Interaction of Pyridoxamine-Pyruvate Transaminase with Carbonyl Derivatives of Pyridoxal*

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Crystalline pyridoxamine-pyruvate transaminase, which catalyzes Reaction 1, has been isolated from a bacterium grown on pyridoxine or pyridoxamine as a sole source of carbon and nitrogen (1, 2). This transaminase contains no pyridoxal phosphate, and hence provides a simpler system for mechanistic studies than do the more complex, pyridoxal phosphate-containing transaminases. The enzyme binds pyridoxamine and pyridoxal with almost equal avidity; the latter is bound in part by an azomethine linkage to the e-amino group of a lysine residue of the enzyme (2).

During the determination of the specificity of this enzyme, a relatively slow formation of pyridoxamine was noted when pyridoxal oxime or certain other carbonyl derivatives of pyridoxal were substituted for pyridoxal as substrate in Reaction 1. Further studies, described herein, reveal that these substances do not undergo transamination directly, but instead interact with the enzyme to form enzyme-bound pyridoxal, which then undergoes transamination. The results are of interest in connection with claims (3-7) that various derivatives of pyridoxal-P, e.g. the hydrazone, oxime, isonicotinoylhydrazone, act per se as coenzymes for certain pyridoxal phosphate-dependent enzymes.

EXPERIMENTAL PROCEDURE

The oxime, hydrazone, semicarbazone, and N'-isonicotinoyl pyridoxal hydrazone were prepared as described previously (8). Other chemicals were from commercial sources. Recrystallized pyridoxamine-pyruvate transaminase (specific activity, 25 units per mg at 37°) was used throughout the investigation. Reaction mixtures are given in the tables and figures. Disappearance of pyridoxal from reaction mixtures was measured by following the decrease in absorbance at 400 mm (pH 7.0) or by the phenylhydrazine method (9); disappearance of derivatives of pyridoxal with carbonyl reagents was measured at 350 mm (pH 8.5) or at 360 mm (pH 7.0). Pyridoxamine does not absorb at these wave lengths. Formation of pyridoxamine was followed at 312 mm (pH 8.5) or at 325 mm (pH 7.0).

Spectra were taken in a Cary model 14 spectrophotometer and absorbance readings were made in a Beckman model DU spectrophotometer. Pyruvate was determined with lactic dehydrogenase as described by Kornberg (10). Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as the standard.

RESULTS

Carbonyl Reagent Derivatives of Pyridoxal as Substrates of Pyridoxamine-Pyruvate Transaminase

Stoichiometry and Rate of Reaction—The oxime, hydrazone, semicarbazone, and isonicotinoylhydrazone of pyridoxal were substituted for pyridoxal as substrates in Reaction 1, and changes in the spectrum were followed with time (Fig. 1). A progressive decrease in absorbance of the pyridoxal derivatives occurred and was accompanied by an increase in absorbance at 310 mm (pH 8.5) or 325 mm (pH 7.0) corresponding to the appearance of pyridoxamine. On treatment with ninhydrin, paper chromatograms of the reaction mixture showed the characteristic orange spot given by pyridoxamine. Rf values in t-butyl alcohol-formic acid-water (70:15:15) and t-butyl alcohol-acetic acid-acetone-water (40:5:35:20) were 0.78 and 0.38, respectively, agreeing with those of authentic pyridoxamine. Pyridoxamine formation was equivalent to the pyridoxal derivative disappearing (Table I). The amount of free pyruvate formed was always less than that of pyridoxamine, apparently because part of the pyruvate reacts with the carbonyl reagent released from the pyridoxal derivative.

The rate of transamination of these pyridoxal derivatives was very slow compared with that of free pyridoxal (Table II).

Affinities of Pyridoxal Derivatives for Transaminase—The Kᵢ values for pyridoxal and several of its derivatives were determined in the customary fashion and are compared in Table III with the Kᵢ values found when these same compounds are added as inhibitors of Reaction 1 to reaction mixtures that contain pyridoxal. Since pyridoxal is the preferred substrate (cf. Table II), comparatively very small amounts of enzyme are used for the latter determinations, and no appreciable transamination of the derivatives occurs in the time period used. Each of these derivatives inhibited transamination of pyridoxal competitively, as judged from Lineweaver-Burk plots. However, the values for the two constants for any given compound, although of the same order of magnitude, are not in good agreement, indicating that the interaction of enzyme with these compounds, like that of several apoenzymes with pyridoxal phosphate (12), is not freely reversible under the assay conditions. It should be noted that the concentrations of the enzyme used for the two types of determinations are very different.

Interaction of Pyridoxal Derivatives with Stoichiometric Amounts

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of Pyridoxamine-Pyruvate Transaminase—Previous investigations (2) of Reaction 1 with stoichiometric amounts of transaminase showed that 2 moles of pyridoxal combine per mole of enzyme (mol. wt. 120,000) in an azomethine linkage involving the ε-amino groups of 2 lysine residues. Absorbance at 410 mp due to this complex immediately disappeared on addition of l-alanine with formation of pyridoxamine so that Reaction 1

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Comparative rates of transamination of pyridoxal and its derivatives

Incubation mixtures had the same composition as that described in Fig. 1 except that variable amounts of the substrates and enzyme were added and ethylenediaminetetraacetate was omitted. Incubation was carried out in a 1-cm silica cell at 23° with 0.0012, 0.315, 0.105, 0.315, and 0.315 mg of enzyme, respectively, in the five reaction mixtures. Values of V_max were calculated from reciprocal plots of substrate concentration with respect to velocity. The velocity was measured by the decrease in absorbance at 350 mp after 2 minutes of incubation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V_max</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal</td>
<td>11.50</td>
<td>100</td>
</tr>
<tr>
<td>Pyridoxal oxime</td>
<td>0.0067</td>
<td>0.058</td>
</tr>
<tr>
<td>Pyridoxal hydrazone</td>
<td>0.15</td>
<td>1.3</td>
</tr>
<tr>
<td>Pyridoxal semicarbazone</td>
<td>0.046</td>
<td>0.40</td>
</tr>
<tr>
<td>N'-Isonicotinoyl pyridoxal hydrazone</td>
<td>0.0051*</td>
<td></td>
</tr>
</tbody>
</table>

* Measured at pH 7.0 (see Table 1).

Affinities of derivatives of pyridoxal for pyridoxamine-pyruvate transaminase

Values were calculated from reciprocal plots of reaction velocity with respect to substrate concentration. For determinations of K_i values, 1.0 ml of reaction mixture contained 50 μmoles of Tris-HCl (pH 8.5), 10 μmoles of l-alanine, 1.4 μg of pyridoxamine-pyruvate transaminase, 0.01 to 0.4 μmole of pyridoxal, and 0.01 to 0.1 μmole of the pyridoxal derivative. The inhibitor was added to the enzyme before pyridoxal, and the reaction was started by addition of l-alanine. Initial velocities were measured at 23° by following the decrease in pyridoxal concentration by the change in optical density at 406 mp, corrected in the case of the isonicotinoyl pyridoxal hydrazone for the absorbance change resulting from nonenzymatic destruction of this compound. For measuring the K_i values of pyridoxamine, 10 μmole of sodium pyruvate replaced alanine in a similar system, and the increase in absorbance at 400 mp was followed. K_i values of the pyridoxal derivatives were measured in the buffer system given in Table I containing varying amounts of the pyridoxal derivatives and 0.315 mg of pyridoxamine-pyruvate transaminase (0.105 mg for the hydrazone). The reaction rate was measured by following the decrease in optical density at 350 mp.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>K_i</th>
<th>K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxamine</td>
<td>1.6</td>
<td>1.6 x 10^5</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Pyridoxal oxime</td>
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<td>6.5</td>
</tr>
<tr>
<td>Pyridoxal hydrazone</td>
<td>13</td>
<td>7.7</td>
</tr>
<tr>
<td>Pyridoxal semicarbazone</td>
<td>4 1</td>
<td>14</td>
</tr>
<tr>
<td>N'-Isonicotinoyl pyridoxal hydrazone</td>
<td>19</td>
<td>9.6*</td>
</tr>
</tbody>
</table>

* At pH 7.0.
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Fig. 2. Schematic representation of the reactions of pyridoxamine-pyruvate transaminase (I) with pyridoxal (II) or its derivatives with carbonyl reagents (IV) to form various complexes discussed in the text and subsequent catalysis of their transamination with alanine to pyridoxamine. For the sake of simplicity, the scheme shows only a single pyridoxal (or derivative) interacting with the transaminase whereas 2 moles of substrate actually complex per mole of enzyme at two apparently equivalent sites (see Reference 2 and Fig. 5).

Fig. 3. The effect of addition of 2 equivalents of various pyridoxal derivatives on the absorbance of pyridoxamine-pyruvate transaminase at 410 nm. To 1.2 mg (0.01 μmole) of pyridoxamine-pyruvate transaminase, 0.02 μmole of the specified pyridoxal derivative was added at zero time. Incubation was conducted in 0.1 ml of 0.05 M potassium phosphate buffer, pH 7.0, at 23°C. a, pyridoxal oxime; b, pyridoxal hydrazone; c, pyridoxal semicarbazone; d, N'-isonicotinoyl pyridoxal hydrazone.

Fig. 4. The effect of addition of stoichiometric and excess amounts of carbonyl reagents on the spectrum of the transaminase-pyridoxal complex. To pyridoxamine-pyruvate transaminase (1.2 mg, 0.01 μmole) and 0.02 μmole of pyridoxal in 1.0 ml of 0.05 M potassium phosphate buffer, pH 7.0, there was added (a) hydroxylamine, (b) hydrazine, (c) semicarbazide, or (d) isonicotinic acid hydrazide in equivalent and excess amounts. Curve 1, pyridoxal-transaminase complex; Curve 2, Curve 1 + 0.02 μmole (2 equivalents) of the carbonyl reagent; Curve 3, Curve 1 + 2.0 μmole (0.2 μmole for hydrazine) of the carbonyl reagent. Higher concentrations of hydrazine produced turbid solutions.

Another route not involving an azomethine intermediate (e.g. Reactions 2α, β, and γ, Fig. 2) is involved. The pure enzyme was largely freed of bound pyridoxal by extensive dialysis against Tris-HCl buffer, pH 8.5, containing L-alanine, and finally against potassium phosphate buffer, pH 7.0, as previously described (2). On addition of 2 equivalents of the various pyridoxal derivatives to this preparation, a gradual change occurred in the absorbance at 410 nm, the absorption maximum of the enzyme-pyridoxal complex (Fig. 3), indicative of a reaction between the transaminase and the pyridoxal derivative in the absence of the co-substrate, L-alanine. The final spectra obtained after these changes had ceased were different from those of the derivatives themselves or from that of the pyridoxal-enzyme complex, and were identical with those obtained when two equivalents of the free carbonyl reagent were added to the pyridoxal-enzyme complex (see Curves 2, Fig. 4). On addition of excess L-alanine, these spectra were converted to that of pyridoxamine, which, unlike pyridoxal, shows almost no shift in absorption maximum when combined with the transaminase. It appears certain, therefore, that the spectra of Curves 2, Fig. 4 represent the sum of the spectra of the enzyme-pyridoxal complex, VI, Fig. 2 (see Curve 1, Fig. 4), and the enzyme-derivative complex, V, Fig. 2 resulting from Reactions 2a and 2b of Fig. 2. Thus the equilibrium represented by Reaction 2b of Fig. 2 does exist and may be approached from either direction. Addition of an excess of carbonyl reagent should force this equilibrium entirely toward the enzyme-pyridoxal derivative complex (V, Fig. 2). When this was done, a further spectral shift occurred to yield spectra (Curves 3, Fig. 4) which, except for that of pyridoxal oxime, were very close to those of the free pyridoxal derivative. That the spectrum obtained in this way was due to the enzyme-bound pyridoxal derivative, rather than to the free compound, was established by titration of the transaminase with pyridoxal oxime and with pyridoxal hydrazone in the presence of an excess of hydroxylamine or of hydrazine, respectively (Fig. 5). A change in the slope occurred in each case at the point where 2
equivalents of pyridoxal derivative were present for each equivalent of the enzyme. Similar titrations were carried out with the semicarbazone and isonicotinoylhydrazone of pyridoxal, but no shift in the absorption maxima or enhancement in the absorbance of these compounds occurred in the presence of the transaminase. However, the affinity of the enzyme for these compounds is nearly as high as that for the oxime and hydrazine (cf. Table III). This fact, together with the isosbestic points present in the spectra of Fig. 4, suggests that these compounds, like pyridoxamine, bind to the enzyme, but no marked shift in spectrum occurs in the absence of further reaction.

These findings indicate formation of the derivative-enzyme complexes, V, by operation of Reaction 2a (Fig. 2), and, as shown in Figs. 3 and 4, their further conversion to an equilibrium mixture of V and VI through operation of Reaction 2b (Fig. 2). The position of the latter equilibrium can be determined approximately by spectrophotometric titration of the pyridoxal-enzyme complex with carbonyl reagents (Fig. 6). For a 5 μM solution of pyridoxal-enzyme complex, more than 140 μM isonicotinic acid hydrazide was required to yield the maximum optical density at 350 μm, whereas as little as 20 μM hydrazine sufficed to give the saturation value. The concentration of hydrazine and semicarbazide required fell between these values. The change of absorbance at 350 μm produced by the addition of 2 equivalents divided by that produced by an excess of hydrazine and semicarbazide required fell between these values. The change of absorbance at 350 μm produced by the addition of 2 equivalents divided by that produced by an excess of a given carbonyl reagent gives directly the fraction of derivative-enzyme complex (V) present when Reaction 2b (Fig. 2) is at equilibrium. The values for the oxime, hydrazide, semicarbazide, and isonicotinoylhydrazone of pyridoxal were 70, 51, 35, and 2%, respectively.

These conclusions can be checked in another way. When the aldime linkage in the pyridoxal-transaminase complex, VI (Fig. 2), is reduced with sodium borohydride, a stable linkage is formed to the enzyme, which is thereby inactivated (2). Similar reduction of complex V, formed between a pyridoxal derivative and the transaminase, does not form a covalent link between the derivative and the enzyme and should therefore not inactivate it. If the change in absorption spectrum with time noted in

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**FIG. 5.** Titration of pyridoxamine-pyruvate transaminase (1.2 mg, 0.01 μmole) with (a) pyridoxal oxime, (b), pyridoxal hydrazone, or (c) pyridoxal. Excess hydroxylamine (0.5 μmole) and hydrazine (0.2 μmole) were present in cuvettes a and b, respectively. All reaction mixtures also contained 60 μmole of potassium phosphate buffer, pH 7.0, per ml. Absorbance was measured at 370 μm for Curve a, 360 μm for Curve b, and 410 μm for Curve c.

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**Fig. 6.** The effects of graded additions of carbonyl reagents on the absorbance of the pyridoxal-transaminase complex at 350 μm. a, hydroxylamine; b, hydrazine; c, semicarbazide; d, isonicotinic acid hydrazide. Pyridoxal-transaminase complex (0.7 mg, 0.01 mmole) in 1 ml of 0.05 M potassium phosphate buffer, pH 7.0, was titrated in each case. Absorbance values are corrected for dilution.

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**FIG. 7.** a, Effect of borohydride reduction on the transaminase activity of an incubation mixture containing pyridoxamine-pyruvate transaminase and N'-isonicotinoyl pyridoxal hydrazone. Curve I, the reaction mixture, containing 1.2 mg (0.01 μmole) of pyridoxamine-pyruvate transaminase and 0.02 μmole of N'-isonicotinoyl pyridoxal hydrazone per ml of 0.05 M potassium phosphate buffer, pH 7.0, was incubated at 23°C. Aliquots of the reaction mixture taken at the indicated times were treated with sodium borohydride (1.0 μmole per ml of incubation mixture), then dialyzed for 20 hours at 3°C against 0.02 M potassium phosphate buffer, pH 7.0. Transaminase activity was measured after dialysis. Curve 2, the reaction mixture contained 10 μmole of isonicotinic acid hydrazide per ml in addition to the components listed for Curve I, and was treated in the same manner. b, inactivation of pyridoxamine-pyruvate transaminase by borohydride reduction as a function of the absorbance at 390 μm. Samples were removed from the incubation mixture described under a, Curve I, at various times, their optical density at 390 μm was measured, and then they were treated immediately with sodium borohydride as described in a. Specific activity was determined after dialysis as described in a.
borohydride reduction should increase with time in a similar way, and addition of excess carbonyl reagent, by reversal of Reaction 2b, should protect the enzyme from inactivation. Exactly these results were obtained (Fig. 7a). The extent of inactivation was also linearly correlated with the increase in absorbance at 390 mp, resulting from reaction between pyridoxal derivative and enzyme (Reaction 2b, Fig. 2). The lower curve describes spectral changes observed in cuvettes to which L-alanine (10 μmoles per ml) was also added at zero time. At times indicated by the arrows, 10 μmoles of L-alanine were added to replicate cuvettes used to obtain the upper curve. Absorbance drops immediately to the point reached in those cuvettes to which L-alanine was present at zero time. The reactions were carried out at 23°.

These results show that each of the derivatives of pyridoxal tested is bound to the enzyme (Reaction 2a, Fig. 2), then slowly converted to enzyme-bound pyridoxal (Reaction 2b), which undergoes transamination with L-alanine (Reactions 1c and 1d). The possibility was considered that in addition to this process, complex V might undergo a direct reaction with alanine by Reaction 3 (Fig. 2) to yield carbonyl reagent and complex VII, from which pyridoxamine would be liberated. Since Reaction 2b proceeds slowly (cf. Fig. 3) one should expect a more rapid disappearance of pyridoxal derivative if Reaction 3 is operative than can be accounted for by Reaction 2b alone. This is not observed (Fig. 8); within experimental error the same amount of reaction occurs if Reaction 2b is allowed to proceed in the absence of alanine, and alanine is subsequently added at times indicated by the arrows, as is observed when alanine is added at zero time. We conclude that Reaction 3 does not contribute significantly to the transamination reaction between pyridoxal derivatives and L-alanine.

**DISCUSSION**

Several workers have shown that derivatives of pyridoxal phosphate in which the aldehyde group was blocked by reaction with carbonyl reagents such as isonicotinic acid hydrazide and semicarbazide could replace pyridoxal phosphate as coenzyme for crude preparations of glutamic-oxaloacetic transaminase, kynureninase, 3,4-dihydroxyphenylalanine decarboxylase, glutamic decarboxylase, and other pyridoxal phosphate enzymes. Their failure to demonstrate liberation of pyridoxal phosphate during the incubation of these compounds with the enzyme preparations led them to conclude that the derivatives acted as such, without transformation to pyridoxal phosphate (3-7). Such a view conflicts with present concepts (12-14) of the role of the carbonyl group in pyridoxal phosphate-dependent reactions of amino acids. It is important, therefore, to reconsider the experimental basis of these findings. Jenkins (see discussion in Gomard (4)) and Snell (see discussion in Makino et al. (5)) both pointed out that such pyridoxal derivatives might interact stoichiometrically with apoenzyme to yield carbonyl reagent and holoenzyme in equivalent amounts, thus explaining the experimental facts and requiring no revision in present concepts. Torchinsky (15, 16) has since presented spectrophotometric evidence that bound pyridoxal phosphate is formed when glutamic-aspartic aminotransaminase is activated by the isonicotinoylhydrazone of pyridoxal phosphate. The present study indicates that similar reactions occur between pyridoxamine-pyruvate transaminase and several different carbonyl reagent derivatives of pyridoxal to yield bound pyridoxal, which then undergoes transamination in normal fashion. The affinity of this transaminase for pyridoxal is even less than that of most apo-enzymes so far studied for pyridoxal phosphate, and, since interaction of the carbonyl group of the coenzyme with an amino group of the protein is a common feature of holoenzyme formation in pyridoxal phosphate enzymes so far studied, reactions fully analogous to Reaction 2b (Fig. 2) would be favored in all of these cases. In the absence of further data it may thus be assumed that all of the activating effects previously reported for the corresponding derivatives of pyridoxal phosphate result from their interaction with the apoenzymes to form pyridoxal phosphate enzymes.

The summation of Reactions 2a, 2b, 1c, and 1d (Fig. 2) catalyzed by pyridoxamine-pyruvate transaminase gives Reaction 2.

\[
P y - C H = N R + l - a l a n i n e + H _ 2 O = \rightarrow
\]

\[
P y v r u v a t e + P y C H _ 2 N H _ 2 + R N H _ 2
\]

From Table I, it is apparent that pyruvate is formed in less than the stoichiometric amounts required by this equation, a result ascribed to occurrence of Reaction 3.

\[
P y r u v a t e + R N H _ 2 \rightarrow C H _ 3 C(=N R) C O O H + H _ 2 O
\]

Since the carbonyl reagent is liberated at the surface of the transaminase, which also has affinity for pyruvate, it is possible that Reaction 3 occurs more efficiently in the presence of the transaminase than would be true for equimolar concentrations of the reactants in the absence of enzyme. This possibility has not been checked. Finally, summation of Equations 2, 3, and 1 gives Equation 4, a partially enzyme-catalyzed transfer of a carbonyl reagent residue from one carbonyl compound to another.

\[
P y - C H = N R + C H _ 2 C O C O O H \rightarrow
\]

\[
C H _ 3 C(=N R) C O O H + P y C H O
\]

If the carbonyl reagent were hydroxylamine, Equation 4 would represent one instance of a transamination reaction. The reac-
tion may be mechanistically related to a relatively sluggish enzymatic transoximation reaction reported several years ago (17) to require pyridoxal phosphate. Previous studies in this laboratory have shown that injected pyridoxal oxime rapidly gives rise to excretion of 4-pyridoxic acid in rats (18), and that incubation of pyridoxal oxime with liver proteins results in a slow enzymatic formation of pyridoxal. The enzymes responsible for the latter reactions have not been purified or fully characterized.

SUMMARY

Pyridoxamine-pyruvate transaminase catalyzes a slow transamination reaction between L-alanine and the oxime, hydrazone, semicarbazone, or N'-isonicotinoylhydrazone of pyridoxal to yield pyridoxamine. These pyridoxal derivatives have affinities for the enzyme approaching those of pyridoxal and pyridoxamine; they undergo transamination at maximal velocities approximately 1% or less of that of pyridoxal. Spectrophotometric studies with stoichiometric amounts of the pure enzyme show that 2 moles of these pyridoxal derivatives bind per mole of protein; this complex then undergoes a slow conversion to an equilibrium mixture with pyridoxal-enzyme complex. This transformation can also be followed by sodium borohydride reduction, which inactivates the pyridoxal-enzyme complex by irreversible fixation of pyridoxal to a lysine residue of the enzyme but does not inactivate the pyridoxal derivative-enzyme complex. Only the pyridoxal-enzyme complex undergoes transamination with L-alanine at a measurable rate; i.e. transamination of the pyridoxal derivatives is obligatorily dependent upon their interaction with enzyme to form the pyridoxal-transaminase complex. These observations support the view that activation of various apoenzymes by the carbonyl reagent derivatives of pyridoxal phosphate occurs only by virtue of their interaction with the apoenzyme to yield enzyme-bound pyridoxal phosphate. The reactions may also be related to the ill-defined process of “transoximation.”

REFERENCES

Interaction of Pyridoxamine-Pyruvate Transaminase with Carbonyl Derivatives of Pyridoxal
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