Reaction of Apoaspartate Aminotransferase with Analogs of Pyridoxal Phosphate*

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ABSTRACT: The interactions of a variety of analogs of pyridoxal phosphate with aspartate aminotransferase have been studied in an attempt to investigate the role of the functional groups of pyridoxal phosphate in binding and in catalysis. Pyridoxal phosphate *N*-oxide, *N*-methylpyridoxal phosphate, *O*-methylpyridoxal phosphate, and pyridoxal phosphate analogs which contain modified substituents in the 5 position (CH_2CH_2-COOH, $CH_2PO_3H_2$, $CH_2OPO_2(CH_3)H$, and $CH(CH_3)OPO_3$-H_2) were found to bind at the pyridoxal phosphate binding site.

This conclusion was based on the observations that (1) the analogs in the presence of apoenzyme have absorption and circular dichroism spectral properties similar to those observed with the holoenzyme. However the pK_A of 6.3 for the native enzyme is shifted to below 4 for the analogs bearing carboxyl or methylphosphonate groups in the 5 position; (2) the bound analogs undergo reversible transamination by L-glutamate; and (3) the analogs are displaced slowly from the apoenzyme by pyridoxal phosphate. Only the enzyme-bound pyridoxal phosphate *N*-oxide and α-5′-*C*-methylpyridoxal phosphate show 1% or more of the activity of pyridoxal phosphate. Some possible explanations for the very low activation of the enzyme by most of these analogs, in spite of their apparent normal binding, are discussed.

The phosphate group of pyridoxal phosphate (I)[1] is often assumed to function by merely providing a "handle" for the binding of the coenzyme to the protein part of an enzyme. That such a binding function exists is demonstrated by the weak affinity for the protein of simpler compounds such as pyridoxal or 5-deoxypyridoxal which lack the phosphate group. Nevertheless, the phosphate may also have an additional function, possibly one of acid–base catalysis. The present work concerns the behavior of analogs of PLP with apoaspartate aminotransferase (EC 2.6.1.1) of pig heart (the α subform of the cytoplasmic enzyme). Several of the analogs (structures II–VII) contain various modified side chains, most of which contain anionic groups, in the 5 position. *N*-Methylated and *O*-methylated PLP and PLP-*N*-oxide as well as the analog of pyridoxamine which bears a propionic acid group in the 5 position (XI), and which is the transamination product of II, were also studied. Both the binding of the analogs to apoenzyme and the catalytic activity of the bound analogs have been examined with the aim of understanding the function of the phosphate group of PLP.

Materials and Methods

Compounds. Pyridoxal phosphate, cysteinesulfinic acid, and α-ketoglutaric acid were purchased from Sigma Chemical Co., and oxaloacetic acid was obtained from Calbiochem. Compounds II, V, VII, and XI (Chart I) were prepared in this laboratory (Tomita and Metzler, 1964; Iwata and Metzler, 1967; Iwata, 1968; a description of the preparation of compound V is in preparation); compounds III, IV, and VI were supplied by Dr. Walter Korytnyk; compounds VIII and X by Dr. Anna Pocker (Pocker and Fischer, 1969); and compound IX by Dr. S. Fukui.

Triethanolamine buffers were prepared from crystalline triethanolamine hydrochloride and sodium hydroxide.

Enzyme. The aspartate aminotransferase used for most of the experiments was donated by Dr. W. T. Jenkins. This was highly purified α subform (Martinez-Carrion *et al.*, 1967). The absorption spectrum above 300 mμ is shown in Figure 1. The acidic and basic forms of the PLP form of the enzyme are related by a pK_A of 6.3 (Jenkins and Sizer, 1957; Jenkins *et al.*, 1959). The spectra of the single ionic forms in Figure 1 were evaluated by the method of Nagano and Metzler (1967). The spectrum of the pyridoxamine phosphate form of the enzyme (E-PMP) was evaluated from that of a mixture of E-PLP (41%) and E-PMP (59%) obtained by reaction of native E-PLP with L-glutamate followed by dialysis. It is almost identical with that obtained by treatment of native E-PLP with cysteinesulfinate (Jenkins and D'Ari, 1966; Leinweber and Monty, 1962). The extinction coefficients in Figure 1 are based on the assumption that the basic form of the PLP–enzyme has a molar extinction coefficient at the maximum at 363 mμ of 8.20×10^3, a "mole" being that amount of enzyme containing 1 mole of bound pyridoxal phosphate.[2] The apparent extinction coefficient of the apoenzyme was estimated by assuming that the bound pyridoxal

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[1] Abbreviations used are: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate.

[2] The value, ϵ 8.2×10^3, has been confirmed approximately in this laboratory by adding to the enzyme sodium hydroxide to a concentration of 0.09 M. The resulting spectrum (above 300 mμ) was that of free PLP. Using the extinction coefficient of PLP in 0.1 N NaOH of 6.60×10^3 at 388 mμ (Peterson and Sober, 1954). The value of the extinction coefficient of the enzyme at 363 mμ was estimated as 8.0×10^3.

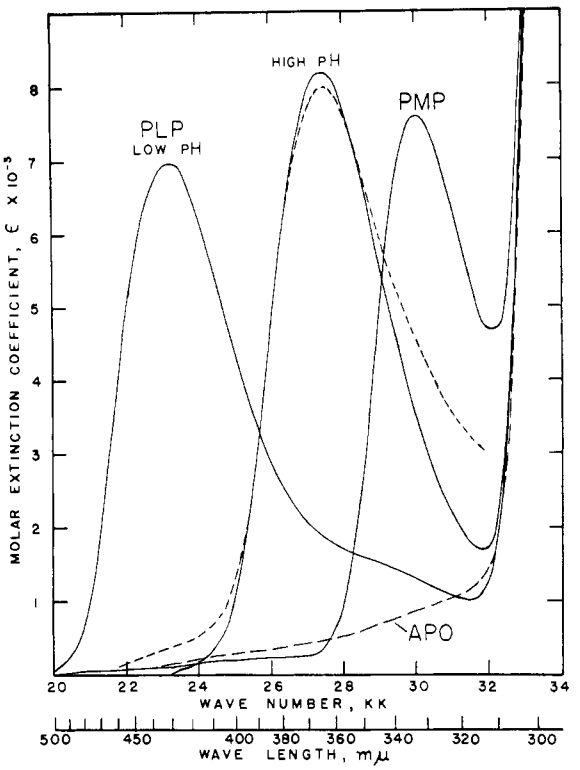
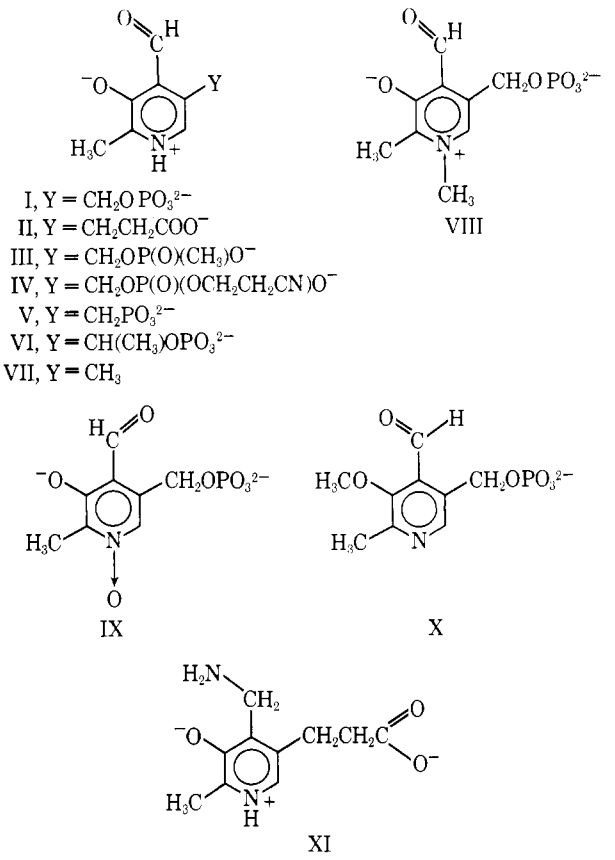


FIGURE 1: Spectrum of native aspartate aminotransferase in the low and high pH forms of the pyridoxal phosphate containing enzyme and in the pyridoxamine phosphate (PMP) form. The dotted line is enzyme reconstituted by combining apoenzyme (5.55×10^{-5} M) with pyridoxal phosphate (6×10^{-5} M). The dashed line marked APO is the spectrum of the apoenzyme. The spectra in this figure have been corrected for very small amounts of turbidity.

CHART I



phosphate has a spectrum similar to that of the Schiff's base of valine and pyridoxal (Metzler, 1957). On this basis, the ϵ of the bound coenzyme at 280 mμ would be 1.90×10^3; that of the basic form of the holoenzyme, 65.46×10^3; and that of the apoenzyme, 63.56×10^3. These values are higher than those of Banks *et al.* (1968) which are 56.4×10^3 and 54.1×10^3, for the holo- and apoenzymes, respectively, if a molecular weight of 40,000 is assumed. The discrepancy is possibly a result of there being some apoenzyme already present in the native holoenzyme used in this work. Thus, our molar extinction coefficients refer always to the coenzyme (or analog) content, and they are to be regarded only as *apparent molar extinction* coefficients throughout the region of the protein absorption band. In estimating molar concentrations of *apoenzyme* used in the experiments described, the extinction coefficient of 54.1×10^3 at 280 mμ was used.

For several of the experiments with compounds II and VII, enzyme prepared in this laboratory by the method of Jenkins (1960) was used. This was a mixture of subforms of the cytoplasmic enzyme containing a substantial fraction of pyridoxal phosphate in the inactively bound form which absorbs maximally at 340 mμ. The absorbance ratio, A_{430}/A_{340} at pH 4.8, was 1.20 (compared with 4.7 for the pure α subform) and A_{280}/A_{363} at pH 8.3 was 10.0 (compared with 7.98 for the pure α subform).

Activity Measurements. The enzyme activity was measured by the direct method of Jenkins *et al.* (1959). Enzyme (0.1 ml) was added with an adder mixer to a cuvet containing 200 μmoles of Tris at pH 8.3, 20 μmoles of α-ketoglutarate, and 20 μmoles of L-aspartate in a total final volume of 3.00 ml. The oxaloacetate formation was followed at 280 mμ in a Beckman Model DU spectrophotometer equipped with a Gilford Model 220 absorbance indicator and automatic recorder. The extinction coefficient of oxaloacetate was taken as 0.57×10^3 M^{-1} cm^{-1} (Velick and Vavra, 1962) and was used to express activity as micromoles of product per minute per milligram of enzyme at 25°. In some experiments L-glutamate or another amino acid was allowed to react with oxaloacetate and the decrease in the concentration of the latter was recorded.

Resolution of Holoenzyme. The method of Scardi *et al.* (1963) was adapted as follows. Holoenzyme stock solution (1 ml; A_{280} 35) was mixed with 24 ml of 0.2 M L-glutamate at pH 8.3 to produce the PMP form of the enzyme. After 10 min 25 ml of 1.0 M phosphate buffer (pH 4.8) was added, the solution was held at 30° for 30 min, and the protein was precipitated by addition of 150 ml of saturated ammonium sulfate solution. The precipitate was collected by centrifugation and was taken up in 1–2 ml of 0.1 M triethanolamine·HCl buffer (pH 8.3). The treatment with glutamate and phosphate and the precipitation were then repeated a second time. This time the precipitate was dissolved in a minimum of 0.1 M triethanolamine·HCl buffer and passed through a short column (1.5 × 10 cm) of Sephadex G-25 to remove residual ammonium sulfate. The protein was eluted with 0.01 M triethanolamine·HCl buffer (pH 8.3). The ratio A_{280}/A_{330} was

TABLE I: Times for Completion of Reaction of PLP and Analogs with Apotransaminase in Cuvet.

Compound	Binding to Apoenzyme pH 8.3	Reaction with Glutamate pH 8.3	Reaction with α-Keto-glutarate pH about 6.5	Act. (% of act. of native enzyme)
I (PLP)	<2 min	<2 min	<2 min (pH 7.1)	100
II (CMDPL)	<2% change after 2 min	<2 min except for slow loss of 420-mμ band	Slow, faster at pH 8.3	<0.2
III	6–7% change after 2 min	80% complete in 2 min	*Very* slow	<0.2
IV (cyanoethyl-PLP)	>20 min	Slow	Slow except for a small portion reacting fast	Variable
V	<2 min	Slow, 430-mμ intermediate	*Very slow*	<0.2
VI (*C*-methyl-PLP)	<2 min	<2 min	<2 min (pH 8.3)	3.0
VII (5-DPL)	slow increase at 430–40 mμ	<2 min for most; 430-mμ band lost slowly	Partial reaction	
VIII (*N*-methyl-PLP)	~10 min	Slow	Very slow	0
IX (PLP-*N*-oxide)	<2 min	<2 min, but a portion reacting more slowly	<2 min	Active
X (*O*-methyl-PLP)		5 min	5 min	1.0

greater than 40. (If it was not, the resolution procedure was repeated once more.)

The apoenzyme was only about 0.2% as active as the holoenzyme in the standard assay; it was activated to 90–99% of the original activity when incubated with an excess of PLP prior to assay. The spectrum is shown in Figure 1. The cause of the small shoulder at about 330 mμ is not known; it was always present in small amounts but apparently caused no catalytic activity.[3]

Reduction of Enzyme. The aldimine linkage of some of the cofactors and the protein was reduced by adding solid sodium borohydride (1 mg/ml of solution) to enzyme in 0.1 M triethanolamine buffer, pH 8.3, 0°. The reduced enzyme was dialyzed against 0.01 M triethanolamine buffer, pH 8.3, overnight.

Spectra. Absorption spectra were measured with a Cary Model 15 recording spectrophotometer equipped with a Cary-Datex digital output system and an IBM card punch. Spectra were corrected for base-line errors and, in some cases, for very small amounts of turbidity and were replotted automatically at the Iowa State University Computation Center. The turbidity correction will be described in a later publication.

Some circular dichroism spectra were measured with a Jouan dichrograph which was modified by Dr. John Foss of this department and which has been described by Johnson and Graves (1966). Its sensitivity was approximately 2×10^{-5} absorbance unit under the conditions employed. A Jasco Model ORD/UV-5 spectrometer with circular dichroism attachment was also used. Fluctuations in the recorder tracings never exceeded $\pm 0.5 \times 10^{-4}$ absorbance unit for either instrument. Both instruments were calibrated to ±1 mμ, but peak positions in the circular dichroism spectra, especially for low, broad peaks, have an uncertainty of up to ±5 mμ. Solutions of enzyme for circular dichroism spectra were from 1 to 2×10^{-4} M in coenzyme or analog.

[3] The shoulder at 330 mμ increased with the age of the apoenzyme preparation. Apparently it was not inactively bound PLP since no PLP was released from the apoenzyme in 0.1 N NaOH. Because of this change upon aging the apoenzyme was prepared in small batches and used as soon after preparation as possible.

Results

Binding of Pyridoxal Phosphate to Apoenzyme. When apotransaminase (5.55×10^{-5} M) was mixed with pyridoxal phosphate (PLP, 6.0×10^{-6} M) at pH 8.3, within 2 min a constant spectrum was obtained which was very similar to that of the native enzyme (Figure 1, Tables I and II). A small amount of absorption in the 430-mμ range is ascribed to "nonspecific binding." This "nonspecific binding" was observed to some extent with all analogs of PLP as well as with PLP itself. The amount of "nonspecific binding" usually decreased with time and the peak of specifically bound coenzyme became sharper, but in the case of PLP no significant change was seen after the initial binding over a 10-min period. The PLP was unintentionally present in a few per cent excess over the enzyme active sites, and the excess probably was in part nonspecifically bound and in part free and absorbing at 390 mμ. When PLP was added to apoenzyme in a 1:2 molar ratio, the spectrum was very similar to that of Figure 1 but the amount of 430-mμ absorption was decreased and the amount of 363-mμ absorption was increased.

The reconstituted PLP–enzyme (hereafter referred to as E–PLP) behaved like the native enzyme in every respect. Addition of 0.018 M L-glutamate at pH 8.3 caused an approximately 75% conversion to forms absorbing at 330 mμ (probably a mixture of E–PMP and ES complexes); subsequent addition of α-ketoglutarate (0.0083 M) and lowering the pH

TABLE II: Peak Positions for Absorption Bands of Coenzyme Analogs.[a]

Compound	Free Compd pH 8.3	Bound to Apoenzyme pH 8.3	pH 5.4	+ Glutamate[b] pH 8.3	+ Glutamate and[c] α-Keto-glutarate pH 6
I (PLP)	390	363	430	332	
II (CMDPL)	391	368	No change	319	323
III	390	370	No change	316	327
IV (cyanoethyl-PLP)	390	369	No change[d]	~317	326
V	369	~335, 359[e] ~420	~431	326	323.5
VI (*C*-methyl-PLP)	389	~375	~425	320	427 (pH 8.3)
VII (5-DPL)	391			321	323
VIII (*N*-methyl-PLP)	398	377	380, ~420	~335	333.5
IX (PLP-*N*-oxide)	392.5	322, ~382, ~408	~420	326	
X (*O*-methyl-PLP)	315	310 (sh)	No change	485	Loss of 485 (pH 8.3)

[a] Peaks are located to the nearest millimicron. When partially "buried," approximate values, indicated by ~, are shown. Wavelengths of peaks in millimicrons. [b] L-Glutamate was added at a concentration of 0.0182 M. [c] Peak appearing immediately after addition of α-ketoglutarate (final concentration 0.0083 M) to the mixture of enzyme, analog, and glutamate (final concentration of glutamate = 0.0167 M). [d] Except for portion of coenzyme which is apparently hydrolyzed to PLP and which absorbs at 430 mμ. [e] The major peak is at 359 mμ; the shoulder at 335 mμ is more pronounced with aged apoenzyme preparations.

TABLE III: Circular Dichroism of Bound PLP and Analogs.

Compound	pH 8.3		pH 5.4		Plus Glutamate pH 8.3	
	λ_{max}[a]	$\Delta A/A$	λ_{max}[a]	$\Delta A/A$	λ_{max}[a]	$\Delta A/A$
I	362 (363)	2.0×10^{-3}	430 (430)	3.2×10^{-3}	330 (332)	1.5×10^{-3}
II	362 (368)	1.6×10^{-3}	As at pH 8.3			
III	365 (370)	1.5×10^{-3}	As at pH 8.3			
IV	369 (370)	1.3×10^{-3}	As at pH 8.3			
V	355 (359), 415 (420)	1.0×10^{-3}	440 (430)	2.0×10^{-3}		
VII	~365 (380)	$\sim 0.2 \times 10^{-3}$				
VIII	375 (377)	2.0×10^{-3}	430 (425)			
IX	380		410 (~420)[b]			

[a] Positions of absorption maxima are given in parentheses. [b] pH 5, Ohishi *et al.* (1968).

to 7.1 partially reversed the reaction, about 50% of the bound coenzyme appearing as the abortive complex of the PLP–enzyme absorbing at 435 mμ. Lesser amounts of the 363-mμ PLP form and the 330-mμ forms were also present.

Binding of Pyridoxal Analogs to Apoenzyme. All of the compounds, II–X, appear to bind specifically to the apoenzyme as indicated by the following.

COMPOUND II (5′-CARBOXYMETHYL-5-DEOXYPYRIDOXAL). When apotransaminase (A_{280} 5.0, mixed subforms) was allowed to react with an excess (2×10^{-4} M) of II at pH 8.3 and was then passed through a 1.5 × 15 cm column of Sephadex G-25, two bands containing compound II emerged. One was not retarded and contained the protein while the other was free II. The protein fraction (E–II) possessed an absorption maximum at 368 mμ, the absorbance ratio, A_{280}/A_{368}, being 10.5. From this we assumed that the amount of II bound was about the same as the amount of PLP originally present.

In another series of experiments various amounts of II, from less than one-half the stoichiometric amount up to approximately a 1:1 molar ratio of II, and enzyme binding sites were examined with the result shown in Figure 2. The solid line labeled "8.3" in Figure 2 is the spectrum of bound II obtained from a solution of 0.29×10^{-4} M II plus 0.67×10^{-4} M apoenzyme at pH 8.3. The contribution of the apoenzyme to the spectrum in the 300–350-mμ region has been subtracted to show more clearly the shape of the peak of bound II. The spectrum of free II is shown for comparison. When the concentration of II in the same solution was raised to 0.57×10^{-4} M the molar extinction coefficient at the peak dropped by about 4% and some increased absorbance was observed in the 400–450-mμ region. Figure 3 (solid

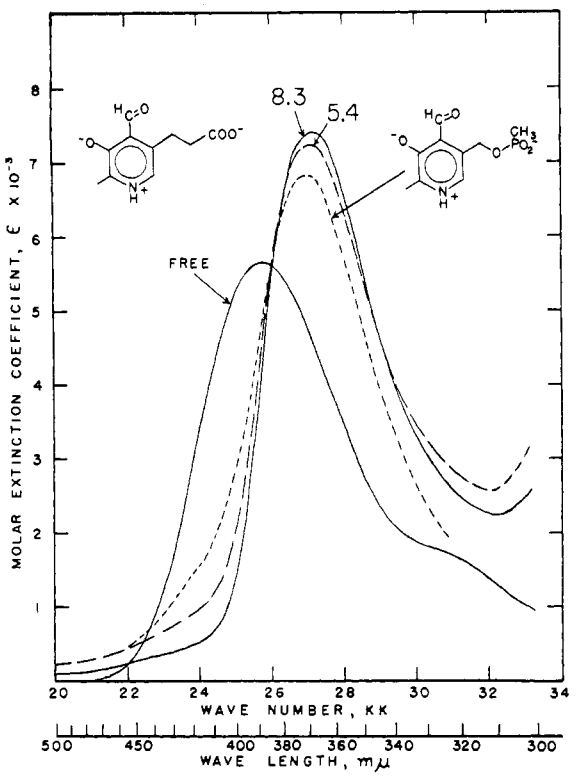


FIGURE 2: Spectra of free (pH 8.3) and bound (pH 8.3 and 5.4) II and of bound III (pH 8.3). The contribution of the apoenzyme to the spectra has been subtracted to show the spectra of the bound analogs more clearly. All spectra at pH 8.3 were obtained in 0.01 M triethanolamine·HCl (pH 8.3) and those at pH 5.4 were obtained in 0.01 M acetate buffer.

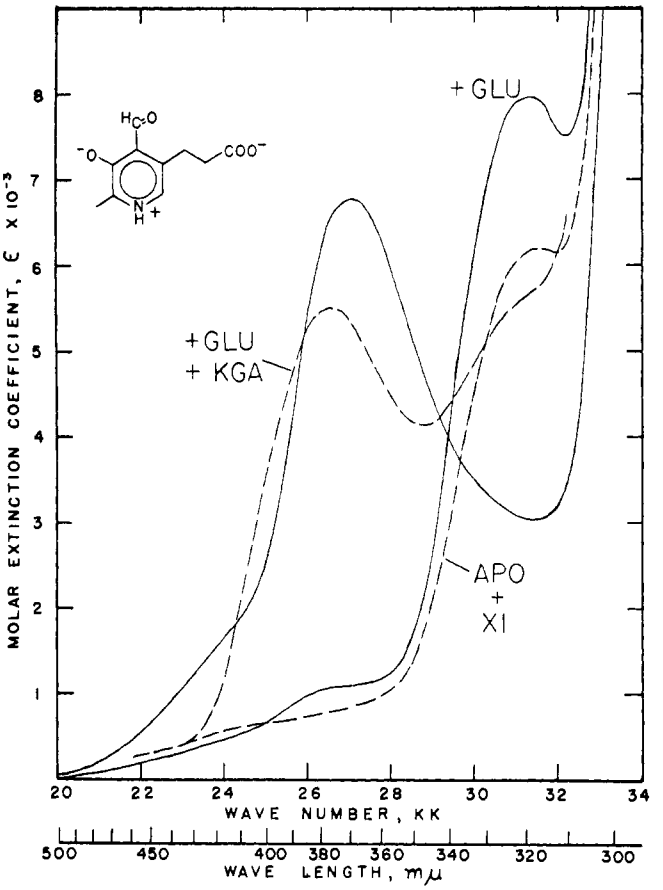


FIGURE 3: Spectra of E–II at pH 8.3 (solid lines) before and after addition of 0.018 M L-glutamate and 29 hr after subsequent addition of 0.0083 M α-ketoglutarate and change of pH to 6.5 (dashed line). The spectrum of apoenzyme plus compound XI is also shown as a dashed line similar in shape to the spectrum of E–II plus glutamate.

line) shows a spectrum obtained with 6×10^{-5} M II plus 5.55×10^{-5} M apoenzyme; the protein absorption has not been subtracted in this case. Again a somewhat lower extinction coefficient at the peak and an increased "nonspecific absorption" around 430 mμ is observed.

Not only is II bound to apotransaminase in a distinctive way with an absorption peak shifted toward lower wavelengths compared with that of free II, but the compound is bound asymmetrically as evidenced by the circular dichroism of E–II, which is shown in Figure 4 and in Table III. The positive circular dichroism band at 362 mμ is associated with the absorption band at 368 mμ and is of similar intensity as the circular dichroism band in the native enzyme (Ivanov *et al.*, 1967) and in reconstituted E–PLP (Table III). A small negative circular dichroism peak at 412 mμ varied with different preparations of apoenzyme; it is apparently associated with the "nonspecifically bound" II.

A striking difference between the native or reconstituted E–PLP and E–II is that the spectrum of the latter does not change with pH down to pH 5.4 (Figure 2). We conclude that the pK_A of the enzyme, normally found at 6.3, is shifted in the E–II to below 4.

COMPOUND III (METHYLPHOSPHONIC ACID OF PYRIDOXAL PHOSPHATE). The binding is a little slower than that of II, but the spectrum, circular dichroism, and other properties of E–III are strikingly similar to those of E–II (Tables I, II, III, and Figures 2 and 3). The shift in the pK_A of the enzyme to below 4 is also observed in E–III, and, as described below, E–II and E–III are both almost lacking in enzymic activity.

COMPOUND IV (CYANOETHYL-PLP). Binding is slow, the spectrum still changing gradually after 20 min, but the characteristic absorption peak of E–IV at 370 mμ, the positive circular dichroism, the relative insensitivity of the spectrum to pH change, and other properties are reminiscent of E–II and E–III. However, a small amount of yellow color (430-mμ absorption) did develop at low pH. More striking, as described below, E–IV gained activity over a period of several hours indicating a possible hydrolysis of the bound coenzyme to PLP. Since E–IV does not absorb strongly at 430 mμ at pH 5, an easy test of apparent conversion into PLP consists of allowing E–IV to stand at room temperature for various times, adding acetate buffer to change the pH to about 5, and observing the increased absorbance at 430 mμ accompanying conversion into PLP. In one such test an apparent 50% conversion into PLP was observed in 4.5 hr at 24°. The conversion was also followed by the activity increase as described below.

COMPOUND V. Binding of this phosphonic acid is rapid, but the spectrum of E–V is complex with bands at both 420 and 359 mμ (Figure 5). A band at about 330 mμ is also

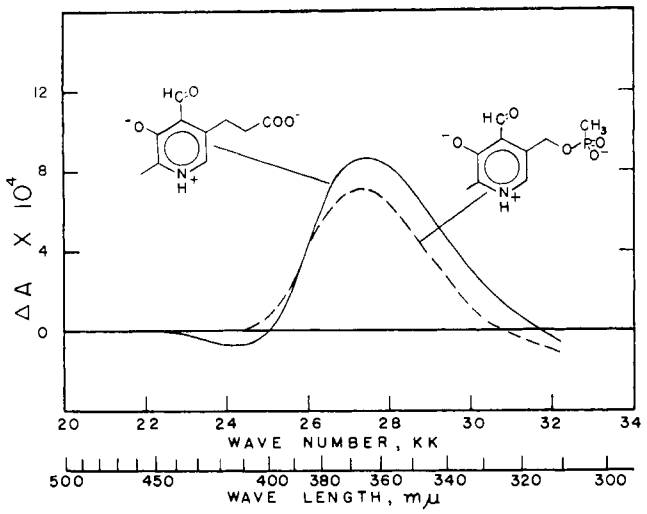


FIGURE 4: Circular dichroism of E–II (solid line) and E–III (dashed line) at pH 8.3. The solutions were 0.93×10^{-4} M in apoenzyme and 1.0×10^{-4} M in compound II or III.

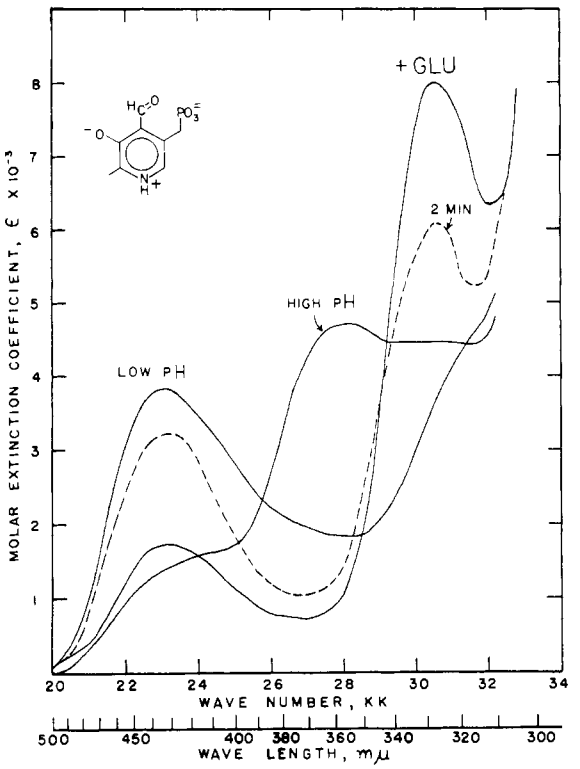


FIGURE 5: Spectra of E–V in high and low pH forms and after addition of 0.018 L-glutamate; dashed line, 2 min after addition of glutamate (pH 8.3); solid line, 35 min after addition of glutamate (pH 8.3).

present, and more so when the apoenzyme preparation has aged for 1 or 2 days. Only the 359-mμ band showed a positive circular dichroism similar in magnitude to that of other bound analogs, while the 430-mμ peak showed a weak negative circular dichroism (Table III and Figure 6). However, E–V behaved much more like E–PLP in developing an intense absorption band at 431 mμ with positive circular dichroism at low pH. The pK of this spectral shift is about 6.1; the protonated and nonprotonated forms of the enzyme are shown in Figure 5.

COMPOUND VI (*C*-METHYL-PLP). Binding is rapid at pH 8.3; the absorption spectrum is again complex (Figure 7) with a maximum at about 378 mμ and shoulders at 320 and 430 mμ. Like E–PLP and E–V, E–VI displays an increase in absorption at 430 mμ at low pH. The pK of this spectral shift is approximately 6.1.

COMPOUND VII (DPL). When apoenzyme and VII are mixed in equimolar concentrations (6×10^{-5} M) only a small change in spectrum is seen, principally an increased absorbance in the 430-mμ region. Thus a weak binding is suggested. However, addition of glutamate led to a rapid and complete turnover to deoxypyridoxamine indicating that some binding at the active site had occurred. In another experiment, an excess of VII (5×10^{-4} M) was allowed to combine with apoenzyme (1.1×10^{-4}, mixed subforms). The circular dichroism of this solution showed two peaks of very low intensity, one at 350–375 mμ (maximum $\Delta A/A = 1.9 \pm 0.5 \times 10^{-4}$). Dialysis of the solution against buffer at pH 8.3 for just 1 hr led to a 50% decrease in the circular dichroism of the peak above 400 mμ but little change in the one at 350–375 mμ. Dialysis was not complete and the spectrum of free VII was still visible after dialysis. These results suggest that VII binds weakly to the active site with a circular dichroism maximum around 365 mμ.

COMPOUND VIII (*N*-METHYL-PLP). This analog was also bound; E–VIII absorbed at 377 mμ at pH 8.3 (Figure 8). The circular dichroism was strong and the spectrum shifted partially to a form absorbing maximally at about 420 mμ at pH 5.4. However, judging by the spectrum, the pK must be a little lower than this. Both forms of E–VIII display a positive circular dichroism (Figure 6).

COMPOUND IX (PLP-*N*-OXIDE). Binding is rapid and the spectrum of E–IX is complex (Figure 8). Ohishi *et al.* (1968) have reported a positive circular dichroism as well as enzymic activity. As with E–VIII, the spectrum shifts with a decrease of pH.

COMPOUND X (*O*-METHYL-PLP). The absorption spectrum of this analog does not change appreciably when the compound is added to apotransaminase (Figure 7). E–X does have a 310-mμ absorbing shoulder, the height of which is less than the sum of the absorbancies of apoenzyme and analog alone at 310 mμ.

COMPOUND XI. This amine, which is the transamination product of compound II, appears to be only weakly bound to the apoenzyme. Whereas the spectrum of the PMP form of the enzyme possesses a maximum at 332 mμ (Figure 1), 7 mμ higher than that of free PMP (Peterson and Sober, 1954), XI absorbs at 319 mμ at pH 8.3, both when free and in the presence of apotransaminase. Furthermore, while the spectrum of E–PMP is independent of pH in the range 4.8–8.9, despite the pK of 8.6 of free PMP (Williams and Nielands, 1954), the spectrum of apoenzyme plus XI was altered by lowering the pH to 6.5 (Table II). At pH 6.5 the peak appeared at 323 mμ, just as with free XI.

Reaction of Bound Analogs with Enzyme Substrates. The reaction of the bound analogs with L-glutamate or L-aspartate was observed spectrophotometrically. In most experiments 0.018 M L-glutamate at pH 8.3 was added to the enzyme and

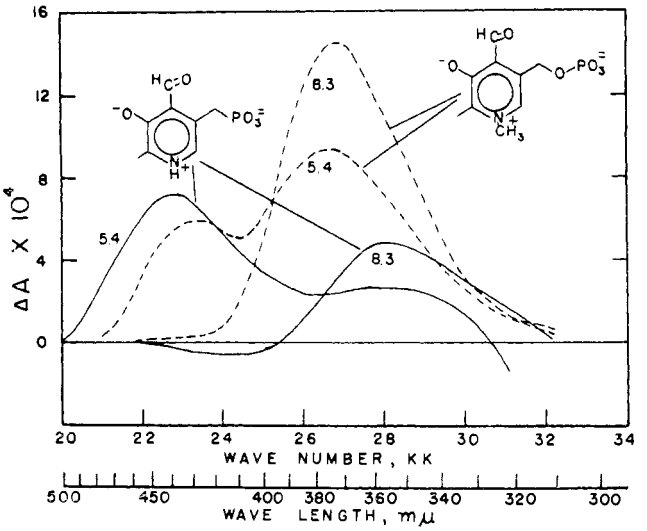


FIGURE 6: The circular dichroism of E–V (solid lines, 0.93 × 10^{-4} M apoenzyme + 1.0 × 10^{-4} M V) and of E–VIII (dashed lines, 0.53 × 10^{-4} M apoenzyme + 0.60 × 10^{-4} M VIII) at pH 8.3 and 5.4.

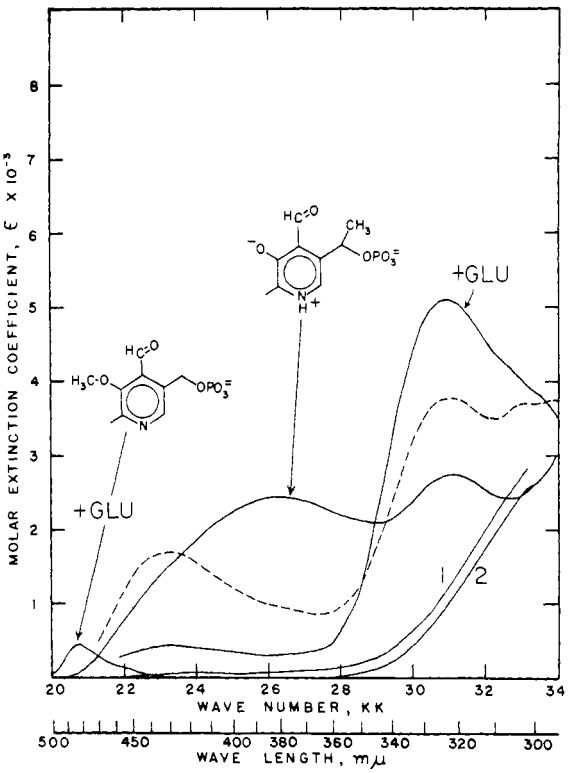


FIGURE 7: Spectra of E–VI and E–X. The spectrum of E–VI is shown at pH 8.3 before and after addition of 0.018 M L-glutamate and after subsequent addition of 0.0083 M α-ketoglutarate at the same pH (dashed line). The spectrum of E–X is shown at pH 8.3 before (line 1) and after (line 2) addition of 0.018 M L-glutamate.

the spectrum was observed over a period of time. In all cases, the band in the 360–380-mμ region which shows the positive circular dichroism and is thought to represent the specifically bound analog disappears and a new band in the 320–330-mμ region appears (Table II). This presumably represents the transamination product analogous to pyridoxamine phosphate. Final spectra following the reaction with glutamate are shown for E–II, E–V, E–VI, and E–X in Figures 3, 5, 7 (curves labeled + Glu). Compounds II, V, VI, and IX react rapidly, the turnover being almost complete in less than 2 min (Table I). The small amount of change after 2 min appears to be due largely to a slow reaction of the nonspecifically bound (430-mμ absorption) analog. In the case of II the spectrum obtained following reaction with glutamate is almost identical with that of a solution of apoenzyme plus the expected transamination product, XI (Figure 4, dashed line).

The absorption band at 319 mμ formed by transamination of E–II with glutamate or aspartate showed very little circular dichroism (as did a mixture of apoenzyme + XI), and when aspartate-treated E–II was passed through a short column of Sephadex G-25, the ratio A_{280}/A_{320} increased from 4.5 to 13.2 indicating a substantial removal of the chromophore, a consequence of the previously mentioned weak binding of XI.

Compound IV, cyanoethyl-PLP, reacts with L-glutamate more slowly. The phosphonic acid (V) also reacts slowly, but with the formation of strong transient absorption band at 430 mμ (with either L-glutamate or L-aspartate; see Figure 5). This band disappears gradually over a period of 40 min. Compound VIII (*N*-methyl-PLP) reacted the slowest of all, the reduction of the 377-mμ peak of the bound analog amounting to only 40% in 25 min.

Addition of glutamate to a mixture of compound X and apoenzyme resulted in a rapid formation of a small peak at 485 mμ and a decrease in the absorption at 310 mμ (Figure 7).

After the reaction of a bound analog with L-glutamate was complete, α-ketoglutarate (0.0083 M) was added and the reverse reaction was observed (Table I, Figure 3). In most cases, the pH was also changed simultaneously to about 6.5; with E–II and E–VI the reaction was also observed at pH 8.3. All of the compounds showed a partial reversal of the transamination reaction with some of the absorption peak of the amine form remaining at equilibrium, along with peaks at longer wavelengths associated with the aldehyde forms. Only E–PLP, E–VI, and E–IX (PLP-*N*-oxide) showed a rapid reversal (Table I); E–II reacted slowly and E–III, E–V, and E–VIII very slowly over a period of hours. E–II reacted several times faster at pH 8.3 than at pH 6.5 in the reverse reaction and it is likely that the same thing was true for other analogs. In the case of E–X (*O*-methyl) the band absorbing at 485 mμ was lost and the shoulder at 310 mμ increased.

When the α-ketoglutarate was first added to the slow-reacting enzyme–analog mixtures, with a change of pH to 6.5, a shift in absorption spectrum was observed (Table II, right-hand column). This appears to be a result of the pH change and can be taken as an indication that the amine forms of III, IV, V, and VII are probably weakly bound to the apoenzyme as is compound XI. Thus the shifts in peak position recorded in Table II are a result of changes in the state of dissociation of the free amine forms of the PLP analogs.

α-Ketoglutarate is known to form a strong "abortive complex" with the PLP form of this transaminase (Jenkins and D'Ari, 1966, 1968). The normal pK_A of 6.3 is raised in

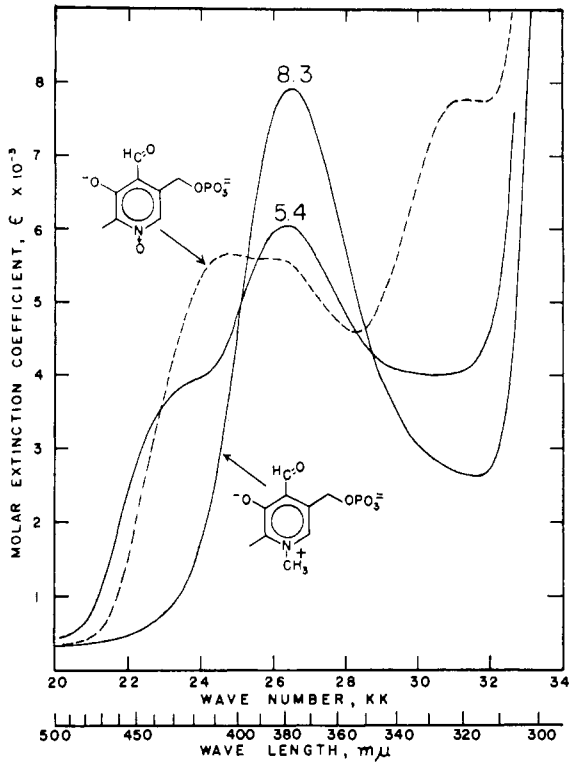


FIGURE 8: Spectra of E–VIII at pH 8.3 and 5.4 (solid lines) and of E–IX at pH 5.4 (dashed line).

this complex to above 7, so that at pH 6.5–7 a predominant form of the PLP–enzyme in the presence of α-ketoglutarate is the protonated abortive complex absorbing maximally at 435 mμ. However, when the amine forms of E–II and E–III react with α-ketoglutarate at pH 6.5, no 435-mμ absorption is observed. Rather, in the case of II, the peak of the bound analog is shifted from 368 to about 380 mμ, presumably as a result of the formation of a nonprotonated abortive complex. The complex with α-ketoglutarate possesses circular dichroism with a maximum at 272 mμ and $\Delta A/A = 7.1 \times 10^{-4}$. At pH 5.4 a distinct circular dichroism peak was observed at 450 mμ, but the enzyme–ketoglutarate complex denatured at this pH and it was not possible to obtain a satisfactory absorption spectrum. Nevertheless, it does appear that the pK of the abortive complex with α-ketoglutarate is higher than in free E–II, as is the case with E–PLP.

A mixture of apoenzyme and XI was also converted by reaction with α-ketoglutarate or oxaloacetate into the ketoglutarate complex absorbing at 368 mμ.

E–IV behaves somewhat differently in the reverse reaction, a small amount of 430-mμ absorption appearing immediately. We believe this a result of some hydrolysis to PLP having taken place and that otherwise E–IV behaves much like E–III.

Relative Catalytic Activities. All of the analogs of PLP tested showed some very slight catalytic activity in the standard assay (Experimental Section and Table I). However, only three showed substantial activity, the *C*-methyl-PLP (VI), PLP-*N*-oxide (IX), and cyanoethyl-PLP (IV). All of the others are 0.2% or less as active as PLP. E–IV gave unexpected

TABLE IV: Kinetic Constants for Enzyme–*C*-Methyl-PLP Complex (E–VI) and for Holoenzyme (E–PLP).

	E–PLP	E–VI
K_m (aspartate)	1.8×10^{-3} M	2.2×10^{-3} M
K_m (glutamate)	3.5×10^{-3} M	4.2×10^{-3} M
V_{max} (Asp → oxaloacetate)[a]	546	16
V_{max} (Glu → ketoglutarate)[a]	900	32

[a] Optical density units per minute per micromole of enzyme.

results. When assayed 5 min after mixing the apoenzyme and analog, the activity was 0.0040 mM/min but it rose gradually to a maximum of 0.0490 mM/min after 320 min at room temperature. Figure 9 shows that the increase of activity with time is a first-order process with a rate constant at pH 8.3 of 1.3×10^{-4} sec^{-1} which is equivalent to a half-life of 88 min for the reactivation.

The low activity observed with II is partially due to the previously mentioned poor binding of the amine form, XI. Thus the activity of E–II ($I \times 10^{-6}$ M) in the presence of five concentrations of XI, from 1×10^{-5} to 9×10^{-4} M, increased linearly with the concentration of XI to a maximum of seven times the activity in the absence of XI. Under these conditions, II was 1% as active as PLP.

Michaelis Constant for E–VI. The Michaelis constants for amino acid substrates and maximum velocities were determined for E–VI in the reaction of L-glutamate with oxaloacetate and in the reverse reaction by the method of Velick and Vavra (1962); the same parameters for the native enzyme were redetermined under the same conditions (Table IV). While the maximum velocities with E–IV were only about $^1/_{30\text{th}}$ those with E–PLP, both Michaelis constants were almost unchanged.

Reactivity of E–II toward Other Substrates and Inhibitors. Of other substances tested as possible substrates only L-valine showed measurable activity when 0.035 mg of E–II was assayed with 20 μmoles of amino acid and 2 μmoles of oxaloacetate in 3 ml of Tris buffer (pH 8.3). L-Valine was 37% as active as L-glutamate, whereas under the same conditions (but with 0.0035 mg of enzyme) E–PLP was only 4% as active with L-valine as with L-glutamate. The amino acids D-glutamate, L-alanine, L-serine, L-methionine, and α-methyl-DL-aspartate were all inactive with both E–PLP and E–II under the conditions of our tests.

Under the same conditions, succinate, glutarate, adipate and 2-methyl-DL-aspartate all inhibited the enzyme (from 37 to 82%) when present in equimolar concentration with the substrate, L-glutamate. Inhibition was somewhat less with E–II than with E–PLP in every case.

Substrate Inhibition. E–II is strongly inhibited by an excess of the substrate, α-ketoglutarate, much more so than is the native E–PLP (Velick and Vavra, 1962). The inhibition may be a result of the strong abortive complex of α-ketoglutarate with E–II.

Reduction with Sodium Borohydride. Reduction of E–II (prepared from mixed subforms) at pH 8.3 (Experimental Section) caused an immediate loss of the 370-mμ peak and

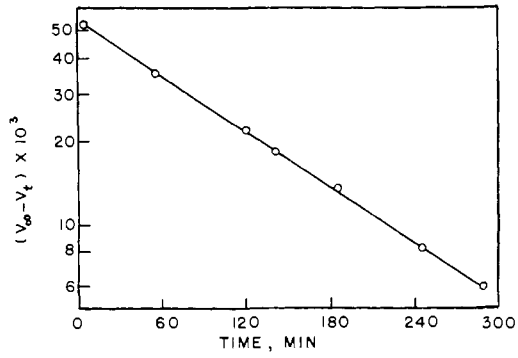


FIGURE 9: First-order plot of activity *vs.* time showing reactivation of E-IV with a half-life of 88 min. Protein (9.4×10^{-7} M) and IV (1.0×10^{-6} M) were incubated in 0.01 M triethanolamine·HCl (pH 8.3) at 23°. Aliquots were removed and assayed (see Experimental Section) at the times designated. V_t is the relative velocity at time t, and v_∞ is the final relative velocity attained.

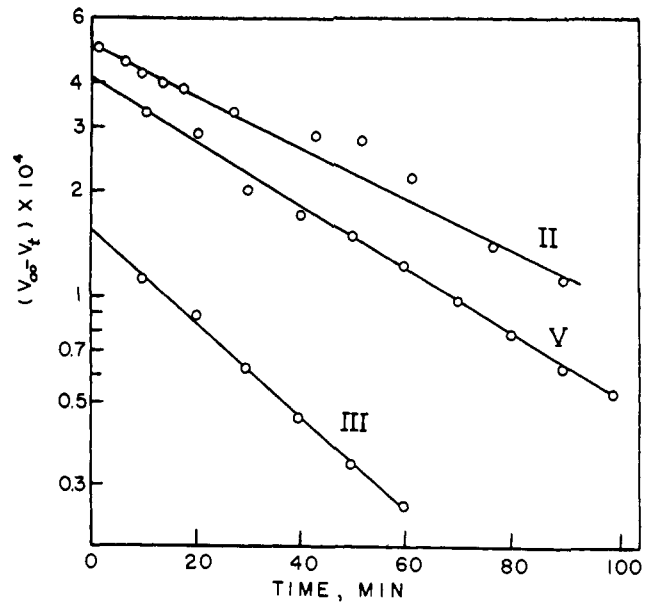


FIGURE 10: First-order plots for the reactivation of E-II, E-III, and E-V by displacement of the analog with an excess of PLP. V_t represents the relative activity at time t, and v_∞ the final activity at equilibrium. PLP (2.5×10^{-5} M) was added to the enzyme-bound analog (1.0×10^{-6} M) in 0.01 M triethanolamine·HCl (pH 8.3) at 25°.

replacement with a band at 332 mμ: circular dichroism, $\Delta A/A = 7.4 \times 10^{-4}$, $A_{280}/A_{332} = 5.03$. Incubation with an excess of PLP followed by dialysis reactivated the reduced E-II only slightly (to 0.16 mM/min per mg). Under the same conditions, a control sample of apoenzyme was activated by PLP to a level of 8.8 mM/min per mg. The spectral characteristics of reduced E-II in the presence of PLP were interesting. A 10-fold excess of PLP was added to 3 ml of reduced E-II (3.27 mg/ml) and the solution was dialyzed against 0.001 M triethanolamine buffer (pH 3) overnight. Under these conditions, the circular dichroism at 332 mμ was destroyed and a small negative peak ($\Delta A/A = 1.67 \times 10^{-4}$) appeared at 414 mμ while the absorption spectrum showed two peaks with maxima at 325 and 390 mμ. No additional activity resulted from this treatment. A second reduction of this complex with sodium borohydride followed by dialysis gave an absorption peak at 325 mμ with a peak in the circular dichroism at 329 mμ (Furbish, 1969).

Displacement of Analogs by PLP. Compounds II, III, and V were all effectively displaced from the enzyme complexes by PLP. Displacement of II was observed by direct spectrophotometry at pH 5.4. Neither bound II nor free PLP absorb strongly at 430 mμ, but bound PLP does at this pH. Addition of 0.54×10^{-5} M PLP to an equimolar concentration of E-II (0.54×10^{-5} M II + 0.62×10^{-5} M apoenzyme) led to a rapid conversion of all of the free PLP into E-PLP absorbing strongly at 430 mμ with a decrease in the 370-mμ absorption of E-II. The reaction was complete within 5 min.

In another experiment, E-II (0.20×10^{-6} M) was mixed with an equivalent amount of PLP at pH 8.3 and the activity of the enzyme was followed by diluting at various times and assaying. The activity increased gradually over a period of time to a maximum of 71% of that observed with an equivalent amount of apoenzyme plus PLP alone. Reactivation occurred over a period of 300 min. The final equilibrium activity suggests that II is bound approximately one-fifth as tightly as PLP at pH 8.3.

In a third type of experiment, compounds II, III, and V were displaced from the enzyme by incubating the enzyme-analog complex at 25° with a large excess of PLP and observing the activity.

Let the rate constants k_1, k_2, and k_3 govern the binding and dissociation of the analog, A, and PLP to the apoenzyme, E.

$$E - A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E + A$$

$$E + PLP \xrightarrow{k_3} E\text{-PLP (active)}$$

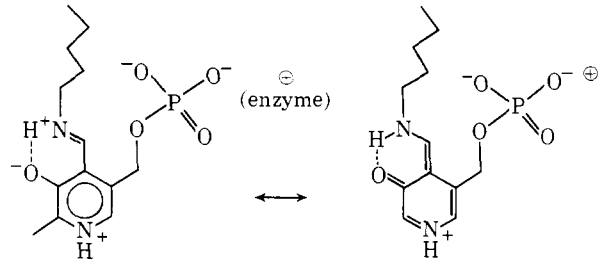
Steady-state treatment shows (Furbish, 1969) that if $k_3[PLP] \gg k_2[A]$, as is true when $[PLP] \gg [A]$, $d[E\text{-PLP}]/dt = k_1[E\text{-A}]$.

Since E-A has almost no activity as compared with E-PLP, a plot of ln (activity at $t = \infty$ − activity at time t) *vs.* time gives a straight line of slope k_1. Figure 10 shows plots of this type for displacement of compounds II, III, and V from the apoenzyme.

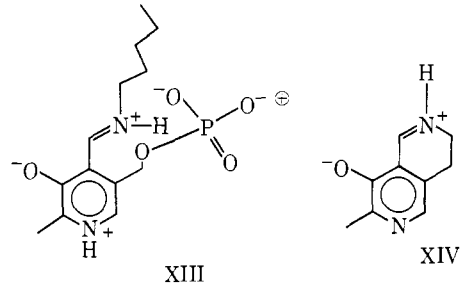
Discussion

Several observations support the conclusion that the various analogs of PLP which we have studied all bind at the PLP binding site of apoaspartate aminotransferase in a nearly normal way. (1) Spectral absorption bands of the free compounds are shifted upon mixing with apoenzyme. At pH 8.3 the shifts vary from 10 to 23 mμ toward shorter wavelengths. Most are in the 20–23-mμ range which approaches the 27-mμ shift observed with PLP. The spectra of bound V, VII, and VIII change with pH in the same way as does the spectrum of bound PLP. (2) The absorption bands of the bound compounds which appear to correspond to the 363-mμ band of bound PLP show positive circular dichroism of the same magnitude as that shown by the native enzyme. Those bound analogs whose spectrum shifts

CHART II



XII



XIII

XIV

to about 430 mμ at low pH (as does bound PLP) also exhibit positive circular dichroism in the shifted band. (3) All bound analogs appear to undergo reversible transamination with L-glutamate and L-aspartate at varying rates. While these reactions are, in most cases, very slow compared with the rate of reaction of bound PLP, they are fast compared with nonenzymic transamination. (4) The bound analogs are displaced from the apoenzyme by PLP, but the displacement is not instantaneous, and enzymic activity is regenerated over a period of 1 hr or more. This suggests that the analogs occupied the PLP binding site. (5) Compound II when bound to the apoenzyme is reduced by sodium borohydride to a form which cannot be reactivated by treatment with PLP.

Binding of PLP to apotransaminase is known to include Schiff's base formation with an ε-amino group of a lysine residue and is also thought to involve electrostatic interaction of the phosphate group with some group on the enzyme. Structure XII (Chart II), shown in two resonance forms, is usually assumed for the low pH, protonated, form. At higher pH, the hydrogen-bonded proton dissociates with a pK of 6.3 to give the dipolar ion form which absorbs at 363 mμ. The pK of 6.3 seems too low for this structure, but the presence of another positively charged site in the enzyme (possibly the binding site for α-carboxyl group of substrate) nearby might lower the pK to 6.3.

An alternative conformation of the bound PLP is XIII, in which the imine double bond lies near the phosphate group.

That XIII might possess the requisite spectral properties is shown by the study of compound XIV (Fisher and Metzler, 1969). Compound XIV absorbs at 430 mμ with a shift upon deprotonation to 364 mμ and a pK of 8.6. Although we are not proposing that structures of type XIII exist in enzymes, it is clear that the possibility must be considered. It is also clear from study of XIV and other Schiff's bases that the state of protonation of the pyridine ring nitrogen has little effect on the spectrum and that it must be considered another unknown in the chemistry of the active site of PLP–enzymes.

The most striking feature of the bound analogs II, III, and IV is that they *do not* undergo a spectral shift to 430 mμ at low pH as does bound PLP. We suggest that this is a result of a shift in the pK of the protonated imine from 6.3 in E–PLP to below 4 in E–II, E–III, and E–IV. This concept is confirmed by the fact that formation of a complex with glutarate, which is known to shift the pK of the native enzyme from 6.3 to 8.7 (Jenkins and D'Ari, 1968), does apparently raise the pK of E–II to about 4.7.[4]

Why does replacement of the phosphate group of pyridoxal phosphate by a carboxylate group (II) or a methylphosphonate group (III) lower the pK by 2.5 units or more? The first possibility is that it is simply a charge effect. Compounds II and III possess one less negative charge in the side chain than does PLP, or compounds V, VI, VIII, or IX. (It may be of significance that complete desalting of ox heart aspartate aminotransferase leads to a shift of the pK from 6.3 to less than 4.5 (Marino *et al.*, 1966), a change which may result from a loss of an anion from the active center (Fasella, 1967).) One of the charged oxygen atoms can be very close to the aromatic ring, and it is possible that a strong electrostatic effect is transmitted through the ring in a structure analogous to XII but with the phosphate turned back above the ring. On the other hand, in structure XIII a strong electrostatic effect on the pK would be expected. However, it is not clear why the pK with compounds II and III would be as low as is observed.

A second possibility for explaining the shift in pK is that binding of the phosphate group to the enzyme is accompanied by a change in the conformation of the protein which favors the easy protonation of the imine group. If this is the case, it is a little surprising that the methylphosphonic acid III cannot induce the conformation change but that phosphonic acid V can, even though its side chain is one atom shorter than that of PLP.

Some of the inactivity of II and III lies in the slow reversal of the transamination, *i.e.*, the slow reaction of the amine form (*e.g.*, XI) with α-ketoglutarate. This in turn is partly a result of the weak binding of the amine forms. For instance, E–II is seven times more active in the presence of a high concentration (9×10^{-4} M) of XI than in its absence. The same has been observed by Wada and Snell (1962) for pyridoxal and pyridoxamine. In the case of pyridoxal and pyridoxamine a maximum turnover number of about 10/min was obtained in the presence of very high concentrations of pyridoxal and pyridoxamine. In our experiments it was not possible to obtain saturation with XI because the high absorbance of XI interferes with the assay. However, we believe it unlikely that the K_m for XI would be as high as that for pyridoxamine (2.4×10^{-3} M, Wada and Snell, 1962). If we achieved half-saturation with XI the maximum activity of II would be only 2% of that of PLP.

Not only are II and III far inferior to PLP as coenzymes for transaminase, but the even more closely related phosphonic acid analog containing the group $CH_2CH_2PO_3^{2-}$ in the 5 position is reported to be 7% or less as active as PLP (Hullar, 1969). The unsaturated phosphonic acid, with the side chain $CH{=}CHPO_3^{2-}$ (*trans*), is 2% or less as active as PLP. Judging by inhibition data, both combine quite tightly

[4] Unpublished work of M. L. F.

with the apoenzyme. These results and our observations with compounds II–V suggest that the phosphate group of pyridoxal phosphate may serve as more than simply a handle for binding the coenzyme to the protein. One possible way in which the phosphate could function is, through its particular spacial arrangement of charged oxygen atoms, to orient certain groups in the protein and to dictate a specific conformation or set of conformations of protein groups essential for catalysis. A second possibility is to participate directly in catalysis.

The transamination reaction must occur in several discrete steps, some of which are depicted in Scheme I. This reaction sequence has been drawn to show a possible way in which the phosphate group might participate as a proton acceptor (step 2) and donor (step 4).[5] The findings of Hullar (1969) suggest that the bridge oxygen, rather than or in addition to the one shown in Scheme I, may participate in the catalysis.

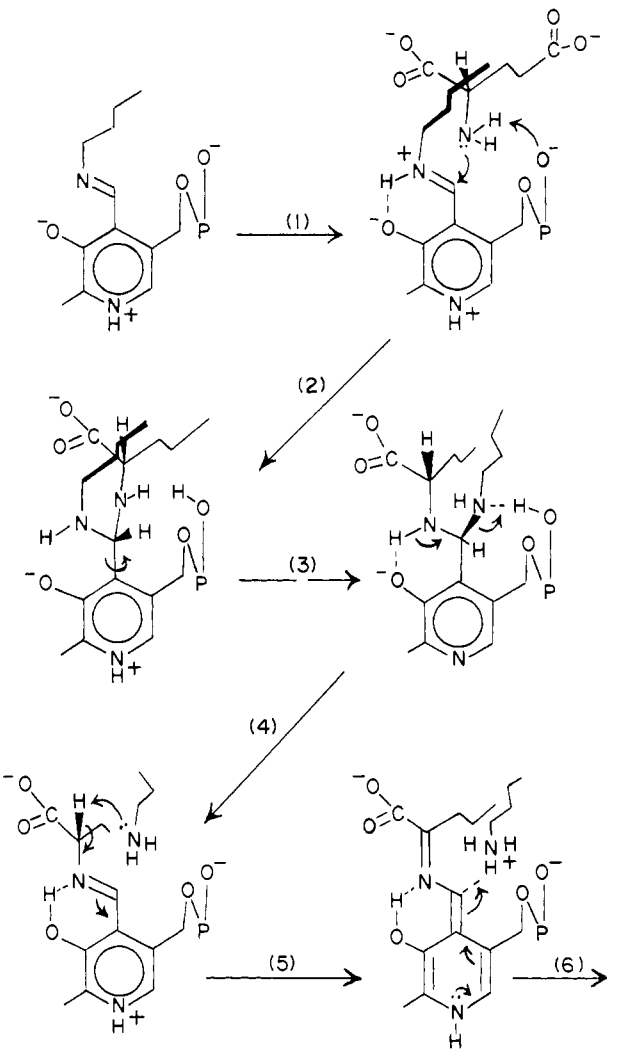
Compound V reacts extremely slowly on the enzyme surface with accumulation of an intermediate absorbing at 435 mμ, probably an ES Schiff's base (product of step 4, Scheme I). This suggests that with this analog, step 5 is extremely slow, but it is not obvious why this should be so. Preliminary studies suggest that V is displaced from the enzyme easily by P_i and it may be that the substrate Schiff's base is not held sufficiently rigidly for efficient catalysis to occur.

Judging by the absorption spectrum and circular dichroism, compound VIII appears to bind normally, but it is the slowest of all the analogs to react and does not yield a detectable concentration of ES Schiff's base.

Karpeisky and Ivanov (1966; Ivanov and Karpeisky, 1969) have emphasized that the state of protonation of the groups at the active site varies for different stages of the reaction; at each stage the groups may be protonated in such a manner as to facilitate the next stage. Thus in step 1 not only does the transfer of a proton to the imine group generate a nucleophic NH_2 group from the original NH_3^+ of the substrate, but it also creates a more electrophilic center in the imine double bond, facilitating step 2. As pointed out by these authors, the state of protonation of the pyridine ring is also crucial. Thus, for step 4, the ring nitrogen may need to be unprotonated. This would have the effect of making the phenolate ion in the 3 position much more nucleophilic. If the deprotonation were accomplished by shifting the proton (*e.g.*, *via* an imidazole bridge) to a position closer to the 5-phosphate group it would also make the protonated phosphate group a better proton donor, the two effects working in concert. The reactivity of PLP-*N*-oxide (IX) may be a consequence of the polarizability and proton-accepting ability of the *N*-oxide group.

[5] Scheme I shows one of several ways in which the reaction could occur. The sequence shown, in which the substrate adds to the C=N bond from the back side, was chosen on the basis of the following. (1) It leads to the correct conformation of the ES Schiff's base required by Dunathan's tentative conclusion regarding stereochemistry of transamination of pyridoxal and glutamate with this enzyme (Dunathan *et al.*, 1968). (2) It leaves the ϵ-NH_2 group on the right side of the substrate to participate in catalysis at step 5, a very attractive idea proposed by Snell and Jenkins (1959). The addition of substrate to a C=N bond rotated about 90° out of the plane of the pyridine ring can also fulfill these criteria. Note that Scheme I can be related to the proposal of Ivanov and Karpeisky (1969) if steps 2 and 3 are combined into a single step involving a rotation of the pyridine ring.

SCHEME I



The small peak at 485 mμ observed with E–X (*O*-methyl-PLP) is probably related to the 490-mμ band formed by reaction of the enzyme with the pseudosubstrate, *erythro*-β-aspartate (Jenkins, 1964). This is thought to be a paraquinoid structure, the product of step 5.

The reactivation of E–IV, the cyanoethyl derivative, is unexpected and suggests the participation of a group of the enzyme in promoting the cleavage of either the O–C bond (elimination of cyanoethylene) or the O–P bond by hydrolysis or by some other displacement mechanism. It is conceivable that the phenomenon is related to the phosphorylation of a threonine residue of apotransaminase by reaction with *N*-pyridoxyl-5-phospho-L-glutamate, an analog of the substrate–coenzyme Schiff's base (Khomutov *et al.*, 1969).

Acknowledgments

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