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Pyridoxamine-Pyruvate Transaminase. 1. Determination of the Active Site Stoichiometry and the pH Dependence of the Dissociation Constant for 5′-Deoxypyridoxal[†]

Penny J. Gilmer,[‡,§] William S. McIntire,[‡] and Jack F. Kirsch*

ABSTRACT: Spectrophotometric titration of pyridoxamine-pyruvate transaminase (EC 2.6.1.30) with pyridoxal at pH 7.15 gives four equivalent binding sites per tetramer. The pH dependence of the equilibrium constant for the association of 5′-deoxypyridoxal with the active site lysine residue was determined spectrophotometrically. These dissociation constants increase with increasing pH over the range pH 7.5–9 and are correlated with the values obtained from fast reactions kinetics (Gilmer, P. J., and Kirsch, J. F. (1977), *Biochemistry 16*

(following paper in this issue)). In addition to this specific reaction at an active site lysine residue, a second slower reaction at non-active site residues is observable at pH values greater than 8. The pH dependencies of the association and dissociation rate constants for this slow reaction were studied over the pH range 8 to 9 after blocking the active site by NaBH₄ reduction of the pyridoxal adduct. The enzyme is stabilized and markedly activated by potassium ion.

Pyridoxamine-pyruvate transaminase (EC 2.6.1.30) (PPT)[1] is unique among pyridoxyl dependent transaminases in that

the vitamin B-6 derivative acts not as a cofactor but is itself a substrate in the enzymatic reaction shown in Scheme I. Wada and Snell (1962) isolated PPT from *Pseudomonas* sp. MA-1 grown on pyridoxamine as the source of carbon and nitrogen. This enzyme is part of the degradative pathway for B-6 compounds utilized by this organism. The enzyme was charac-

[1] Abbreviations used are: 5′-deoxy-PL, 5′-deoxypyridoxal; PPT, pyridoxamine-pyruvate transaminase; PL, pyridoxal; PM, pyridoxamine; PLP, pyridoxal 5′-phosphate.

SCHEME I

E + PM ⇌ E · PM

$$E \cdot PM + \text{pyruvate} \rightleftharpoons EXY$$

$$EXY \rightleftharpoons E \cdot PL + \text{L-Ala} \quad (1)$$

$$E \cdot PL \rightleftharpoons E + PL$$
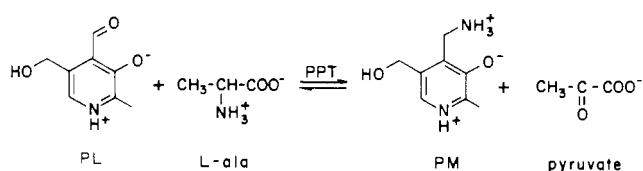
terized by Dempsey and Snell (1963) and shown to contain an active site lysine residue which forms a Schiff base with the substrate, pyridoxal. Ayling and Snell (1968a,b) carried out extensive steady-state and stopped-flow studies at pH 8.85 and determined that the kinetic mechanism was ordered with the pyridoxyl derivative binding first and the three-carbon substrate second (eq 1).
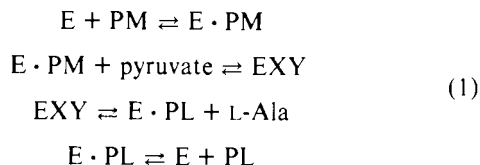
The ease of purification and the mechanistic simplicity of this half transaminase taken together with the fact that various vitamin B-6 analogues can be substituted for pyridoxal and pyridoxamine make this enzyme an attractive choice for a detailed examination of its mechanism of action by rapid reaction methods. Preparatory to such an investigation (Gilmer and Kirsch, 1977), the studies reported here were undertaken in order to determine the number of active sites per tetramer and the pH dependence of the enzyme–substrate dissociation constants. A preliminary account of this work has been presented (Kury and Kirsch, 1973).

Experimental Section

Materials and Methods. Potassium pyrophosphate was obtained from Ventron; 5'-deoxy-PL was a gift from Professor E. E. Snell and was purified by sublimation at 56 °C (mp 109–109.5 °C (lit. mp 111.5–113 °C, Mühlradt and Snell, 1967)). Contaminating material that would not sublime at that temperature was identified as 5'-deoxypyridoxol (mp 179 °C (lit. mp 181–182 °C, Heyl et al., 1953)). Thin-layer chromatography of the purified 5'-deoxy-PL on an Eastman 6060 silica gel chromatogram showed one spot of R_f 0.45 with chloroform as eluent.

All experiments were performed in buffer solutions prepared from freshly degassed glass-distilled water. The ionic strength was adjusted with potassium chloride and the pH determined on a Radiometer type PHM 4C meter.

The difference spectra were determined using split cells with the reference cell containing 5'-deoxy-PL in one compartment and PPT in the other and with the sample cell containing the two components in both compartments. The difference spectra and the determinations of the binding constant, K_d, of PPT for 5'-deoxy-PL were obtained on a Unicam spectrophotometer Model SP-800A at 25 °C.

Growth of Bacteria. PPT, an inducible enzyme, was obtained from the pure culture of *Pseudomonas* MA-1 grown on pyridoxol as the prime carbon source. The cells were grown as described previously (Guirard and Snell, 1971) except that a 200-L fermentor at 30 °C was used. The fermentation required 55–65 h to attain the maximum growth of the cells using a 2.5% inoculum and 4 ft³/min aeration.

Purification of PPT. The purification procedure of Ayling and Snell (1968a) was followed except that a Manton-Gaulin cell mill was used to break the cells. A solution of 700 mL of cold 0.02 M potassium phosphate buffer, pH 7.0, was used to

suspend 300 g of wet cells. The mill was initially chilled by running through 3 L of ice water. The slurry of cells was passed through the mill once and the eluate chilled in an ice bath. The temperature was not allowed to go above 30 °C. The mixture was then cooled to 20 °C and run through the mill a second time following the same procedure. A very thick gelatinous mass forms if the partially broken cells are cooled below 20 °C after only one to two times through the mill. The slurry is passed through the mill a total of four to five times.

The assay for enzyme activity employed the phenylhydrazine method of PL determination (Dempsey and Snell, 1963). Specific activity was 19–22 μmol of PL min⁻¹ mg⁻¹ at 37 °C. The enzyme was shown to be at least 90% homogeneous by isoelectric focusing (O'Farrell, 1975). Two minor bands, corresponding to more acidic proteins, were detectable.

Resolution of the Enzyme. Since the enzyme is crystallized in the presence of PL, it is necessary to remove all of it before adding substrate analogues to the resolved enzyme in any determination of dissociation or equilibrium constants. PL-free enzyme was obtained by a procedure similar to that of Dempsey and Snell (1963) by dialyzing at 4 °C 1–2 mL of enzyme at a concentration 10–20 mg/mL against 200 mL of 0.05 M Tris (Tris-HCl), 1 mM L-alanine, pH 8.50 (for 8 h), followed by 200 mL of 0.05 M Tris (Tris-HCl), pH 8.50 (for 8 h), and finally by two 200-mL changes (8 h each) of the buffer to be used in the experiment.

Determination of the pH Dependence of K_H. The pH dependence for K_H, the equilibrium constant for the formation of 5'-deoxy-PL hydrate from free aldehyde and water, was calculated using the spectral data and the microscopic dissociation constants reported by Johnson and Metzler (1970). The reported value for the pK_a of 5'-deoxy-PL of 8.14 is a composite of the microscopic dissociation constants of the aldehyde and hydrate forms (Johnson and Metzler, 1970).

Absorbancy. The optical density of a 1 mg/mL solution at 280 nm of the pure resolved enzyme was determined by three independent techniques: (1) the specific refractive increment method using the dialyzing buffer as the reference solution (where 40.5 fringes is equivalent to 10 mg/mL for any nonheme protein) (Richards et al. 1968; Babul and Stellwagen, 1969); (2) the Kjeldahl method of nitrogen determination (Ma and Zuazaga, 1942), based on a N content of 16.4% calculated from the amino acid composition shown in Table I (16.0% N) and on an ammonia concentration of ca. 0.4% N for a molecular weight of 148 000 (Kolb et al., 1968); and (3) amino acid analysis (see below). The A_{280} (1 mg/mL) were: 1.3 ± 0.1 (Kjeldahl), 1.24 ± 0.02 (fringe count), and 1.25 ± 0.03 (amino acid analysis). The absorbance at 280 nm (1 cm pathlength) at a concentration of 1 mg/mL in 0.02 M potassium phosphate buffer is thus 1.25 ± 0.05. The value of the extinction coefficient at 280 nm used by Ayling and Snell (1968a) was 0.975 for 1 mg/mL, so the present figure represents an upward revision.

Trace amounts of PL raise the absorbance at 280 nm; therefore the protein concentration is routinely determined by the method of Lowry et al. (1951). The concentration of the standard bovine serum albumin stock solution was determined by the absorbance at 280 nm which equals 0.667 for 1 mg/mL (Lever, 1972). It was found that [PPT] determined by A_{280} = [PPT] (determined by Folin) × 0.97. The molar concentration of PPT is given in terms of the 37 000 molecular weight subunit unless specified in terms of the tetramer.

Amino Acid Analysis. A solution of PPT (A_{280} = 0.885) was prepared in 1.00 mL of 0.02 M potassium phosphate buffer, pH 7.00, which contained about 25 nmol of each of three internal amino acid standards, α-aminobutyric acid,

norleucine, and ε-aminocaproic acid (Cavins and Friedman, 1970).

Three 0.2-mL aliquots of the above enzyme solution were hydrolyzed in 0.2 mL of 12 N HCl containing a small crystal of phenol at 100 °C for periods of 24, 48, and 72 h. The hydrolysates were analyzed on a Beckman Model 120C amino acid analyzer. An unhydrolyzed enzyme sample was prepared by diluting 0.2 mL of the original enzyme solution into 2.3 mL of citrate application buffer. This solution was used to obtain the ninhydrin color values for each internal standard and to correct for systematic error.

α-Aminobutyric acid eluted between alanine and valine with the acidic elution buffer, norleucine eluted between leucine and tyrosine with the neutral buffer, and ε-aminocaproic acid eluted before histidine with the basic buffer. The results of the amino acid analyses and extinction coefficient calculation are given in Table I of the microfilm edition.

Preparation of N-Pyridoxyl-PPT. Resolved PPT (67.5 μM) was incubated with PL (310 μM) in 0.01 M potassium phosphate, pH 7.15, ionic strength = 0.15 for 1 min at 25 °C. The PL was then fixed covalently to the PPT at the active site lysine residue by addition of $NaBH_4$ to a final concentration of 100 μM. The yellow color of the Schiff base was bleached by the borohydride treatment. The solution was dialyzed against two 200-mL changes of 0.05 M potassium pyrophosphate (pH 8.55), ionic strength = 0.5. The ultraviolet spectrum of the dialyzed N-pyridoxyl-PPT has peaks at 280 and 325 nm (in 0.02 M potassium phosphate, pH 7.00) with a ratio of peak heights (A_{280}/A_{325}) of 7.0. The 325-nm peak is due to the pyridoxyl moiety on the enzyme. The enzyme was totally inactivated by this treatment. A control sample of PPT treated only with $NaBH_4$ in the absence of PL had 76% of the original enzyme activity.

Kinetics of the Slow Reaction of 5'-Deoxy-PL with N-Pyridoxyl-PPT. The dialyzed N-pyridoxyl-PPT was diluted into 0.05 M potassium pyrophosphate buffer to give a final concentration of the enzyme of 20 μM with a final pH of 7.98, 8.56, or 8.93. Small aliquots (3–7 μL) of 5'-deoxy-PL stock solution (0.060 M in ethanol) were added to 1.0 mL of the N-pyridoxyl-PPT. The kinetics were followed at 430 nm at 25 °C. Semilog plots of the exponential traces were linear over at least 1.5–2 half-lives.

The Effect of Potassium Ion on PPT Stability and Activity. The enzyme was observed to lose activity over a period of 12 h at 25 °C at a concentration of 50 μg/mL in a sodium containing buffer (0.01 M sodium phosphate buffer, pH 7.15, μ = 0.15 (NaCl)) but was stable at concentrations of 6 to 70 μg/mL when maintained in a potassium containing buffer (0.01 M potassium phosphate buffer, pH 7.15, μ = 0.15 (KCl) or 0.02 M potassium phosphate buffer, pH 7.0).

The enzyme activity was determined as a function of potassium ion concentration using the phenylhydrazine activity assay (Dempsey and Snell, 1963). The assay buffer is sodium pyrophosphate ([Na^+] = 0.407 M). The addition of small amounts of potassium phosphate to this buffer leads to a substantial enhancement of enzyme activity, e.g., 70% at 0.03 M potassium phosphate. Higher concentrations of potassium phosphate result in a gradual diminution of activity. Because of the stabilization and activity enhancement effected by potassium ion, the kinetic and equilibrium studies reported herein were carried out in 0.05 M potassium pyrophosphate buffers at ionic strength 0.35–0.5 except where noted.

Results and Discussion

Two Classes of Binding Sites. A PPT–5'-deoxy-PL difference spectrum taken as described in the Experimental Section
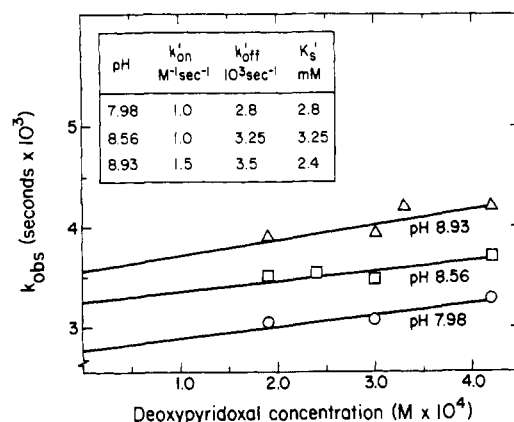

FIGURE 1: Rates of reaction of 5'-deoxy-PL with N-pyridoxyl-PPT. Conditions: N-pyridoxyl-PPT = 20 μM and [5'-deoxy-PL] = 180–500 μM in 0.05 M potassium pyrophosphate buffers, μ = 0.5, 25 °C. The reactions were initiated by the addition of 5'-deoxy-PL to the enzyme solution. (Insert) Table of derived rate and equilibrium constants.

indicated large initial difference peaks at 285, 380, and 435 nm whose amplitudes increased by about one-third within 30 min after the initial spectrum was taken. The initial differences are due to the reaction of 5'-deoxy-PL at the active site lysine residue, while the subsequent slow change can be attributed to the reaction of 5'-deoxy-PL with non-active site lysine residues.

Slow Reaction of 5'-Deoxy-PL with PPT. The slow reaction can be studied in isolation after blocking the PL adduct of the active site by reduction with $NaBH_4$. This reaction was carried out at pH 7.15 where reaction with non-active site lysines is negligible. The slow reaction of 5'-deoxy-PL with PPT probably represents Schiff base formation with non-active site lysine residues because of the spectral similarity of the product with model compounds (Johnson and Metzler, 1970).

The dissociation constant for 5'-deoxy-PL at the active site is at least a factor of 80 less than that at the non-active sites in the pH range studied. Therefore, the slow reaction is detectable only when [5'-deoxy-PL$_t$] > [E$_t$] or when the active site lysine residues are otherwise blocked. It appears from the magnitude of the absorbance changes assuming an average $\Delta\epsilon_{430}$ = 3750 for the Schiff base (Gilmer and Kirsch, 1977) that 35–45 non-active site lysine residues out of the total of 60 per tetramer are available to react with 5'-deoxy-PL in the time range studied.

The association and dissociation rate constants for the slow reaction of N-pyridoxyl-PPT with 5'-deoxy-PL were studied as a function of pH. Pseudo-first-order kinetics were obtained for at least 1.5–2 half-times indicating that all of the reacting non-active site lysines react with nearly the same rate constant. All symbols are primed for the slow reaction (eq 2)

$$N\text{-pyridoxyl-PPT} + 5'\text{-deoxy-PL} \underset{k'_{off}}{\overset{k'_{on}}{\rightleftharpoons}}$$

$$N\text{-pyridoxyl-PPT}-5'\text{-deoxy-PL}$$
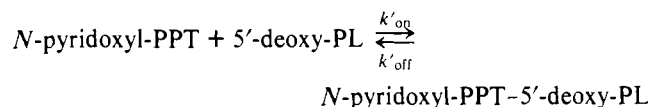
where

$$K'_s = \frac{k'_{off}}{k'_{on}} \qquad (2)$$

The rate constants for a reversible second-order reaction run under pseudo-first-order conditions can be obtained from a plot of k_{obsd} vs. ligand concentration with the slope = k'_{on} and the y intercept = k'_{off} (Jencks, 1969) (Figure 1).

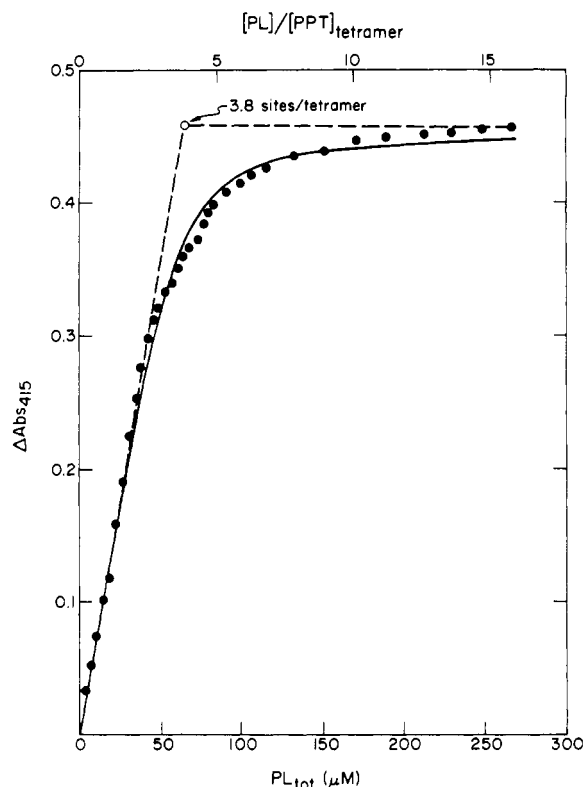*Spectrophotometric Determination of the Number of Catalytic Sites per Tetramer.* PPT was titrated spectropho-

FIGURE 2: Spectrophotometric titration of PPT. PPT (2.5 mg) in 1 mL of 0.01 M sodium phosphate buffer at pH 7.15, μ = 0.15 at 25 °C, was titrated with successive additions of PL. The curve represents the nonlinear least-squares fit to eq 4 with K and PPT_t as adjustable parameters. The minimizing values were K = 3.2 μM and PPT_t = 63.8 μM. The latter figure gives 3.8 binding sites per tetramer based on a molecular weight of 148 000.

tometrically at high protein concentrations with PL at pH 7.15 where PL is found to exhibit the highest affinity for the enzyme. The slow reaction of PL with nonactive site lysines is insignificant at neutral pH. For the 2 → 1 association given in eq 3
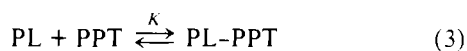
$$PL + PPT \underset{}{\overset{K}{\rightleftharpoons}} PL\text{-}PPT \qquad (3)$$

the concentration of liganded enzyme [PL-PPT] is expressed by eq 4

[PL-PPT] =
$$\frac{K + PL_t + PPT_t - \sqrt{(K + PL_t + PPT_t)^2 - 4PL_tPPT_t}}{2}$$
$$(4)$$

Figure 2 shows a plot of ΔAbs_{415} (corresponding to a maximum in the PPT + PL vs. PL-PPT difference spectrum) vs. PL_t. The equivalence point is reached at a stoichiometry of 3.8 active sites per tetramer, based on a molecular weight of 148 000 for the tetramer (Kolb et al., 1968). This new value of one active site per monomer represents an upward revision from the previously determined value of one site per dimer at pH 8.85 measured under conditions of lower enzyme concentration (1 mg/mL) (Ayling and Snell, 1968a) and calculated assuming a lower extinction coefficient for the protein.

The enzyme was also titrated spectrophotometrically at pH 8.85 at a concentration of 2.3 mg/mL. The observed titration curve was biphasic and showed approximately four high affinity sites reacting within the mixing time followed by addi-

tional PL reacting with a slower reaction rate and having a weaker affinity for PPT (see above).

Four binding sites have also been determined by spectrofluorimetric titration at pH 7.0 (J. F. Kirsch and H. Winkler, unpublished results). Still further evidence for four active sites per tetramer is provided by an analysis of the amplitudes of temperature-jump experiments as a function of concentration (Gilmer and Kirsch, 1977). In all cases simple hyperbolic binding isotherms were obtained indicating the absence of any substantial amount of positive or negative cooperativity.

The following enzymes that contain PLP as a natural cofactor have one PLP binding site per subunit (Snell and Di-Mari, 1970): arginine decarboxylase (10 subunits); aspartate β-decarboxylase (12 subunits); aspartate aminotransferase (2 subunits); phosphorylase a (4 subunits); serine dehydratase (1 subunit); tryptophanase (4 subunits); L-threonine dehydratase (degradative) AMP dependent (4 subunits); O-acetylserine sulfhydrylase A (2 subunits); tryptophan synthetase B protein (2 subunits); cystathionine γ-synthetase (4 subunits); L-glutamate decarboxylase (6 subunits); and L-histidinol phosphate transaminase (2 subunits) (Henderson and Snell, 1973). Only L-threonine dehydratase (biosynthetic) (4 subunits and 2 PLP binding sites) has been reported to have an unequal number of PLP binding sites and subunits (Burns and Zarlengo, 1968; Zarlengo et al., 1968).

The pH Dependence of the 5'-Deoxy-PL-PPT Dissociation Constant. The dissociation constants for the binding of 5'-deoxy-PL to the active site of PPT were obtained by spectrophotometric titration using the difference absorption peak at 430 nm due to aldimine formation (Johnson and Metzler, 1970) (eq 5).

$$E + 5'\text{-deoxy-PL} \rightleftharpoons E\text{-}5'\text{-deoxy-PL}$$

where

$$K_d = \frac{[\overline{5'\text{-deoxy-PL}}][\overline{E}]}{[E\text{-}5'\text{-deoxy-PL}]} \qquad (5)$$

The determination of the endpoint is especially difficult at the higher pH values because of the time-dependent slow reaction of 5'-deoxy-PL with nonactive site lysine residues on the enzyme (see above). The endpoints were successfully obtained in separate experiments by adding a 16-fold excess of 5'-deoxy-PL to the resolved enzyme at the same concentration as in the spectrophotometric titration experiments and recording the instantaneous change in absorbance due to aldimine formation at the active site. Where the affinity of 5'-deoxy-PL for the enzyme was weakest, at the higher pH values, the enzyme concentrations employed were approximately 25 μM, and 10-mm pathlength cells were used. At lower values of pH the enzyme concentration was reduced to 2 μM because of the higher affinity for 5'-deoxy-PL, and a 50-mm pathlength cell was employed to increase the absorption changes. The data were plotted according to eq 6 which follows from eq 5 assuming equivalent sites (Figure 3).

$$\frac{[\overline{5'\text{-deoxy-PL}}]}{[E\text{-}5'\text{-deoxy-PL}]} = \frac{[\overline{5'\text{-deoxy-PL}}]}{[E_t]} + \frac{K_d}{[E_t]} \qquad (6)$$

where

$$[E\text{-}5'\text{-deoxy-PL}] = \Delta Abs_{430} \frac{[E_t]}{\Delta Abs_{430\,max}}$$

and

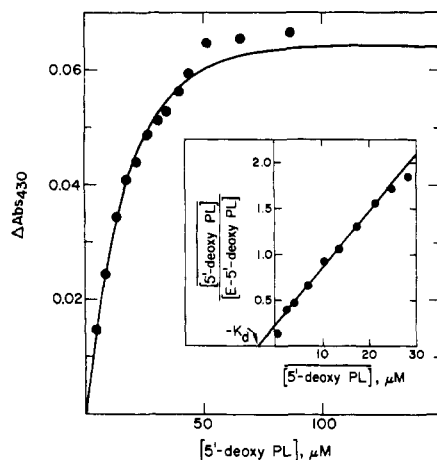$$[\overline{5'\text{-deoxy-PL}}] = [5'\text{-deoxy-PL}]_t - [E\text{-}5'\text{-deoxy-PL}]$$

FIGURE 3: Spectrophotometric determination of the PPT-5'-deoxy-PL dissociation constant at pH 8.70 in 0.05 M potassium pyrophosphate buffer, $\mu = 0.5$, 25 °C, 10-mm pathlength cell. The endpoint was determined by addition of excess 5'-deoxy-PL to an identical solution of PPT (16.5 μM) and recording immediately the observed change in absorbance ($\Delta Abs_{430} = 0.0640$). (Inset) The data from the spectrophotometric titration curve are replotted in linear form according to eq 6 to give K_d for the PPT-5'-deoxy-PL complex at pH 8.70. The three points at highest 5'-deoxy-PL concentration indicated on the titration curve are omitted from the inset because they are higher than the endpoint of the active site reaction due to a contribution from the slow reaction of 5'-deoxy-PL reacting at nonactive site residues (see text).

The term $[\overline{5'\text{-deoxy-PL}}]$ includes the unligated aldehyde, S_A, and its hydrate, S_H, i.e.

$$[\overline{5'\text{-deoxy-PL}}] = [\overline{S}_A] + [\overline{S}_H] \qquad (7)$$

A plot of $[5'\text{-deoxy-PL}]/[E\text{-}5'\text{-deoxy-PL}]$ vs. $[\overline{5'\text{-deoxy-PL}}]$ is linear with a slope equal to $1/[E_t]$ and the abscissa intercept is equal to $-K_d$ (inset in Figure 3).

K_d was determined over the limited pH range 7.5 to 9.0. It was not possible to determine this value accurately below pH 7.5, because the dissociation constant is so small that the low enzyme concentration needed for an accurate determination of K_d gives too small a change in absorbance on binding 5'-deoxy-PL. The determination of K_d at pH values greater than 8.5 is complicated by the slow reaction of 5'-deoxy-PL at nonactive site lysines causing an error in the endpoint and consequently in the calculated concentration of E-5'-deoxy-PL.

The K_d obtained from spectrophotometric determination is not identical to the dissociation constant, K_s, obtained from temperature-jump studies (Gilmer and Kirsch, 1977) because of the equilibrium between the aldehyde and the hydrate forms of 5'-deoxy-PL (eq 8 and 9).

$$K_d = \frac{[\overline{E}][\overline{5'\text{-deoxy-PL}}]}{[E\text{-}5'\text{-deoxy-PL}]} \qquad (8)$$

from spectrophotometric determination

$$K_s = \frac{[\overline{E}][\overline{S}_A]}{[E\text{-}5'\text{-deoxy-PL}]} \qquad (9)$$

from temperature-jump determination

Equation 10 follows from eq 8 and 9 and from $K_H = [S_H]/[S_A]$ and eq 7

$$K_s = \frac{K_d}{(K_H + 1)} \qquad (10)$$

The values of K_H calculated from the spectrophotometric data of Johnson and Metzler (1970) vary from 0.25 at pH 7.5 to
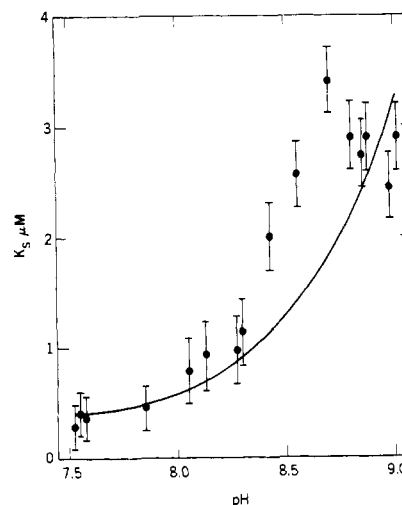

FIGURE 4: Plot of K_s vs. pH for the binding of 5'-deoxy-PL to PPT. All determinations were made in 0.05 M potassium pyrophosphate buffer, $\mu = 0.35$-0.5 at 25 °C. The experimental points were obtained from the spectrophotometric determinations of K_d and from the pH dependence of K_H (Johnson and Metzler, 1970). The solid line shows the pH dependence of K_s calculated from kinetic determinations of k_{on} and k_{off} (where $K_s = k_{off}/k_{on}$) (Gilmer and Kirsch, 1977).

0.13 at pH 9. Essentially similar values have been obtained in this laboratory from the ratios of the areas of the aldehydic proton to the aromatic C_6 proton as a function of pH by proton magnetic resonance (B. Simmons, unpublished results). The values of K_s calculated from the experimental determinations of K_d at each pH and from the dependence of K_H on pH (Johnson and Metzler, 1970) are plotted as a function of pH in Figure 4. The curve in Figure 4 is calculated from kinetic data and does not reflect a single ionizable group (Gilmer and Kirsch, 1977).

Potassium Ion Effect. The activating effect of potassium ion on PPT activity has not been previously reported. Several PLP enzymes are activated by monovalent cations: tryptophanase, L-serine hydrolase (adding indole), L-serine hydrolase (deaminating), and L-threonine hydrolase (deaminating) (Suelter, 1970). The enzyme tryptophanase absolutely requires potassium ion for enzymatic activity. In the presence of this ion there is a pH-dependent change of the PLP-enzyme ($pK_a = 7.2$) from the protonated, enzymatically inactive form, (λ_{max} 420 nm), to the deprotonated active form of the enzyme (λ_{max} 337 nm) (Morino and Snell, 1967). The 420-nm peak is larger in the absence of potassium ions. Therefore, potassium ion might lower the pK_a of the transition of the 420 nm to the 337 nm form. Suelter (1970) has suggested that monovalent cations might activate enzymes by stabilizing a specific form of a coenzyme keto-enol tautomeric equilibrium.

Supplementary Material Available

Table I (amino acid composition and absorbancy of PPT) (2 pages). Ordering information is given on any current masthead page.

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Pyridoxamine-Pyruvate Transaminase. 2. Temperature-Jump and Stopped-Flow Kinetic Investigation of the Rates and Mechanism of the Reaction of 5'-Deoxypyridoxal with the Enzyme[†]
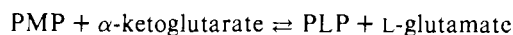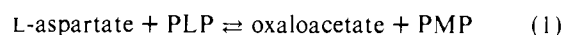
Penny J. Gilmer[‡] and Jack F. Kirsch*

ABSTRACT: The kinetics and mechanism of enzymatic Schiff base formation and hydrolysis were investigated by rapid reactions measurements of 5'-deoxypyridoxal with pyridoxamine-pyruvate transaminase (EC 2.6.1.30). The dissociation rate constant, k_{off}, was determined as a function of pH over the range pH 7-9 by a stopped-flow method in which the nascent free enzyme was trapped by the potent bisubstrate analogue inhibitor, N-pyridoxyl-L-alanine. The values of k_{off} increase with pH and are dependent upon a pK_a (app) of 8.35 which is assigned to the pyridine nitrogen of the Schiff base formed between 5'-deoxypyridoxal and an ϵ-amino group of the active site lysine. The rate-determining step in the dissociation reaction is assigned to the separation of the components of the Michaelis complex by diffusion. A temperature-jump investigation of the pH dependence of the association rate constant, k_{on}, showed a maximum at pH 8.15. This is engendered by a mechanism involving formation of a productive Michaelis complex only when the active site ϵ-amino group is unprotonated and 5'-deoxypyridoxal is in its neutral zwitterionic form. The pK_a of the lysine ϵ-amino group has a kinetically determined pK_a of 8.2. Analysis of the amplitudes of the temperature-jump experiments confirms that the enzyme has 4 active sites per tetramer and gives values of -14.4 kcal/mol and -24.2 eu for the enthalpy and entropy of the association reaction, respectively.

The wealth of spectral detail accompanying transformations between the various intermediates involved in pyridoxal phosphate dependent enzyme catalyzed transamination has stimulated important efforts to elucidate the mechanistic details of this reaction by the temperature-jump technique. The prototypic enzyme for these investigations has been aspartate aminotransferase studied by Hammes and Haslam (1968, 1969), Giannini et al. (1975), Fasella and Hammes (1967), and Czerlinski and Malkewitz (1965). This enzyme catalyzes the reactions shown in eq 1.

$$\text{L-aspartate} + \text{PLP} \rightleftharpoons \text{oxaloacetate} + \text{PMP} \qquad (1)$$

$$\text{PMP} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{PLP} + \text{L-glutamate}$$

Each of the two equations shown in eq 1 is itself composed of several steps which include carbinolamine or *gem*-diamine formation and decomposition, and the interconversion of aldimine and ketimine forms of the amino acid–B-6 adducts (Snell and DiMari, 1970). These are reflected, for example, in the 11 relaxation times observed for the interaction of the