Mechanism of Action of Pyridoxamine Pyruvate Transaminase*

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ABSTRACT: Crystalline pyridoxamine pyruvate transaminase was employed as a model system for study of the kinetics and mechanism of transamination. This enzyme has no coenzyme requirement and catalyzes the reversible reaction: pyridoxamine (A) + pyruvate (B) $\rightleftharpoons$ pyridoxal (C) + L-alanine (D). Initial rate studies showed that the forward reaction followed the rate equation: $v = V_m/(1 + K_A/[A] + K_B/[B] + K_{AB}/[A][B])$. The several mechanisms which conform to this equation were distinguished by the following criteria. (a) The type of product inhibition: pyridoxal inhibited competitively with respect to pyridoxamine, and *vice versa;* all other product inhibitions were not of the competitive type. This is consistent only with a mechanism (eq i) involving

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4(-B)}{\overset{k_3(+B)}{\rightleftharpoons}} EXY \underset{k_6(+D)}{\overset{k_5(-D)}{\rightleftharpoons}} EC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + C \quad \text{(i)}$$

ordered addition of substrate to enzyme (E) in which pyridoxamine or pyridoxal combines first, followed by formation of a ternary complex (EXY). (b) The equilibrium constant: K_{eq} determined from the Haldane equation for this mechanism, 1.53, agreed closely with the directly determined value of 1.21. (c) The relationship between kinetic parameters for forward and reverse reaction was consistent with the postulated mechanism. (d) The degree of product inhibition: the velocity constants calculated from experimentally obtained kinetic parameters were used to calculate the per cent inhibition expected on addition of a given concentration of product. Calculated and observed values were in good agreement. Direct spectrophotometric measurement of the rate of binding of pyridoxal to enzyme in a stopped-flow apparatus, and of the dissociation constants of the enzyme–pyridoxal and enzyme–pyridoxamine complexes, gave values that agreed with those calculated from steady-state kinetics. This result indicates that the rate law based on steady-state assumptions is valid and provides an independent proof that pyridoxamine (and pyridoxal in the reverse reaction) is the first substrate to combine with enzyme. It also shows that the enzyme–pyridoxal complex measured in the stopped-flow apparatus is either a true reaction intermediate or is in rapid equilibrium with the true intermediate. The mechanism of the pyridoxamine pyruvate transaminase reaction established by these studies, in which pyridoxal and pyridoxamine are substrates, is analogous to that proposed previously for other transaminase reactions in which pyridoxal phosphate and pyridoxamine phosphate serve as coenzymes with the major difference that the coenzymes of the latter reactions do not leave the enzyme surface following the isomerization and cleavage of the enzyme-bound azomethine intermediates.

Pyridoxamine pyruvate transaminase has been isolated in crystalline form from a soil bacterium, tentatively designated as *Pseudomonas* MA-1, grown on pyridoxine or pyridoxamine as sole carbon source (Wada and Snell, 1962). This inducible enzyme catalyzes reaction 1. The ease with which the enzyme can be purified and its unique properties provide an ideal system for studying the mechanism of transamination. Like nonenzymatic model reactions, it has the advantage of simplicity, in that the reaction catalyzed appears analogous to a half-reaction catalyzed by enzymes such as glutamate oxaloacetate transaminase. At the same time, the metal ion

$$\underset{A}{\text{pyridoxamine}} + \underset{B}{\text{pyruvate}} \underset{b}{\overset{a}{\rightleftharpoons}} \underset{C}{\text{pyridoxal}} + \underset{D}{\text{alanine}} \quad (1)$$

catalyst of the nonenzymatic reactions (Metzler and Snell, 1952) is replaced by a protein. Unlike other transaminases, pyridoxamine pyruvate transaminase has no coenzyme requirement (Wada and Snell, 1962). On the other hand, its properties resemble closely those of pyridoxal phosphate dependent transaminases. Its substrate, pyridoxal, forms a Schiff base with the ϵ-amino group of a lysine residue, as does pyridoxal phosphate in glutamate oxaloacetate transaminase (Dempsey and Snell, 1963); free sulfhydryl groups, which are necessary for the activity of most pyridoxal phosphate enzymes, are also necessary for pyridoxamine pyruvate transaminase function (Fujioka and Snell, 1965); the molecular weight and number of substrate binding sites are similar to glutamate oxaloacetate transaminase (Dempsey and Snell, 1963). Pyridoxamine pyruvate transaminase is specific in its substrate requirements; only pyru-

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vate functions as amino group acceptor in reaction 1a (Wada and Snell, 1962). The turnover number of the enzyme is similar to that of the holoenzyme of glutamate oxaloacetate transaminase, and the reaction is fully reversible. Because of these similarities to the more complex pyridoxal phosphate requiring transaminases, pyridoxamine pyruvate transaminase provides a favorable system for studying the mechanism of transamination. The present paper is a kinetic and equilibrium approach to the determination of the reaction mechanism of this enzyme.

Experimental Procedure

Growth of Bacteria. Pseudomonas MA-1 was grown in pyridoxine-containing medium at 30°, as described by Wada and Snell (1962). All components of the medium were autoclaved together. Stock cultures were stored in liquid medium at 4° or in frozen medium at −10°. For large-scale production, cells were grown for up to 3 weeks in a biogen; 85% of the cells was harvested daily, leaving a 15% inoculum for the freshly added, sterile medium. Continuous harvesting was impractical due to the long generation time (about 6 hr) of the organism when grown on the inducing medium.

Purification of Enzyme. The cells were disrupted in a refrigerated Eppenbach colloid mill (Model QV-6, Gifford-Wood Co., Hudson, N. Y.) using a procedure adapted from Garver and Epstein (1959). Wet cells (300 g) and 350 ml of Superbrite glass beads (Minnesota Mining and Manufacturing Co., 0.12-mm diameter) were diluted to a total volume of 800 ml with 0.02 M phosphate buffer (pH 7.0). The slurry was milled for 90 min using a mill clearance of 0.036 in. The purification procedure was similar to that previously described (Wada and Snell, 1962), except that the acetone fractionation step was found to be unnecessary. From 80 to 90% of the enzyme activity precipitated between 45 and 60% saturation (4°) with ammonium sulfate. The enzyme was crystallized directly from this fraction. All enzyme preparations were recrystallized three times. The enzyme gave a single symmetrical peak in the ultracentrifuge, and a single band on starch gel electrophoresis at pH 8.0 and on acrylamide gel electrophoresis at pH 8.3. The specific activity in 0.1 M Tris·HCl, pH 8.5 and 37°, with 3 mM pyridoxamine and 3 mM pyruvate as substrates was 25 μmoles of pyridoxal formed/min per mg of enzyme. The concentration of the pure enzyme was determined spectrophotometrically. Pure enzyme (1 mg/ml) has an absorbance at 280 mμ of 0.975 (1-cm light path).

Preparation of Pyridoxal-Free Enzyme. Crystalline pyridoxamine pyruvate transaminase contains variable amounts of bound pyridoxal. For the experiments using substrate concentrations of enzyme, pyridoxal-free enzyme was required. This was obtained by dialyzing against three changes of 0.01 M borate–1.0 mM cysteine (pH 8.0) each for 12 hr, and three changes of 0.02 M potassium phosphate (pH 7.0) each for 12 hr. Microbiological assay showed that approximately 0.4 mole of vitamin B_6, in the form of pyridoxal and pyridoxine, still remained per mole of enzyme. Treatment with charcoal, dialysis against EDTA, and repeated recrystallization from solutions containing penicillamine did not reduce the vitamin B_6 content further. Dialysis after 24-hr incubation with a 100-fold excess of radioactive pyridoxine removed the radioactivity but not the residual bound vitamin B_6. This irreversibly bound vitamin B_6 also appears to be bound nonspecifically, since (a) addition of alanine or cysteine had no effect on the absorption spectrum of the enzyme, and (b) the number of active sites determined by substrate titration was not affected.

Rate Measurements for Steady-State Kinetics. All rates were measured in 0.05 M sodium pyrophosphate buffer (pH 8.85) at 25°. The rate was determined by measuring the absorbance change at 390 mμ due to pyridoxal formation or utilization. A cuvet with a 10-cm light path was used with a water jacket to maintain the temperature at 25°. The rates were recorded on a Cary 14 spectrophotometer using a tenfold expanded scale. After attainment of the steady-state phase (which occurs within 10 sec), the initial velocity was measured at each level of substrate at four different enzyme concentrations, ranging between 0.04 and 0.4 μg/ml. The rate, which usually remained constant for at least 5 min, was plotted as a function of enzyme concentration and this plot, which was linear, was used to calculate the initial velocity in micromoles of pyridoxal formed or consumed in 1 min per mg of enzyme. The nonenzymatic transamination between pyridoxamine and pyruvate was negligible in the concentrations used for the enzymatic reaction.

Results

Number of Binding Sites. The molecular weight of pyridoxamine pyruvate transaminase, determined by both high- and low-speed equilibrium runs, assuming a value for the partial specific volume of 0.73, is 150,000 (H. Kolb, R. D. Cole, and E. E. Snell, to be published). By assuming stoichiometric binding of pyridoxal to enzyme, the number of binding sites was previously approximated at two (Dempsey and Snell, 1963). This number can be accurately determined, without this assumption, by use of eq 2a for the dissociation constant of

$$K_{diss} = \frac{([E_0] - Z)([S_0] - nZ)}{Z} \qquad (2a)$$

the enzyme–pyridoxal complex, where concentrations are designated by brackets and $[E_0]$ = total enzyme, $[S_0]$ = total substrate, n = the number of binding sites, and $Z = [E_0]$ (fraction of binding sites bound to S). The fraction of enzyme sites bound to substrate is determined as the ratio, $\Delta A_{obsd}/\Delta A_{max}$, where ΔA_{obsd} and ΔA_{max} are the observed and maximum values of the absorbance change on addition of S. nZ is then the molar concentration of bound S. Rearranging eq 2a into linear form gives eq 2b, similar to that derived by Stockell (1959) from equa-

$$\frac{[S_0]}{Z} = \frac{K_{diss}}{([E_0] - Z)} + n \qquad (2b)$$

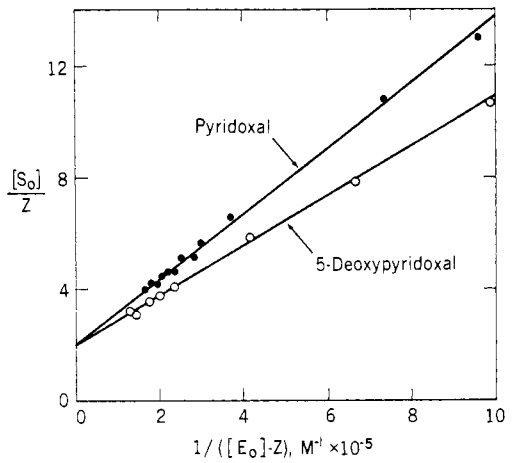

FIGURE 1: Number of binding sites per molecule of pyridoxamine pyruvate transaminase. The equation is given in the text. Enzyme (1.35 mg), previously dialyzed free of substrate, was titrated in 0.05 M sodium pyrophosphate buffer (pH 8.85) at 25° with 0.005–0.1 mM of either pyridoxal at 415 mμ or 5-deoxypyridoxal at 430 mμ. 5-Deoxypyridoxal is also a substrate of pyridoxamine pyruvate transaminase (Ayling and Snell, 1968).

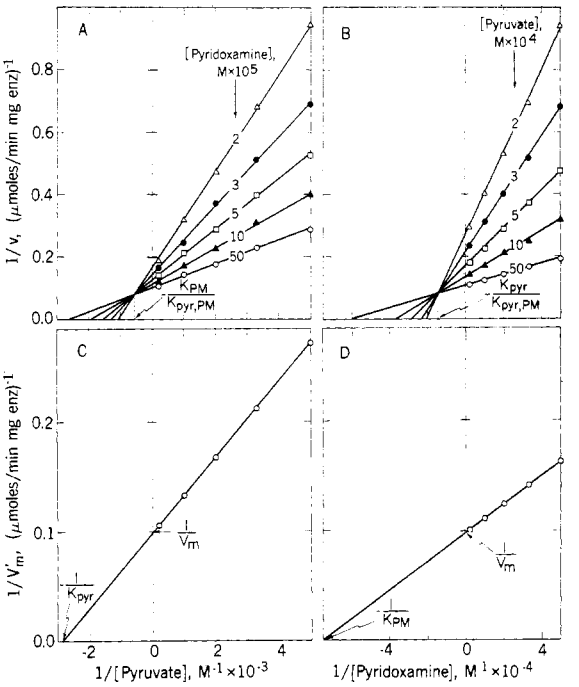

FIGURE 2: Initial velocities of pyridoxal formation from pyridoxamine and pyruvate in reaction 1a catalyzed by pyridoxamine pyruvate transaminase. (A,B) Double-reciprocal plots at various levels of substrates, as indicated. Conditions of assay are given under Experimental Procedures. (C,D) Secondary plots of apparent maximal velocities (from parts B and A, respectively) as a function of substrate concentration.

tions derived by Klotz (1946) for the binding of ions by proteins. A plot of $[S_0]/Z$ *vs.* $1/([E_0] - Z)$ gives n, the number of binding sites as the intercept on the ordinate. The results (Figure 1) confirm the presence of two binding sites per molecule of mol wt 150,000.

Steady-State Kinetics

The Rate Equation. Multiple double-reciprocal plots of initial rate measurements of transamination in the forward direction (eq 1a) are shown in Figure 2A,B. In each case, the lines produced by varying one substrate at several fixed concentrations of the other substrate meet in the left upper quadrant. The intercepts on the ordinate, which increase linearly with the reciprocal of the second substrate concentration, are plotted in Figure 2C,D, giving the Michaelis constants and maximum velocity. The primary plots for the reaction measured in the reverse direction (eq 1b) are of the same form as for the forward direction (Figure 3A,B). The Michaelis constants and maximum velocity for the reverse reaction (eq 1b) are determined in the secondary plots (Figure 3C,D). The relationships observed in Figures 2 and 3 are given by the rate eq 3, where v is the observed ve-

$$v = \frac{V_m}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}} \quad (3)$$

locity, V_m is the maximum velocity, A and B represent the two substrates, K_A and K_B their Michaelis constants, and K_{AB} the combined constant (Alberty, 1953). The kinetic constants for the reaction in both directions are summarized in Table I.

Several different mechanisms give the same rate equation as that derived for pyridoxamine pyruvate transaminase (Alberty, 1953): (a) random order of addition of substrates to enzyme to form a ternary complex when equilibrium conditions apply, (b) compulsory sequence of addition of substrates to enzyme with formation of rate-determining ternary complexes, and (c) ordered addition of substrates to enzyme with only binary complexes being significant in determining the rate. Three methods employing steady-state kinetic data have been used to distinguish between these three possible mechanisms.

RELATIONSHIP BETWEEN THE KINETIC PARAMETERS AND THE EQUILIBRIUM CONSTANT FOR THE OVER-ALL REACTION (Alberty, 1953). For all three mechanisms eq 4 applies, where $V_f = V_m$ for the forward reaction (1a), V_r the corresponding value for the reverse reaction (1b), and $K_{PL,Ala}$[1] and $K_{PM,Pyr}$ represent the combined constant (K_{AB}) for reaction 1a and 1b, respectively. In addition,

$$K_{eq}' = V_f K_{PL,Ala}/V_r K_{PM,pyr} \quad (4)$$

the last of these mechanisms also satisfies eq 5, where K_{PL}, K_{Ala}, K_{PM}, and K_{pyr} are the Michaelis constants

$$K_{eq}' = V_f^3 K_{PL} K_{Ala}/V_r^3 K_{PM} K_{pyr} \quad (5)$$

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: PL, pyridoxal; PM, pyridoxamine; E, pyridoxamine pyruvate transaminase; OAA, oxalacetate; α-KG, α-ketoglutarate; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate.

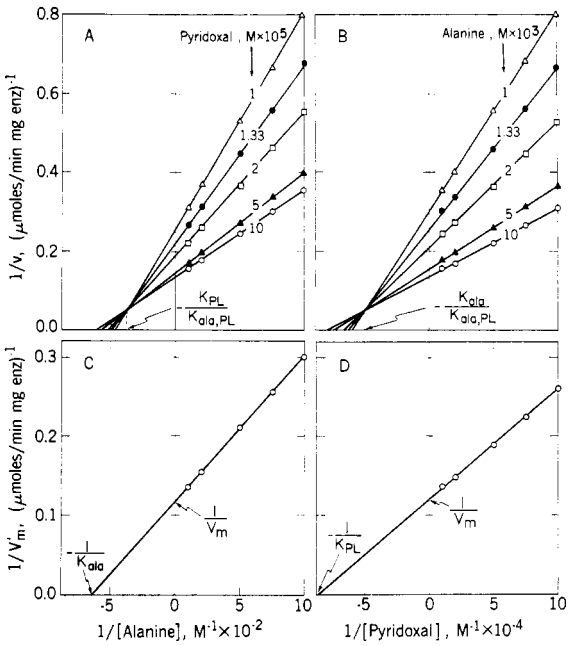

FIGURE 3: Initial velocities of pyridoxal disappearance in the reaction between pyridoxal and alanine (reaction 1b) catalyzed by pyridoxamine pyruvate transaminase. (A,B) Double-reciprocal plots at various levels of substrates. Conditions of assay are the same as for Figure 2. (C,D) Secondary plots of apparent maximal velocities (from parts B and A, respectively) as a function of substrate concentration.

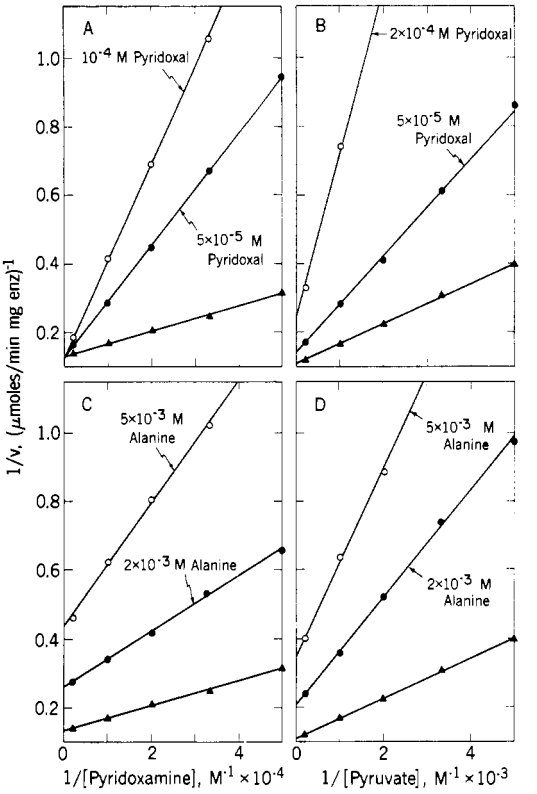

FIGURE 4: Inhibition of reaction 1a between pyridoxamine and pyruvate catalyzed by pyridoxamine pyruvate transaminase by addition of the products, pyridoxal (parts A and B) or L-alanine (parts C and D). In parts A and C, the pyridoxamine concentration was varied, and the initial pyruvate concentration was held constant at 1 mM. In parts B and D, the pyruvate concentration was varied, and the initial pyridoxamine concentration was held constant at 0.1 mM. The lower curve in A–D is the reaction rate in the absence of added product. Conditions of assay are the same as for Figure 2.

for pyridoxal, L-alanine, pyridoxamine, and pyruvate, respectively.

The equilibrium constant for pyridoxamine pyruvate transaminase was determined by calculating the concentration of reactants from the absorbance at 390 mμ due to pyridoxal. For this purpose, 150 μg of enzyme was used in the presence of 0.25–1 mM reactants, and the reaction was followed until no further absorbance change occurred. At 25°, in 0.05 M sodium pyrophosphate buffer (pH 8.85), the average of four measurements, each with different initial concentrations of reactants, gave a value for K_{eq}' = [pyridoxal][alanine]/[pyridoxamine][pyruvate] = 1.21 ± 0.05. Substituting the kinetic constants from Table I into eq 4 and 5 gives a calculated value of K_{eq}' of 1.58 from eq 4 and 7.0 from eq 5. The close agreement between the directly determined value and that given by eq 4 and the disagreement with the value given by eq 5 suggest that the mechanism involving only binary complexes may be eliminated as a possibility.

TABLE I: Kinetic Parameters for the Reversible Reaction Catalyzed by Pyridoxamine Pyruvate Transaminase.[a]

Reaction 1a		Reaction 1b	
K_{PM}	0.013 mM	K_{PL}	0.012 mM
K_{pyr}	0.35 mM	K_{Ala}	1.6 mM
$K_{pyr,PM}$	2.4×10^{-8} M²	$K_{Ala,PL}$	3.2×10^{-8} M²
V_f	10.1 μmoles min⁻¹ mg of enzyme⁻¹	V_r	8.4 μmoles min⁻¹ mg of enzyme⁻¹

[a] V_f is the maximum velocity for the forward reaction (reaction 1a, see text) and V_r is the maximum velocity for the reverse reaction, 1b.

RELATIONSHIP BETWEEN THE KINETIC PARAMETERS FOR THE FORWARD AND REVERSE REACTIONS (Dalziel, 1957). For a mechanism involving ordered addition of substrates with formation of a ternary complex, the relationship shown in eq 6 applies. If the reaction sequence

$$K_A K_B / K_{AB} V_f < 1/V_r \quad (6)$$

involves only binary complexes, then eq 7 applies.

$$K_A K_B / K_{AB} V_f = 1/V_r \quad (7)$$

For the forward reaction of pyridoxamine pyruvate transaminase, the ratio $K_{PM}K_{pyr}/K_{PM,pyr}V_f$ has a value of 0.0145, and $1/V_r$ = 0.09. For the reverse reaction,

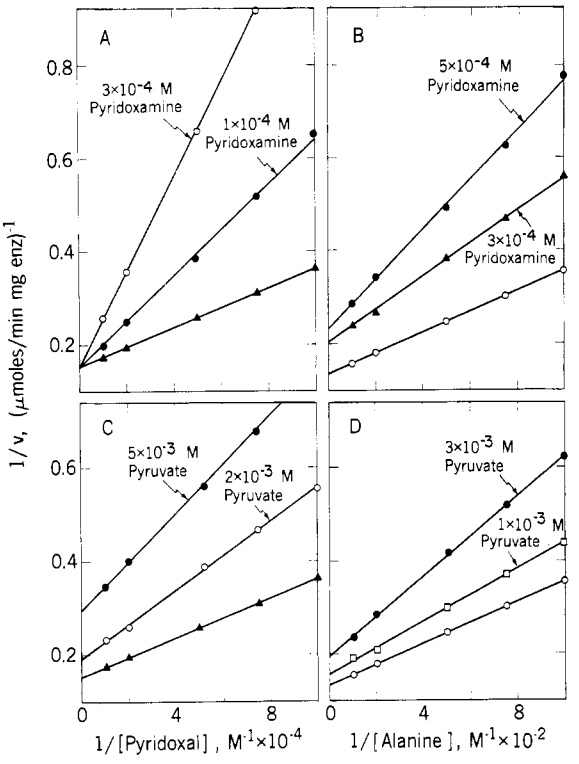

FIGURE 5: Inhibition of reaction 1b between pyridoxal and alanine catalyzed by pyridoxamine pyruvate transaminase by addition of the products, pyridoxamine (parts A and B) or pyruvate (parts C and D). In parts A and C, pyridoxal concentration was varied, and L-alanine was constant at 5 mM. In parts B and D, L-alanine was varied, and pyridoxal was constant at 0.1 mM. The lower curve in A–D is the reaction rate in the absence of added product. Conditions of assay are the same as for Figure 2.

the corresponding ratio, $K_{PL}K_{Ala}/K_{PL,Ala}V_r$, has a value of 0.0525, and $1/V_f = 0.0745$. The relationship for the reaction in each direction agrees with eq 6 and not with eq 7, thus eliminating the binary complex mechanism.

THE EFFECT OF ADDED PRODUCT ON THE INITIAL STEADY-STATE RATE. The effects of adding products on the initial rate of reaction 1a are shown in Figure 4. The addition of pyridoxal decreases V_m', the apparent maximum velocity, with respect to pyruvate (Figure 4B) but has no effect on V_m' with respect to pyridoxamine (Figure 4A); *i.e.*, pyridoxal is a competitive inhibitor of pyridoxamine, but not of pyruvate. The addition of alanine decreases V_m' with respect to both pyridoxamine (Figure 4C) and pyruvate (Figure 4D). Similar results are observed for the reverse reaction, 1b (Figure 5). Pyridoxal is competitively inhibited by pyridoxamine (Figure 5A), but not by pyruvate (Figure 5C). V_m' for alanine is decreased by both pyridoxamine (Figure 5B) and pyruvate (Figure 5D).

The derivation of complete rate equations (Dixon and Webb, 1964) for each of the three possible mechanisms, including all terms in which one or the other product appears, shows that each mechanism results in a different pattern of inhibition (Alberty, 1958). These are summarized in Table II, together with the observed effects with pyridoxamine pyruvate transaminase. The results with the transaminase are consistent only with mechanism 2; *i.e.*, a compulsory sequence of addition of substrates with the formation of ternary complexes, as indicated by eq 8a–d, where substrates are represented by A and B, products by C and D, and the ternary complex(es) by EXY. The values of the kinetic parameters in terms of velocity constants and product concentrations for this mechanism are given by eq 9a–d. The apparent

TABLE II: The Effect of Added Products on the Apparent Maximum Velocity of Reaction 1.[a]

Substrate Varied	Product Added	Inhibition by Products: Theoretical for Mechanism[a] 1	2	3	Observed for[b] PPTA
A	C	+	+	+	+
	D	+	−	−	−
B	C	+	−	−	−
	D	+	−	+	−
C	A	+	+	+	+
	B	+	−	−	−
D	A	+	−	−	−
	B	+	−	+	−

[a] Mechanism: (1) random, (2) ordered with ternary complexes, and (3) ordered with binary complexes. PPTA = pyridoxamine pyruvate transaminase. A or C is the first substrate to combine in ordered sequence mechanisms. For PPTA, A = pyridoxamine, B = pyruvate, C = pyridoxal, and D = alanine. Competitive inhibition is denoted by (+); (−) indicates that the inhibition is not competitive. [b] Data are shown in Figures 4 and 5.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \quad (8a)$$

$$EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EXY \quad (8b)$$

$$EXY \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EC + D \quad (8c)$$

$$EC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + C \quad (8d)$$

$$V_f = \frac{[E_0]k_5k_7}{[D]k_6 + k_7 + k_5} \quad (9a)$$

$$K_A = \frac{k_5k_7 + [C]k_5k_8}{[D]k_1k_6 + k_1k_7 + k_1k_5} \quad (9b)$$

$$K_B = \frac{k_5k_7 + k_4k_7 + [D]k_4k_6}{[D]k_3k_6 + k_3k_7 + k_3k_5} \quad (9c)$$

$$K_{AB} = \frac{k_2k_4k_7 + k_2k_5k_7 + [D]k_2k_4k_6 + [C](k_2k_5k_8 + k_2k_4k_8)}{[D]k_1k_3k_6 + k_1k_3k_7 + k_1k_3k_5} \quad (9d)$$

maximum velocity for substrate A, $1/V_m' = (1 + K_B/[B])/V_f$, contains terms in D but not in C, whereas the apparent maximum velocity for substrate B, $1/V_m' = (1 + K_A/[A])/V_f$, contains terms in C as well as D. This means that the addition of product C will not affect $1/V_m'$ of A, but will affect $1/V_m'$ for B, whereas addition of product D will affect the apparent maximum velocities of both substrates. Since V_m' for both substrates was affected by adding alanine (Figure 4C,D), this product in reaction 1a is represented by D in eq 9a–d. Therefore, the other product, pyridoxal, is represented by C, the last product to dissociate from enzyme. The V_m' for pyruvate was altered by addition of pyridoxal, but V_m' for pyridoxamine was not (Figure 4A,B); hence, pyridoxamine is represented by A, the first substrate to combine with enzyme, and pyruvate by B. The mechanism shown in eq 8a–d is symmetrical in the forward and reverse direction so that analogous effects of products result for the reverse reaction (*cf.* Figure 5).

Calculation of Velocity Constants. When those terms containing product are set equal to zero, as is the case during initial rate measurements in the absence of added product, eq 9a–d reduce to 9e–h, respectively, for the forward reaction (1a) and to eq 9i–l for the reverse reaction (1b). The numerical values of these eight kinetic parameters have been determined from initial rate studies under steady-state conditions (Table I). The value for $[E_0]$ was calculated using a molecular weight of 150,000, with two independent, noninteracting binding sites per molecule. From these values, the eight individual velocity constants listed in Table III have been calculated from suitable combinations of eq 9e–l.

$$V_f = \frac{[E_0]k_5k_7}{(k_5 + k_7)} \quad (9e)$$

$$K_{PM} = \frac{k_5k_7}{k_1(k_5 + k_7)} \quad (9f)$$

$$K_{pyr} = \frac{k_7(k_4 + k_5)}{k_3(k_5 + k_7)} \quad (9g)$$

$$K_{PM,pyr} = \frac{k_2k_7(k_4 + k_5)}{k_1k_3(k_5 + k_7)} \quad (9h)$$

$$V_r = \frac{[E_0]k_2k_4}{(k_2 + k_4)} \quad (9i)$$

$$K_{PL} = \frac{k_2k_4}{k_8(k_2 + k_4)} \quad (9j)$$

$$K_{Ala} = \frac{k_2(k_4 + k_5)}{k_6(k_2 + k_4)} \quad (9k)$$

$$K_{PL,Ala} = \frac{k_2k_7(k_4 + k_5)}{k_6k_8(k_2 + k_4)} \quad (9l)$$

TABLE III: Velocity Constants for Reaction 1 Catalyzed by Pyridoxamine Pyruvate Transaminase Calculated from Steady-State Data.[a]

k_1	9.5×10^5 M^{-1} sec^{-1}	k_5	44 sec^{-1}
k_2	66 sec^{-1}	k_6	3×10^4 M^{-1} sec^{-1}
k_3	4.7×10^4 M^{-1} sec^{-1}	k_7	18 sec^{-1}
k_4	12.5 sec^{-1}	k_8	9.0×10^5 M^{-1} sec^{-1}

[a] Velocity constants are defined by reaction 8; details are in the text.

Calculation of Product Effects from Velocity Constants. An independent check of the values of the velocity constants has been obtained by calculating the expected apparent maximum velocity of reactions 1a and b in the presence of added product by substituting the values of the velocity constants (Table III) into eq 10a–d and comparing the calculated values with those observed. The equations for apparent maximum velocity in the presence of added product (eq 10a–d) are obtained using the expressions derived for the kinetic parameters given in eq 9a–d. The reciprocal of the apparent maximum velocity of reaction 1a when pyridoxamine is the varied substrate, the pyruvate concentration is held constant, and alanine is the product added is given by eq 10a. When pyridoxal is added, $1/V_m'$ is

$$\frac{1}{V_m'} = \left(\frac{[Ala]k_6([pyr]k_3 + k_4)}{[E_0][pyr]k_3k_5k_7}\right) + \left(\frac{[pyr]k_3k_7 + [pyr]k_3k_5 + k_5k_7 + k_4k_7}{[E_0][pyr]k_3k_5k_7}\right) \quad (10a)$$

given by eq 10b. When pyruvate is the varied substrate

$$\frac{1}{V_m'} = \frac{[pyr]k_3k_7 + [pyr]k_3k_5 + k_5k_7 + k_4k_7}{[E_0][pyr]k_3k_5k_7} \quad (10b)$$

and pyridoxamine is constant, the apparent maximum velocity of reaction 1a in the presence of added alanine is given by eq 10c, and with pyridoxal as the added product by eq 10d. The second term on the right side of eq

$$\frac{1}{V_m'} = \left(\frac{[Ala]k_6}{[E_0]k_5k_7}\right) + \left(\frac{[PM]k_1(k_5 + k_7) + k_5k_7}{[E_0][PM]k_1k_5k_7}\right) \quad (10c)$$

TABLE IV: Calculated and Observed Values of Apparent Maximum Velocities of the Reaction of Pyridoxamine Pyruvate Transaminase in the Presence or Absence of Added Product.[a]

Varied Substrate	Concn of Constant Substrate (mM)	Added Product	Product Concn (mM)	$1/V_m'$ Calcd	$1/V_m'$ Obsd
PM	pyr (1)	0	0	0.133	0.133
		Ala	2	0.255	0.260
		Ala	5	0.436	0.435
		PL	0.05	0.133	0.133
		PL	0.1	0.133	0.133
pyr	PM (0.1)	0	0	0.111	0.111
		Ala	2	0.207	0.206
		Ala	5	0.350	0.350
		PL	0.05	0.144	0.143
		PL	0.2	0.243	0.246
PL	Ala (5)	0	0	0.156	0.156
		pyr	2	0.338	0.192
		pyr	5	0.610	0.297
		PM	0.1	0.156	0.156
		PM	0.3	0.156	0.156
ala	PL (0.1)	0	0	0.133	0.136
		pyr	1	0.230	0.158
		pyr	3	0.273	0.198
		PM	0.3	0.193	0.196
		PM	0.5	0.233	0.232

[a] Observed values are taken from Figures 4 and 5. Apparent maximum velocities are expressed in terms of micromoles of pyridoxal formed or utilized per minute per milligram of enzyme.

$$\frac{1}{V_m'} = \left(\frac{[PL]k_8}{[E_0][PM]k_1k_7}\right) + \left(\frac{[PM]k_1(k_5 + k_7) + k_5k_7}{[E_0][PM]k_1k_5k_7}\right) \quad (10d)$$

10a–d is the expression for $1/V_m'$ in the absence of product. Analogous expressions for the effect of product on the apparent maximum velocity of reaction 1b are obtained by substituting pyridoxamine for pyridoxal, pyruvate for alanine, k_8 for k_1, k_7 for k_2, k_6 for k_3, k_5 for k_4, and *vice versa*.

Values for $1/V_m'$ predicted from these equations and observed values (from Figures 4 and 5) are compared in Table IV. There is very close agreement for all inhibitions except those by pyruvate with respect to pyridoxal (Figure 5C) and with respect to alanine (Figure 5D). Pyruvate inhibits to an extent only 30% of that predicted in each case. The analogous inhibition in the other direction, *i.e.*, that by alanine with respect to pyridoxamine (Figure 4C) and pyruvate (Figure 4D), shows complete agreement with the predicted values. Therefore, it appears that the anomalous results with pyruvate, although not yet understood, may result from complexing of pyruvate with alanine and pyridoxal, but are not due to an inconsistency in the mechanistic formulation.

Direct Measurement of Constants

From the preceding results it can be concluded that pyridoxamine pyruvate transaminase reacts with its substrates in a compulsory order, pyridoxamine (or pyridoxal) combining first, followed by formation of a rate-determining ternary complex. The validity of this conclusion is dependent on the basic steady-state assumptions made in deriving the rate equation. These assumptions can be verified by measuring individual constants by independent methods and comparing the resulting values with those calculated from steady-state kinetic data. For this purpose, we have measured directly the dissociation constants for the enzyme–pyridoxamine and enzyme–pyridoxal complexes and the rate of binding of pyridoxal with the enzyme. These measurements give independent values for k_1/k_2, k_7, and k_8.

SPECTROPHOTOMETRIC DETERMINATION OF ENZYME–SUBSTRATE DISSOCIATION CONSTANTS. The dissociation constant for the enzyme–pyridoxamine complex was determined by measuring the absorption of the complex at 335 mμ in a cuvet of 1-cm light path, thermostated at 25°. The solutions to be titrated contained between 1 and 5 mg of enzyme per ml of 0.05 M sodium pyrophosphate buffer (pH 8.85). Pyridoxamine solution (2–10 μl) was added to both enzyme and reference blank (buffer) solutions. Increase in the absorbance at 335 mμ, corrected for the slight initial absorbance of the

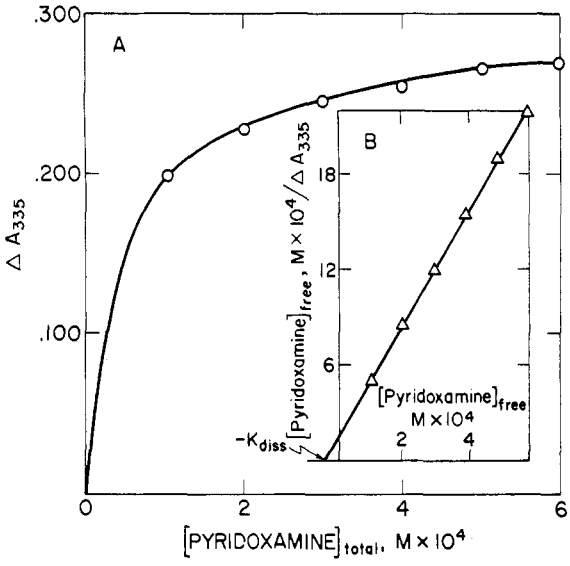

FIGURE 6: Spectrophotometric titration and the determination of the dissociation constants of pyridoxamine pyruvate transaminase. (A) Spectrophotometric titration of pyridoxamine pyruvate transaminase with pyridoxamine. ΔA_{335} refers to the change in absorbance at 335 mμ as a result of formation of the enzyme–pyridoxamine complex read above a blank containing an equal concentration of pyridoxamine. The solution to be titrated contained 1.3 mg of enzyme, previously dialyzed free of substrate (see text), in 1 ml of 0.05 M sodium pyrophosphate buffer (pH 8.85). Measurements were made in a cuvet of 1-cm light path. (B) Determination of the dissociation constant of the enzyme–pyridoxamine (E–PM) complex. The data from A are replotted in linear form according to the equation

$$\frac{[PM]_{free}}{[E\text{–}PM]} = \frac{[PM]_{free}}{[E_0]} + \frac{K_{diss}}{(E_0)}$$

[E–PM] is plotted in terms of ΔA_{335}. $[PM]_{free} = [PM]_{total} - [C]\Delta A_{335}/\Delta A_{max}$, where ΔA_{max} is the absorbance change at completion of titration and [C] is the concentration of enzyme binding sites. In this graph, [C] is 0.0173 mM and ΔA_{max} is 0.290.

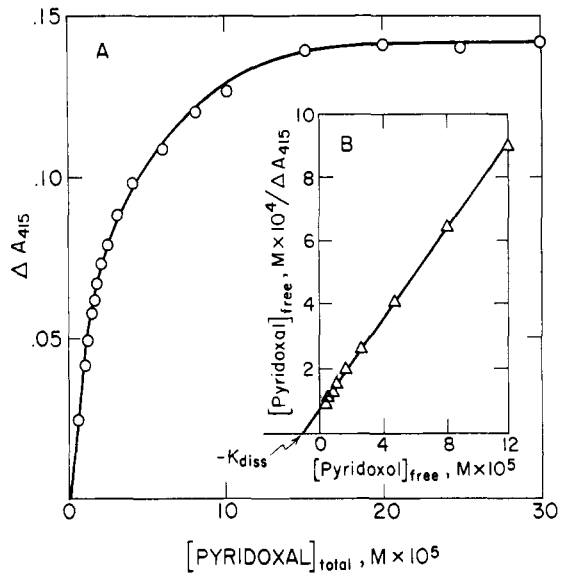

FIGURE 7: Spectrophotometric titrations studies. (A) Spectrophotometric titration of pyridoxamine pyruvate transaminase with pyridoxal. Conditions and methods are the same as for Figure 6a, except for the difference in wavelength. (B) Data taken from part A are replotted in linear form to give the dissociation constant of the enzyme–pyridoxal complex. The concentration of complex is plotted in terms of ΔA_{415}.

enzyme, is plotted in Figure 6A against total pyridoxamine concentration. The data are replotted in linear form in Figure 6B, from which $K_{diss} = 5 \times 10^{-5}$ M is obtained as the intercept on the negative abscissa. The steady-state kinetic value ($= K_{pyr,PM}/K_{pyr}$; see Table I), is 7.0×10^{-5} M.

Conditions for the determination of K_{diss} for the enzyme–pyridoxal complex (E–PL) were similar, except that the enzyme was titrated with pyridoxal at 415 mμ, the maximum in the difference spectrum of E–PL *vs.* PL. The data (Figure 7) yield a value for K_{diss} of 1×10^{-5} M. The steady-state kinetic value ($= K_{Ala,PL}/K_{Ala}$) is 2×10^{-5} M.

The simple hyperbolic curves resulting from spectrophotometric titration of pyridoxamine pyruvate transaminase with substrate (Figures 6A and 7A) indicate that the dissociation constants remain constant as the substrate concentrations increase, implying that the two binding sites are equivalent and that there is no interaction between them.

RATE OF ENZYME–SUBSTRATE COMPLEX FORMATION. The velocity constant for the rate of binding of pyridoxal to enzyme was determined using a Durrum-Gibson stopped-flow apparatus (Durrum Instrument Co., Palo Alto) at 25°. The rate was measured over a range of enzyme concentrations from 1.5 to 7.5 mg per ml and pyridoxal concentrations from 0.02 to 0.1 mM in 0.05 M sodium pyrophosphate buffer (pH 8.85). The reaction was followed by measuring the rate of change in transmittance at 415 mμ due to formation of E–PL. The velocity was measured under second-order conditions rather than as a pseudo-first-order constant. This was necessary since saturating concentrations of pyridoxal gave a very high background absorbance and a rate beyond the range of the instrument. Optimal concentrations for maximizing sensitivity in both the vertical (per cent transmission) and horizontal (time scale) directions were approximately 0.02 mM enzyme and pyridoxal. The time course of such a reaction is shown in the inset of Figure 8. Data from two such traces are plotted in Figure 8 according to the second-order kinetic equation for a reversible reaction (eq 11a) in its integrated

$$\frac{d[E\text{–}PL]}{dt} = k_7([E_0] - [E\text{–}PL])([PL_0] - [E\text{–}PL]) - k_8[E\text{–}PL] \quad (11a)$$

form, eq 11b, where $[PL_0]$ is the total concentration of pyridoxal and other terms are as defined previously. C_1

$$\log \frac{(C_1 - [E\text{–}PL])}{(C_2 - [E\text{–}PL])} = \left(\frac{C_1 - C_2}{2.3}\right)k_8 t + \log \frac{C_1}{C_2} \quad (11b)$$

and C_2, composed of the constants $[E_0]$, $[PL_0]$, and

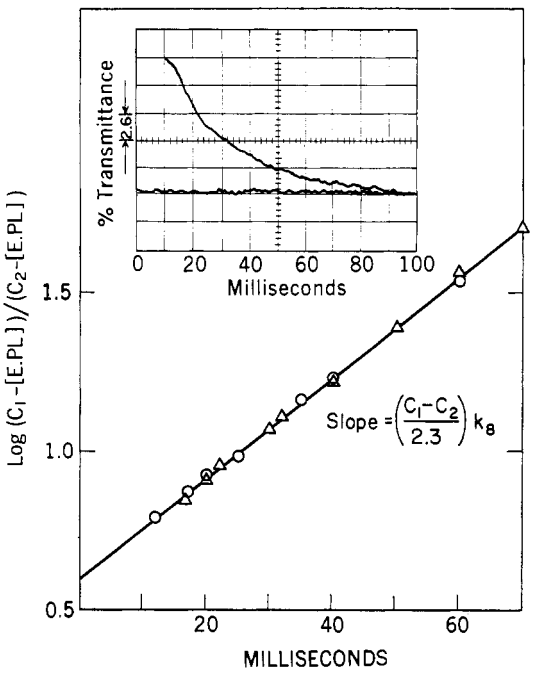

FIGURE 8: Rate of formation of enzyme–pyridoxal complex. The upper curve in the inset shows the time course of a reaction in which the initial concentrations of reactants after mixing were 0.0173 mM enzyme, previously dialyzed free of substrate, and 0.025 mM pyridoxal in 0.05 M sodium pyrophosphate buffer (pH 8.85) at 25°. The lower curve in the inset shows the per cent transmittance at the end of the reaction. The data from this and another similar tracing are used in the second-order plot to determine the velocity constant for combination of pyridoxal with enzyme (k_8). The equation is given in the text.

K_{diss}, are the two solutions of the expression

$$\frac{([E_0] + [PL_0] + K_{diss}) \pm \sqrt{([E_0] + [PL_0] + K_{diss})^2 - 4[E_0][PL_0]}}{2}$$

The value of K_{diss} = 0.01 mM is taken from Figure 7. The value of k_8, calculated from Figure 8, is 1.2×10^6 M^{-1} sec^{-1}. Hence k_7, which is equal to $K_{diss}k_8$, has a value of 12 sec^{-1}. The corresponding steady-state kinetic values are 0.9×10^6 M^{-1} sec^{-1} and 18 sec^{-1}, respectively (Table III).

Discussion

A comparison of constants calculated from steady-state kinetics with those measured directly is given in Table V. The correctness of the velocity constants calculated from steady-state data depends on using the appropriate rate equation and on the validity of the basic assumptions made in deriving the equation. The dissociation constants determined by spectrophotometric titration agree closely with the steady-state kinetic values. In addition to indicating the validity of the steady-state assumption and the resulting rate law, this agreement implies that the number and types of sites do not change upon dilution and that the substrate binds only at the catalytic site.

TABLE V: Comparison of Kinetic Parameters Obtained from Steady-State Kinetic Measurements with Those Directly Measured.[a]

Parameter	Kinetic	Direct
$K_{eq} = \frac{V_f K_{PL,Ala}}{V_r K_{PM,pyr}}$	1.58	1.21
K_{diss} for E–PM complex (k_2/k_1)	7.0×10^{-5} M	5.0×10^{-5} M
K_{diss} for E–PL complex(k_7/k_8)	2.0×10^{-5} M	1.0×10^{-5} M
Rate of E–PL complex formation (k_8)	0.9×10^6 M^{-1} sec^{-1}	1.2×10^6 M^{-1} sec^{-1}
Rate of E–PL complex breakdown (k_7)	18.0 sec^{-1}	12.0 sec^{-1}

[a] See text for details.

The reaction kinetics of the E–PL complex are also in good agreement with the predictions of steady-state theory. The kinetics of its formation agrees quantitatively with the rate computed from the over-all reaction based on measurements of pyridoxal formation or utilization. This agreement has been obtained without assuming the formation of complexes of enzyme with alanine. Therefore, by use of the appropriate conditions, the sequence of interaction of the enzyme with its substrates has been verified.

Direct measurement of the kinetics of formation of the enzyme–pyridoxal complex showed this reaction to be of second order. If the formation of the complex absorbing at 415 mμ (designated here as E′–PL) were preceded by the rapid formation of another binary complex, E–PL, *i.e.*, if $E + PL \overset{fast}{\rightleftharpoons} E\text{–}PL \overset{slow}{\rightleftharpoons} E'\text{–}PL$, the observed reaction kinetics would be of first order. Since they follow a second-order rate law, there is either only one binary complex formed before the formation of the ternary complex or, if there is more than one, the first step is rate limiting. The agreement of the rate of formation of the binary complex absorbing at 415 mμ with the rate calculated from the over-all reaction kinetics indicates that it is either a true reaction intermediate or is in rapid equilibrium with the real intermediate, *i.e.*

$$E + PL \overset{slow}{\rightleftharpoons} E\text{–}PL \overset{Ala}{\rightleftharpoons} EXY \text{ etc.}$$
$$\Big\downarrow \text{fast}$$
$$E'\text{–}PL$$

where E′–PL is the complex which absorbs at 415 mμ.

Studies of pyridoxal analogs show the latter to be the case (Ayling and Snell, 1968).

The mechanism proposed for pyridoxamine pyruvate transaminase is consistent with that previously proposed for pyridoxal phosphate dependent transaminases, such as glutamate oxaloacetate transaminase (Jenkins and Sizer, 1957; Snell and Jenkins, 1959; Velick and Vavra, 1962). This is illustrated by the following comparison of the reaction of pyridoxamine pyruvate transaminase (a–d) with that of glutamate oxaloacetate transaminase (b′–b″). Glutamate oxaloacetate transaminase reacts first with oxaloacetate to form a ternary complex followed by isomerization of the complex (reaction b′) and release of aspartate with retention of pyridoxal phosphate on the enzyme (reaction c′). Reactions b′ and c′ are precisely analogous to reactions b and c between pyridoxamine–enzyme and pyruvate to form pyridoxal–enzyme and alanine, catalyzed by pyridoxamine pyruvate transaminase. The sole apparent difference in the two reactions lies in the firm binding of pyridoxamine phosphate and pyridoxal phosphate by the former enzyme. In the same way, the second half of the over-all reaction catalyzed by glutamate oxaloacetate transaminase (reactions c″ and b″) is entirely analogous to the reverse reaction of pyridoxamine pyruvate transaminase (reactions c and b). It is clear from this formulation why pyridoxamine pyruvate transaminase, in contrast to the more usual transaminases, does not require pyridoxal phosphate as a coenzyme. The function for which the coenzyme is usually required is filled completely by the corresponding unphosphorylated substrates, pyridoxamine and pyridoxal. Since these substrates are readily dissociable, a study of the specificity of pyridoxamine pyruvate transaminase for its pyridine substrates should provide information concerning the role of individual substituents on the pyridine ring in coenzyme function. Such a study is presented in an accompanying paper (Ayling and Snell, 1968).

$$E + PM \rightleftharpoons E\text{–}PM \quad (a)$$

$$E\text{–}PM + pyr \rightleftharpoons E\text{–}PM\text{–}pyr \rightleftharpoons E\text{–}PL\text{–}Ala \quad (b)$$

$$E\text{–}PL\text{–}Ala \rightleftharpoons E\text{–}PL + Ala \quad (c)$$

$$E\text{–}PL \rightleftharpoons E + PL \quad (d)$$

$$\text{Sum: } PM + pyr \rightleftharpoons PL + Ala$$

$$E\text{–}PMP + OAA \rightleftharpoons E\text{–}PMP\text{–}OAA \rightleftharpoons E\text{–}PLP\text{–}Asp \quad (b')$$

$$E\text{–}PLP\text{–}Asp \rightleftharpoons E\text{–}PLP + Asp \quad (c')$$

$$E\text{–}PLP + Glu \rightleftharpoons E\text{–}PLP\text{–}Glu \rightleftharpoons E\text{–}PMP\text{–}\alpha\text{-}KG \quad (c'')$$

$$E\text{–}PMP\text{–}\alpha\text{-}KG \rightleftharpoons E\text{–}PMP + \alpha\text{-}KG \quad (b'')$$

$$\text{Sum: } OAA + Glu \rightleftharpoons Asp + \alpha\text{-}KG$$

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