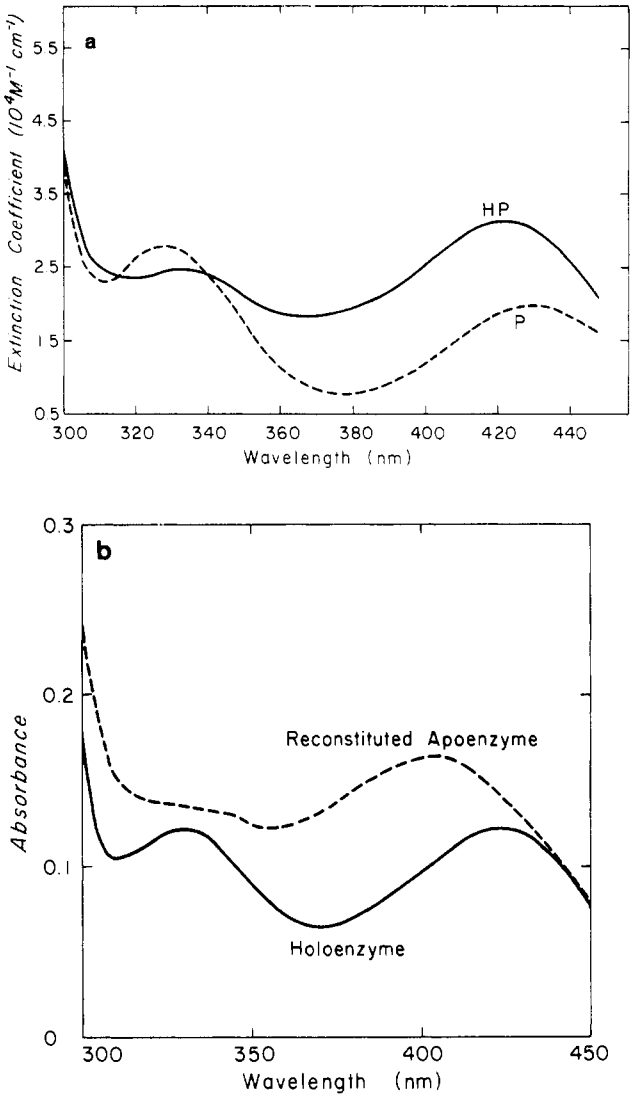


FIGURE 2: (a) Computer-analyzed spectra of the low pH (HP, —) and high pH (P, - - -) forms of holotryptophanase (5.3×10^{-6} *M*) from *B. alvei*. Titration was from pH 6.28 to 9.05 at 9°. (b) Comparative absorption spectra of holotryptophanase and of reconstituted apoenzyme. The absorption spectrum of 1.0 mg of holotryptophanase/ml (—) was recorded after extensive dialysis against 0.05 *M* potassium phosphate (pH 7.7) containing 10^{-4} *M* pyridoxal-P. Concentrated pyridoxal-P was added to a sample of apotryptophanase to a final concentration of 10^{-4} *M* pyridoxal-P. The absorption spectrum of 1.0 mg of reconstituted apotryptophanase per ml (- - -) was recorded after extensive dialysis against 0.05 *M* potassium phosphate (pH 7.7) containing 10^{-4} *M* pyridoxal-P.

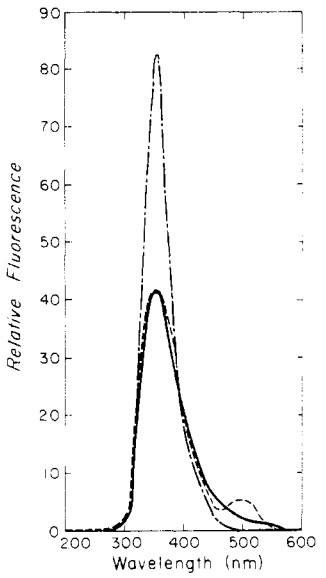


FIGURE 3: Comparative fluorescence emission spectra of holotryptophanase, apotryptophanase, and apotryptophanase reconstituted with pyridoxal-P. Excitation was at 280 nm; emission was scanned at 28° from 200 to 600 nm. All three samples contained 0.11 mg of enzyme/ml. Holotryptophanase (- - -) was dialyzed against 0.01 *M* potassium phosphate (pH 7.8) supplemented with 10^{-5} *M* pyridoxal-P. Apotryptophanase (- · - ·) was dialzyed against 0.01 *M* potassium phosphate (pH 7.8). Apotryptophanase was reconstituted with pyridoxal-P (—) to a final concentration of 10^{-5} *M* pyridoxal-P in 0.01 *M* potassium phosphate (pH 7.8).

the spectra were recorded electronically to evaluate the pK_a. Native holotryptophanase was titrated from pH 6.3 to 9.1. Computer analysis of the spectra yielded a pK_a of 7.89 ± 0.019; computed spectra for the high and low pH forms of the enzyme are shown in Figure 2a.

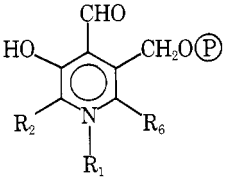
Reconstituted enzyme was titrated from pH 6.5 to 9.3 and a pK_a of 8.40 ± 0.030 was obtained. A difference of 0.5 in pK_a values is significant and strongly suggests that reconstituted enzyme differs from native holoenzyme in the interaction of pyridoxal-P with the protein.

A comparison of the spectra of holoenzyme and reconstituted apoenzyme at pH 7.7 was made (Figure 2b). The experimentally plotted spectrum of reconstituted enzyme at pH 7.7 resembles the computer-calculated spectrum for protonated holotryptophanase. Such a resemblance is not unexpected. Since reconstituted enzyme has a pK_a of 8.4 as compared to 7.9 for holoenzyme, substantially greater amounts of reconstituted enzyme are protonated at pH 7.7. A shift favoring the protonated form of the enzyme explains the greater intensity of the longer wavelength peak in the spectrum of reconstituted enzyme and could also explain a blue shift of 5–10 nm. However, the observed blue shift of 25 nm cannot be explained on the basis of the difference in pK_a values alone.

The fluorescence emission spectra for native holoenzyme and reconstituted enzyme also show that these two enzyme forms are not identical. Excitation of holotryptophanase at 280 nm yields two distinct peaks, one at 350 nm due to tryptophan fluorescence and one at 500 nm reflecting protein bound pyridoxal-P excited by way of an energy transfer from tryptophan (Figure 3). In both holoenzyme and reconstituted enzyme the 350-nm peaks were identical. However, native holoenzyme has a peak at 500 nm while reconstituted enzyme has only a shoulder in the 500-nm region. These differences in the fluorescent spectra support the idea that reconstituted enzyme is not identical with holoenzyme and that the lack of identity is reflected by the interaction of pyridoxal-P with the protein.

Catalytically Active Pyridoxal-P Analogs. Values for the affinity of apotryptophanase for five pyridoxal-P analogs are given in Table I. All five analogs restored activity to the apoenzyme. Increased bulkiness of the substituted group at C_2 of pyridoxal-P decreased the affinity of apoenzyme for the analog. Blocking the pyridine nitrogen with an oxygen molecule decreased the affinity of enzyme for coenzyme more than an order of magnitude while addition of a methyl group on the neighboring C_6 had no significant effect. Comparison of the K_m for coenzyme values with those obtained for *E. coli* tryptophanase (Snell, 1970) indicates that although the *B. alvei* enzyme has less affinity for pyridoxal-P than the *E. coli* enzyme, the *B. alvei* enzyme has a

Table I: Activation of *B. alvei* Apotryptophanase with Pyridoxal-P Derivatives.



Pyridoxal-P Derivative	R_1	R_2	R_6	K_m for Cofactor (μM)	K_m for Cofactor[c] (μM)
Pyridoxal-P	H	CH_3	H	1.6[a]	0.71
2-Norpyridoxal-P	H	H	H	2.1[b] (K_{m_1}) 1.9 (K_{m_2})	3.8
2'-Hydroxypyridoxal-P	H	CH_2OH	H	10.6[a]	59
2'-Methylpyridoxal-P	H	CH_2CH_3	H	8.8[a]	11
6-Methylpyridoxal-P	H	CH_3	CH_3	2.3[a]	7.7
N-Oxide of pyridoxal-P	O	CH_3	H	22.2[a]	

[a] The K_m values were determined by computer analysis. The K_m is given as an average of K_m values obtained using two different programs: (1) an iterative program for fitting the data points to a hyperbola, and (2) a program that computes K_m from V vs. V/S (Eadie plot). [b] The K_m values were determined graphically. [c] Snell (1970).

Table II: Kinetic Constants for the Tryptophanase and Tryptophanase Subsidiary Reactions Catalyzed by Analog Enzymes.

	Reaction					
	Tryptophanase		Tryptophan Synthetase[a]		Serine Dehydratase	
	Rel V_{max}	K_m(Trp) 10^{-3} M	Rel V_{max}	K_m(Ser) 10^{-1} M	Rel V_{max}	K_m(Ser) 10^{-1} M
Enzymes						
Holotryptophanase		0.27[b] 0.30[c]		1.44[b] 1.59[c]		1.29[b] 1.25[c]
Analog Enzymes						
Pyridoxal-P	1.00	0.33	1.00	0.98	1.00	1.32
2-Norpyridoxal-P	1.29	2.31				
2'-Hydroxypyridoxal-P	0.87	0.58				
2'-Methylpyridoxal-P	0.35	0.84	0.60	2.80	0.30	2.76
6-Methylpyridoxal-P	0.38	0.38	1.13	1.69	0.72	3.59
N-Oxide of pyridoxal-P	0.71	2.08	0.26	4.32	0.18	0.54

[a] Tryptophan synthetase activity was measured using indole and serine as cosubstrates. Indole concentration was held constant and the serine concentration varied. The K_m determined was for serine. [b] Hoch and DeMoss (1973). [c] Data are from this investigation.

greater affinity for the analogs especially 2'-hydroxypyridoxal-P.

Titration of apotryptophanase with 2'-hydroxy, 2'-methyl, and 6'-methyl analogs gave values of 0.99, 0.85, and 1.01 mol of respective coenzyme bound per 52,000 g of enzyme. The 2-nor, 2'-hydroxy, 2'-methyl, 6-methyl, and *N*-oxide analogs of pyridoxal-P were able to replace pyridoxal-P with varying degrees of efficiency as coenzymes for tryptophanase in the three tryptophanase-catalyzed reactions studied (Table II). These analogs also restore activity to *E. coli* tryptophanase (Snell, 1970). The *E. coli* 2'-methyl enzyme differs markedly from the *B. alvei* enzyme and is barely active having only 4% of holoenzyme activity but with a K_m (Trp) of 0.2 mM.

As was shown in Figure 2b, the binding of pyridoxal-P to apotryptophanase results in absorption maxima at 343 and 405 nm. As shown in Figure 4, the spectra of 2'-hydroxy-, 2'-methyl-, and 6-methylpyridoxal-P analog enzymes differ significantly from reconstituted enzyme and from each other.

Coenzymatically Inactive Pyridoxal-P Analogs. 4-Deoxypyridoxine-P, isopyridoxal-4'-P, pyridoxal, pyridoxamine, pyridoxine, and 4-deoxypyridoxine (2 μM to 2 mM) were unable to restore detectable catalytic activity to apotryptophanase; 0.2 mM pyridoxamine-P also did not restore activity.

Pyridoxal does not activate apotryptophanase but pyridoxal does bind to apoenzyme and competitively inhibits pyridoxal-P with a K_i of 11 mM. Pyridoxal binding was also followed using spectrophotometric titration; only 0.63 mol of pyridoxal bound per 52,000 g of apoenzyme. Fluorescence quenching of tryptophan fluorescence by pyridoxal plateaued at 22.1%. When pyridoxal-P was added incrementally to maximally quenched pyridoxal analog enzyme, further quenching of tryptophan fluorescence resulted with a total Q_{max} of 45.6% (Figure 5).

When apotryptophanase was dialyzed against pyridoxal, the pyridoxal analog enzyme formed had two absorption bands at 325 and 380 nm (Figure 4). Free pyridoxal has one band at 315 nm. The 325-nm peak intensity of pyridoxal analog enzyme was sensitive to pH in the range from pH 7.5 to 8.5 when titrated from pH 6.57 to 8.95 but the spectrum failed to show the decrease in the long wavelength peak and the increase in the short wavelength peak characteristic of holotryptophanase and reconstituted tryptophanase.

Tryptic digestion and mapping of tritium-labeled sodium borohydride reduced pyridoxal analog enzyme yielded a single titrated peptide indicating that pyridoxal binds to apoenzyme by way of a reducible covalent linkage.

Discussion

As shown in the data reported in this investigation, we have increased our knowledge about the binding of pyridoxal-P to *B. alvei* apotryptophanase. We have established using spectral techniques that apotryptophanase prepared by cysteine treatment binds 1 mol of pyridoxal-P per subunit. The same value is obtained for the binding of coenzyme modified at C_2 as in 2'-methyl or 2'-hydroxy analogs or at C_6 in 6-methylpyridoxal-P. However, the value reduces to 0.5 mol per subunit or 1 mol per dimer when apoenzyme binds pyridoxal.

The data obtained with the pyridoxal analog argue in favor of a complex cofactor binding process in which the 5'-phosphate ester has a vital role. We have shown that pyridoxal binds to apoenzyme at pyridoxal-P sites but that pyridoxal occupies only two or at most three of the four pyridoxal-P sites. Since there is no evidence that the primary structures of the pyridoxal-P binding sites differ, we propose that (1) the binding differences reflect differences in the overall conformation of the multiple subunit protein and (2) the initial interaction of pyridoxal with apoprotein is profoundly different from interaction with pyridoxal-P. We know from studies in our laboratory (Isom and DeMoss, 1975) that under the coenzyme binding conditions used in this report, *B. alvei* apoenzyme is a dimer with each dimer containing two pyridoxal-P sites. The presence of the phosphate ester in pyridoxal-P must orient the cofactor at the

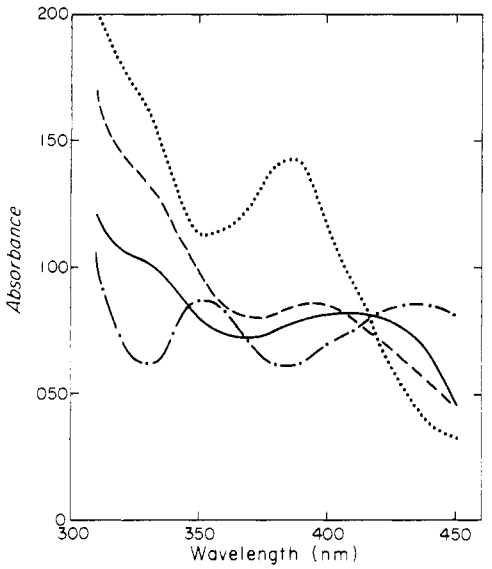


FIGURE 4: Comparative absorption spectra of apotryptophanase reconstituted with pyridoxal-P analogs. The absorption spectra of 1 mg of enzyme/ml were recorded after extensive dialysis against 0.05 *M* potassium phosphate (pH 7.6) containing 10^{-4} *M* pyridoxal (. . .), 2′-methylpyridoxal-P (- - -), 6-methylpyridoxal-P (- · · ·), or 2′-hydroxypyridoxal-P (—).

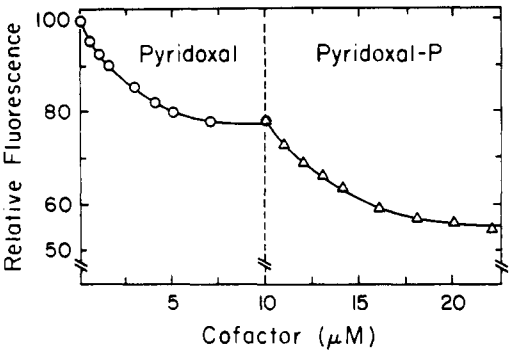


FIGURE 5: Fluorescence quenching of *B. alvei* apotryptophanase with pyridoxal followed by fluorescence quenching with pyridoxal-P. Excitation was at 280 nm; emission was measured at 350 nm 10 min after each addition. Temperature was 29°. Apotryptophanase was diluted to 0.085 mg of protein/ml of 0.1 *M* potassium phosphate (pH 7.8) before titration with pyridoxal (○) and pyridoxal-P (△). At the final point the sample contained 10 μM pyridoxal-P.

active site in a manner resulting in a conformational change that promotes filling of all the remaining sites and which favors aggregation to tetramer. The lack of a phosphate group at C_5 results in an unfavorable conformational change such that the remainder of the coenzyme sites are not filled and the enzyme remains a dimer. Failure of pyridoxal to promote tetramer formation is reported in a separate paper (Isom and DeMoss, 1975). It is also possible that the conformation the protein assumes when 2 or 3 mol of either pyridoxal or pyridoxal-P is bound is capable of binding more pyridoxal-P molecules but not pyridoxal. The pyridoxal binding suggests that all the pyridoxal-P sites within this multisubunit protein do not function identically which is in agreement with the results from studies on hybrid tryptophanase molecules from *E. coli*. That is, pyridoxal-P binding to one protomer leads to a structural change that is transmitted to other protomers (Skrzynia et al., 1974). Also, two or three pyridoxal-P molecules are required to form an enzyme with stability similar to that of holoenzyme (Raibaud and Goldberg, 1973).

We found that no analog modified at C_4 tested in this study was bound by apotryptophanase thereby confirming an absolute requirement for a Schiff's base bond between the aldehyde of the coenzyme and an amino group of the protein. Other points of attachment between coenzyme and protein were studied using analogs modified at N_1, C_2, and C_6 to form analog enzymes. Prior to studying the properties of these analog enzymes, apoenzyme was reconstituted with the natural coenzyme pyridoxal-P in order to compare its properties to native holoenzyme, and determine if the resolution procedure significantly altered the protein. Since no significant differences were observed between reconstituted enzyme and holoenzyme with regard to substrate binding, V_{max} values, sedimentation value, or tryptic peptide maps, when studying these parameters, analog enzymes can be compared to holoenzyme. However, spectroscopy showed that reconstituted enzyme differs from holoenzyme. Therefore, any interpretation of the spectra of analog enzymes requires comparison to reconstituted enzyme.

Since the state of protonation of the pyridine nitrogen directly affects ionization of the phenolic hydroxyl, we indirectly examined binding at the pyridine nitrogen by looking at ionization of the C_3 hydroxyl. For native holoenzyme we determined a pK_a of 7.9. The pK_a for free pyridoxal-P imines is 10.5 (Sizer and Jenkins, 1963) while methylated pyridoxal imines have pK_a values near 8.0 (Johnston et al., 1963). The pyridine nitrogen of pyridoxal-P may interact with a proton donating group at the active center of apotryptophanase. When the group in the protein donates a proton to the nitrogen atom of the coenzyme, the pK_a is reduced from 10.5 to 7.9, and behavior is similar to that of methylated pyridoxal-P imines in the model system.

We have calculated a pK_a of 8.4 for reconstituted enzyme. The active center of the enzyme appears to become irreversibly altered during resolution. One possible explanation for a higher pK_a value is that the structure of apoprotein becomes distorted so that the distance between the proton donating group and the ring nitrogen is altered.

We also used the *N*-oxide of pyridoxal-P to examine binding at the nitrogen position. The oxygen atom does not prevent binding completely, but the K_m for the *N*-oxide analog is 14-fold the K_m for pyridoxal-P which supports the existence of a bond between the pyridine nitrogen and the protein.

We next examined binding at the C_2 position. The kinetic data for analogs strongly suggest that the methyl group of pyridoxal-P is in close contact with apotryptophanase since small alterations in size cause large changes in binding and activity. For example, replacement of the methyl group by a hydrogen atom results in sigmoidal activation kinetics for 2-norpyridoxal-P. The 2-nor enzyme also has a sevenfold lower affinity for tryptophan. That is, deletion of the methyl group eliminates the tight bond orienting the coenzyme thereby resulting in the need for higher concentrations of substrate. Once 2-nor enzyme is saturated with tryptophan, it has 29% more activity than holotryptophanase. Catalytic activity superior to holoenzyme is also found with the 2-nor analog forms of both aspartate and arginine decarboxylase and of aspartate aminotransferase (Morino and Snell, 1967a; Tate and Meister, 1969) but not with *E. coli* tryptophanase (Morino and Snell, 1967a). *E. coli* 2-nortryptophanase has a K_m(Trp) only 2.5-fold that of holoenzyme but has only 60% as much activity as holoenzyme. In this report we have shown a few ways in which studies on the

binding and function of analogs are useful not only in understanding the mechanism of coenzyme binding but as probes able to reveal differences among enzymes as similar as the *E. coli* and *B. alvei* tryptophanases.

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