Inhibition of Pyridoxal Kinase by the Pyridoxal-γ-aminobutyrate Imine*

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Previous studies have demonstrated that pyridoxal kinase is inhibited by γ-aminobutyrate, a neurotransmitter of the vertebrate central nervous system, but the mechanism and significance of this inhibition have remained obscure. The present study was undertaken to clarify the mechanism of this inhibition. The results show that there was little inhibition of pyridoxal kinase by γ-aminobutyrate at low concentrations of pyridoxal (<100 μM), but that the inhibition became stronger as the concentration of pyridoxal was raised. Similar results were obtained when β-alanine and ε-aminocaproic acid were used as inhibitors, but glycine did not inhibit. Conventional models of enzyme inhibition did not fit the inhibition data and γ-aminobutyrate did not inhibit when pyridoxamine was the substrate suggesting that it did not inhibit by interacting directly with the enzyme. Depletion of the substrate, pyridoxal, by formation of the pyridoxal-γ-aminobutyrate imine and direct inhibition of pyridoxal kinase by the pyridoxal-γ-aminobutyrate imine were considered as alternative mechanisms. To distinguish between the two mechanisms, the equilibrium constant for pyridoxal-γ-aminobutyrate imine formation was determined and used to calculate the concentrations of free pyridoxal and pyridoxal-γ-aminobutyrate imine under assay conditions. The values of Kd at pH 6.2, 7.3, and 8.0 (μ = 0.2 M, 37°C) were 0.20 ± 0.03 M−1, 3.3 ± 0.3 M−1, and 14.5 ± 0.7 M−1, respectively. The association constant for the pyridoxal-glycine imine was 0.85 ± 0.12 M−1 (pH 6.2). The results showed that the pyridoxal concentration was not appreciably reduced by γ-aminobutyrate indicating that inhibition was not the result of substrate depletion. This conclusion was supported by the failure of glycine to inhibit the enzyme. The calculated pyridoxal-γ-aminobutyrate imine behaved as a simple noncompetitive inhibitor with respect to pyridoxal thereby supporting the hypothesis that pyridoxal kinase was inhibited by the imine. The relationship of the inhibition of pyridoxal kinase by pyridoxal-γ-aminobutyrate imine to the synthesis of γ-aminobutyrate is discussed.

Pyridoxal kinase (EC 2.7.1.35), pyridoxal:ATP 5'-phosphotransferase, catalyzes the transfer of a phosphate group from ATP to the 5'-hydroxymethyl group of the B6 vitamins (1). Pyridoxal kinase catalyzes the phosphorylation of the three forms of vitamin B6 (pyridoxal, pyridoxamine, pyridoxine) at approximately equal rates. However, the Michaelis constants for the three forms vary widely. For pyridoxal kinase from beef brain, the Kd for pyridoxal is the lowest of the three (2).

In studies with pyridoxal kinase from human brain (3) and rat brain (4), Snell and co-workers demonstrated that carboxyl reagents inhibit the enzymatic production of pyridoxal-P. Moreover, they showed that the adducts which formed between the carboxyl reagents and pyridoxal were more potent inhibitors than the parent carboxyl reagents. The same carboxyl reagents did not inhibit the enzyme when pyridoxine was substituted for pyridoxal as substrate, but pyridoxine phosphorylation was strongly inhibited when the isolated adducts were added to the assay solution (4). These observations suggested that the adducts, not the carboxyl reagents, were the true inhibitors of pyridoxal kinase. In another report, pyridoxal kinase from guinea pig brain was similarly inhibited by a Schiff base isolated from phenylketonuric urine (5). The Schiff base was identified as pyridoxylidene-β-phenylethylamine (6).

The inhibition of brain pyridoxal kinase by biogenic amines has been studied somewhat inconclusively in recent years. Several investigators have suggested that inhibition of pyridoxal kinase by compounds such as GABA, histamine, nor-epinephrine, tyramine, dopamine, and serotonin might represent a feedback regulatory mechanism for the synthesis of these amines in brain (7-9). Several studies have demonstrated that these compounds do indeed inhibit pyridoxal kinase, but little understanding of the mechanism of inhibition by these compounds has been forthcoming from these reports (7, 8, 10). In most instances, it has been postulated that GABA, serotonin, tyramine, and histamine inhibit pyridoxal kinase by directly interacting with the enzyme. The possibility that pyridoxal kinase was inhibited by a Schiff base formed between pyridoxal and the inhibitory amine has been discounted (8, 10).

This study was undertaken to investigate the possibility that GABA inhibits pyridoxal kinase by first reacting with pyridoxal to form a Schiff base. The association constant for the formation of the pyridoxal-GABA adduct was measured under conditions similar to that of the pyridoxal kinase assay medium. This equilibrium constant was used for subsequent analysis of kinetic data obtained for the inhibition of pyridoxal kinase by GABA. In addition, the effect of GABA on pyridoxal kinase activity was measured when pyridoxamine was substituted for pyridoxal as the variable substrate. The experiments indicate that GABA does not inhibit the enzyme directly and provide evidence that the pyridoxal-GABA imine is a noncompetitive inhibitor of pyridoxal kinase.

**EXPERIMENTAL PROCEDURES**

*Materials—Adult bovine brains were obtained from the United States Department of Agriculture, Beltsville, Md. The whole brains were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: GABA, γ-aminobutyrate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid).
were placed on ice immediately after excision and homogenized within 1 h.

Pyridoxal-HCl, pyridoxal 5’-phosphate, pyridoxamine di-HCl, pyridoxamine 5’-phosphate-HCl, γ-aminobutyric acid, β-alanine, δ-amino-n-valeric acid, adenosine 5’-triphosphate (disodium salt), Glutathione (reduced form), ethylenediamine tetracetic acid (disodium salt), DEAE-cellulose, and Dowex 50W were obtained from Sigma Chemical Co. Piperazine-N,N’-bis(2-ethanesulfonic acid) (di-sodium salt) was purchased from Calbiochem. Coomassie Brilliant Blue G-250 was purchased from Eastman Kodak Co., Rochester, N.Y. Utrapure ammonium sulfate was purchased from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were analytical grade reagents.

**Protein Purification**—Pyridoxal kinase was purified by the method of Neary and Diven (11) through the Sephadex G-100 column chromatography step. The average purification at this step in two purification steps was 100-fold. Low yields in subsequent steps and the instability of the highly purified enzyme rendered additional purification impractical. The actual values varied about 5% on a daily basis. GABA had little or no effect on the fluorescence of the pyridoxal-P-CN derivative of GABA. The separation of pyridoxamine-P and pyridoxamine by the cation exchange resin was validated by running solutions of known concentrations through small columns of Dowex 50W-X8 (400 mesh) prepared by heating the suspended beads with 0.01 M NaOH for several hours at 90°C. After cooling, the beads were washed in turn with distilled water, 4 M HCl, distilled water, 1 M NaOH, and distilled water until the pH of the eluate was neutral.

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**Determination of Association Constants**—Measurement of the equilibrium constant for the formation of the pyridoxal-GABA adduct and the pyridoxal-glycine adduct was made with a Cary 14 UV-visible spectrophotometer equipped with a thermostatted cuvette chamber (37°C). Spectra were taken of solutions containing 0.1 mm or 0.05 mm pyridoxal and concentrations of GABA or glycine ranging from 0 to 1.0 mM. The pH was controlled with buffers of constant ionic strength (μ = 0.2 M) prepared according to the method of McElvain (13). Variation in the ionic strength due to variable concentrations of amino acid or chemical reaction was considered negligible in comparison to the ionic strength of the buffer.

The time required for the adduct formation reaction to reach equilibrium was measured by monitoring the absorbance at 410 nm after mixing the reactants in a constant temperature cuvette chamber (37°C) of a Gilford spectrophotometer. For all reactant concentrations studied, the reaction half-time was 3 min or less.

Calculation of the association constant (Kₐ) for each system was done by fitting Equation 2 to the experimental data,

\[
\Delta A_{10} = \frac{\Delta A_{10}}{K_a + [S]} 
\]

where ΔA₁₀ is the difference between the absorbance at 410 nm of a pyridoxal solution and the absorbance of the same pyridoxal solution containing an amino acid, S is the molar concentration of the variable reactant (the amino acid), ΔA₁₀ is the maximum possible \( \Delta A_{10} \) and \( K_a \) is the dissociation constant for the system being studied; \( K_a = (K_a)^{-1} \). Equation 2 is applicable to a single, binary chemical reaction where the system is arranged so that a large molar excess of one reactant (the amino acid) is added to a low concentration of another reactant (pyridoxal). The data for the reaction (pyridoxal + amino acid) in the forward direction fitted by means of Cleland’s hyper program (14) on a Digital Equipment Co. PDP 11/34 Computer. This program was written to estimate kinetic parameters for the Michaelis-Menten rate equation, but was easily adapted to the calculation of \( K_a \) since Equation 2 and the Michaelis-Menten equation have the same mathematical form.

**Calculation of Equilibrium Concentrations of Assay Solution Complexes and Components**—An iterative numerical technique was used to calculate the equilibrium concentrations of the various components of the assay solution (Zn²⁺, ATP⁻, K⁺, etc.) and the corresponding complexes formed from these components (Zn²⁺-ATP⁻, HA⁺⁺, etc.). The calculations were done on the Digital Equipment Co. PDP 11/34 Computer.
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Co. PDP 11/34 Computer using a program (referred to as COMPLX) kindly supplied by A. C. Storer and A. Cornish-Bowden. There are at least eight simultaneous equilibria in an assay solution used for the kinetic experiments described here. The important reactions and the corresponding association constants are listed in Table I. It was assumed in compiling this information that the $K_a$ for ZnHATP is zero (11). It was also assumed that the Pipes buffer does not form a complex with Zn$^{2+}$ to an appreciable extent, since it is known that Pipes does not form complexes with Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, or Cu$^{2+}$ (20).

Unfortunately, several association constants are known only at temperatures other than $37^\circ$C. Since their values are small, the associated error in using these values at $37^\circ$C was negligible. COMPLX was used to calculate concentrations of the pyridoxal-GABA imine for the analysis of some experiments.

RESULTS

Reaction between Pyridoxal and Amino Acids—Pyridoxal is known to react readily with $\alpha$-amino acids to form the associated imine adducts (19, 21–26). Since GABA should react with pyridoxal in the same manner, we measured the equilibrium constant for the formation of the pyridoxal-GABA imine under conditions similar to the pyridoxal kinase assay (see "Experimental Procedures"). At pH 6.2, 7.3, and 8.0 the association constants were $0.20 \pm 0.03 \text{ M}^{-1}$, $3.3 \pm 0.3 \text{ M}^{-1}$, and $14.5 \pm 0.7 \text{ M}^{-1}$, respectively. The pH dependence of $K_a$ is consistent with the formation of the Schiff base with the release of a proton. To demonstrate that glycine reacts with pyridoxal under the conditions of the assay, the equilibrium constant for the formation of the pyridoxal-glycine adduct was determined at pH 6.2. The $K_a$ was computed to be $0.69 \pm 0.12 \text{ M}^{-1}$, a value nearly 5 times that for the pyridoxal-GABA imine at the same pH.

Inhibition of Pyridoxal Kinase by Excess Zn$^{2+}$—Pyridoxal kinase requires a divalent cation for activity; for the mammalian brain enzyme Zn$^{2+}$ has been reported to be the most effective cation among those tested (2, 11). Experiments designed to determine the optimum conditions for the pyridoxal kinase assay demonstrated that Zn$^{2+}$ ion concentrations up to 50 $\mu$M stimulated enzyme activity (1.0 mM ATP). Above this concentration Zn$^{2+}$ was inhibitory (Fig. 1). A similar situation was observed when Mg$^{2+}$ was substituted for Zn$^{2+}$. In comparison to the activation by Zn$^{2+}$, the peak of activity was lower in amplitude and occurred at a higher total Mg$^{2+}$ concentration. These results are similar to earlier reports of divergent cation activation of pyridoxal kinase (2, 11). The inhibitory effect of Zn$^{2+}$ or Mg$^{2+}$ indicated that the results of experiments in which ZnATP$^2-$ or MgATP$^-$ was the variable substrate would be difficult to interpret. We found this to be the case, and thus concentrated our efforts on determining the inhibitory effect of GABA with respect to pyridoxal at a fixed ZnATP$^2-$ concentration.

Inhibition of Pyridoxal Kinase Activity—Initial experiments were designed to measure the inhibitory effect of GABA on pyridoxal kinase activity with pyridoxal as the variable substrate (39 $\mu$M ZnATP$^2-$). The enzyme reaction was linear with time up to 15 min at a GABA concentration of 2 mM and pyridoxal concentrations as high as 400, $\mu$M. Initial velocities were collected for a wide range of GABA and pyridoxal concentrations (Fig. 2). For the GABA concentrations tested there was little or no inhibition at low pyridoxal concentrations, whereas the enzyme was strongly inhibited by GABA at high concentrations of pyridoxal. Conventional types of enzyme inhibition which involve the direct interaction of the inhibitor with the enzyme did not fit the data in Fig. 2. With this in mind, two alternative models which involve Schiff base adduct formation between pyridoxal and GABA were consid-

![Fig. 1. Pyridoxal kinase activity as a function of the concentration of Zn$^{2+}$ or Mg$^{2+}$. Pyridoxal kinase was incubated for 10 min in the presence of 0.1 M Pipes buffer (pH 6.0), 0.1 M KCl, 1.0 mM ATP, 600 $\mu$M pyridoxal, and variable concentrations of zinc acetate (○) or magnesium acetate (△). The enzyme concentration in 0.2 ml of assay volume was 3.8 units/ml (42 $\mu$g/ml of protein). Each point represents the average of two determinations; the deviation of the points from the mean was usually no greater than 7%.

![Fig. 2. Pyridoxal kinase activity as a function of the concentration of pyridoxal in the presence of increasing concentrations of GABA. Pyridoxal kinase was incubated for 10 min at $37^\circ$C in a total volume of 0.2 ml with 0.1 M Pipes buffer (pH 6.0), 0.1 M KCl, 50 $\mu$M zinc sulfate, 1.0 mM ATP, and 0 to 10 mM GABA. The GABA concentrations were: 0 mM (○), 1 mM (●), 2 mM (●), 5 mM (●), 7.5 mM (●), 10 mM (△). The enzyme concentration was 5 units/ml (45 $\mu$g/ml of protein). The pyridoxal-P concentrations were determined by measuring the pyridoxal-P-CN fluorescence and correcting for the interfering pyridoxal-CN fluorescence as outlined under "Experimental Procedures." Each point is the average of two determinations. The deviation of the experimental values from the mean was 5% or less.

### Table I

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$K_a$ (Temp.)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Zn$^{2+}$ + HATP$^{2-}$ = ZnHATP$^-$</td>
<td>$3.95 \times 10^3$</td>
<td>(37) 16</td>
</tr>
<tr>
<td>2. Zn$^{2+}$ + ATP$^{2-}$ = ZnATP$^-$</td>
<td>$5.47 \times 10^3$</td>
<td>(37) 16</td>
</tr>
<tr>
<td>3. H$^+$ + HATP$^{2-}$ = H$_2$ATP$^-$</td>
<td>$8.12 \times 10^3$</td>
<td>(37) 16</td>
</tr>
<tr>
<td>4. H$^+$ + ATP$^{2-}$ = HATP$^-$</td>
<td>$1.03 \times 10^3$</td>
<td>(37) 17</td>
</tr>
<tr>
<td>5. K$^+$ + ATP$^{2-}$ = KATP$^-$</td>
<td>$1.4 \times 10^3$</td>
<td>(30) 18</td>
</tr>
<tr>
<td>6. Na$^+$ + ATP$^{2-}$ = NaATP$^-$</td>
<td>$1.5 \times 10^3$</td>
<td>(30) 18</td>
</tr>
<tr>
<td>7. Zn$^{2+}$ + PAL$^+$ = ZnPAL$^+$</td>
<td>$2.1 \times 10^2$</td>
<td>(25) 19</td>
</tr>
<tr>
<td>8. PAL$^{2-}$ + GABA$^-$ = PAL-GABA$^-$</td>
<td>$2.0 \times 10^4$</td>
<td>(37) 6</td>
</tr>
</tbody>
</table>

*The abbreviations used are: PAL, pyridoxal; PAL-GABA, pyridoxal-GABA imine adduct.*

* Determined at pH 6.2.
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Effect of glycine on pyridoxal kinase activity

Pyridoxal kinase was incubated for 10 min at 37°C in 0.1 M Pipes buffer (pH 6.0), containing 0.1 M KCl, 50 μM zinc sulfate, 1.0 mM ATP, and the indicated concentrations of pyridoxal and glycine. The enzyme concentration was 3.81 units/ml (42 μg/ml of protein). Pyridoxal-P concentration was determined as described under "Experimental Procedures," except that no corrections were necessary since glycine had no effect on the pyridoxal-CN or the pyridoxal-P-CN fluorescence. Each value is the average of two determinations. Deviation of the experimental values from the mean was 3% or less.

<table>
<thead>
<tr>
<th>Glycine (mM)</th>
<th>Pyridoxal (μM)</th>
<th>0</th>
<th>100</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>105</td>
<td>184</td>
<td>371</td>
<td>440</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>203</td>
<td>388</td>
<td>466</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>107</td>
<td>202</td>
<td>391</td>
<td>461</td>
<td></td>
</tr>
</tbody>
</table>

The K_i for the pyridoxal-GABA adduct was approximately 0.1 μM.

Indirect evidence that pyridoxal kinase is inhibited by the pyridoxal-GABA imine rather than directly by GABA was provided by experiments designed to examine the effects of GABA when pyridoxamine was substituted for pyridoxal as substrate. Preliminary experiments showed that pyridoxamine-P production was linear with enzyme concentration and time up to 20 min of incubation at pH 6.0. Using these conditions, enzyme activity was measured at 0.5, 5, and 10 mM pyridoxamine and 0, 0.5, 2, and 5 mM GABA. Pyridoxamine was tested over this wide range of concentrations because GABA was effective as an inhibitor only at the higher concentrations of pyridoxal. We observed no significant inhibition of the enzyme by any concentration of GABA at any of the pyridoxamine concentrations tested. The reaction rates (±S.E.) at 10 mM pyridoxamine and 0, 0.5, 2, and 5 mM GABA were 57.2 ± 0.70, 57.5 ± 0.70, 57.5 ± 2.3, and 58.3 ± 3.5 mnole of pyridoxamine-P/min/mg of protein, respectively, and the rates at 0.5 mM pyridoxamine and the same concentrations of GABA were 5.38 ± 0.93, 6.20 ± 0.70, 5.87 ± 0.23, and 6.04 ± 0.47, respectively. Since GABA and pyridoxamine cannot react to form a Schiff base adduct, the failure of GABA to inhibit under these conditions strongly supports the idea that GABA does not inhibit by direct interaction with the enzyme.

Experiments were conducted to test the effect of glycine on pyridoxal kinase activity since glycine should inhibit equally as well or better than GABA if the inhibition by GABA was attributable to substrate depletion only. Initial velocities were measured at glycine concentrations of 2.0 and 7.5 μM and pyridoxal concentrations from 50 to 500 μM (Table II). The data show that glycine does not inhibit pyridoxal kinase at concentrations at which GABA is a very potent inhibitor indicating that inhibition of the enzyme by GABA was not the result of depleting the substrate, pyridoxal, by Schiff base formation. This conclusion was substantiated by computing the concentration of the pyridoxal-GABA adduct as a function of GABA and pyridoxal concentrations (pH 6.0, 37°C, μ = 0.2 M). The calculations indicated that the concentration of the imine was very low at pH 6.0. For instance, the-adduct concentration was 0.50 μM when the pyridoxal concentration was 250 μM and the GABA concentration was 10 mM. It is clear that under the conditions of the assay the concentration of free pyridoxal would change by only a very small percentage after the reaction of pyridoxal with GABA.

Inhibition of Pyridoxal Kinase by β-alanine and δ-Aminovaleric Acid—Since glycine failed to inhibit pyridoxal kinase, β-alanine, and δ-aminovaleric acid were tested for their...
p-alanine or 8-aminovaleric acid. Above doxal, it is not possible to analyze these results as was done alanine. It was possible to rank them in order of potency; GABA inhibited the enzyme in a manner similar to that of GABA. Very little inhibition was detected at low concentrations of either of these two compounds.

Similar results were obtained when p-alanine or 8-aminovaleric acid were substituted for GABA as the inhibitor, but glycine did not inhibit. The lack of correspondence between the inhibition data and conventional models of enzyme inhibition suggested that the enzyme and GABA did not interact directly. This conclusion was supported by the finding that GABA did not inhibit when pyridoxamine was substituted for pyridoxal as the substrate. The similarity of the results for GABA, p-alanine, and 8-aminovaleric acid inhibition suggested that these compounds inhibited the enzyme by the same mode of action.

In view of these results, we considered two mechanisms for GABA inhibition of pyridoxal kinase which involve Schiff base formation between GABA and pyridoxal. The first was a mechanism in which pyridoxal kinase is inhibited by the pyridoxal-GABA adduct. The second mechanism was substrate depletion. According to the substrate depletion hypothesis, the reaction rate is slowed because of a substantial decrease in pyridoxal concentration due to the formation of an inert adduct between the substrate and the so-called "inhibitor." This inhibition is only apparent since there is no direct interaction of the enzyme with either the so-called inhibitor or the adduct that forms. For the present system, calculation of the adduct concentration as a function of the pyridoxal and GABA concentration showed that only small amounts of adduct actually form at pH 6.0. The amount of pyridoxal available to bind to the enzyme is not greatly different with or without GABA present at any of the concentrations used in these experiments. This was confirmed by the failure of glycine to inhibit pyridoxal kinase. Comparison of the association constants measured under the same conditions showed that glycine and pyridoxal react to a greater extent than do GABA and pyridoxal. Since glycine did not inhibit the enzyme, it appears that the effective concentration of pyridoxal did not change to an extent that could significantly affect the forward rate of reaction when glycine was added to the reaction mixture.

Our evidence provides direct support for the mechanism in which the pyridoxal-GABA imine is the true inhibitor of pyridoxal kinase. In this mechanism, the adduct that forms from the reaction of the substrate and the so-called inhibitor binds to the enzyme to produce a catalytically inactive form of the enzyme. By this scheme, the low degree of inhibition at 2.0 mM GABA and 100 pM pyridoxal can be explained by the low concentration of adduct formed under these conditions; the calculated concentration was 0.04 pM. The greater amount of inhibition observed at 2 mM GABA and 500 pM pyridoxal can be attributed to the larger concentration of adduct under these conditions (0.20 pM). Thus, the apparent inhibition of pyridoxal kinase by GABA can be explained more accurately on the basis of an indirect effect of GABA on the enzyme. GABA must first react with pyridoxal to form an imine adduct which then interacts directly with the enzyme as a noncompetitive inhibitor with respect to pyridoxal.

Since glycine failed to inhibit pyridoxal kinase, we investigated the inhibitory potential of p-alanine and 8-aminovaleric acid, two compounds that are structural homologues of GABA and glycine. Both of these compounds inhibited pyridoxal kinase, and since it is expected that they also react with pyridoxal to form a Schiff base adduct, we concluded that they probably inhibited by the same mode of action as GABA. Thus, it seems contradictory that glycine lacked any inhibitory potential for pyridoxal kinase. However, because glycine is an a-amino acid and p-alanine, GABA, and 8-aminovaleric acid are o-amino acids, glycine probably forms an imine adduct that differs from the imines of pyridoxal and p-alanine, GABA, or 8-aminovaleric acid in its ability to interact with the enzyme site responsible for inhibition of activity. This difference may be rationalized on the basis that Schiff base adducts formed from the condensation of a-amino acids and pyridoxal are known to chelate divalent cations with very large affinities (19, 21–23). The equilibrium constant of formation of a 1:1:1 complex (pyridoxal:glycine:Zn(II)) is 2.7 x 10^{10} M^{-1}; the value for the 2:2:1 complex is 7.3 x 10^{16} M^{-1} (19). The

**FIG. 4.** Inhibition of pyridoxal kinase by o-amino acids. A, inhibition of pyridoxal kinase by p-alanine. Pyridoxal kinase was incubated for 13 min in 0.1 M Pipes buffer (pH 6.0) containing 0.1 M KCl, 50 mM zinc acetate, 1.0 mM ATP, and no p-alanine ( ), 2.0 ( ), and 7.5 mM ( ) p-alanine. The enzyme concentration was 3.8 units/ml (42 pg/ml). Pyridoxal-P concentrations were determined as outlined under "Experimental Procedures." Fluorescence of the pyridoxal-P-CN and pyridoxal-CN derivatives were not different in the presence and absence of the concentrations of p-alanine used for this experiment. Each point represents the average of two determinations; the deviation of the experimental points from the mean was 5% or less. B, inhibition of pyridoxal kinase by 8-aminovaleric acid. Pyridoxal kinase was incubated under the same conditions as (A) except 8-aminovaleric acid was used at 0 ( ), 2.0 ( ), and 7.5 mM ( ). Fluorescence of the pyridoxal-P-CN derivative was not affected by 8-aminovaleric acid, but fluorescence of the pyridoxal-CN derivative was enhanced by this compound. Corrections were made for this in the same manner as described for GABA under "Experimental Procedures." Each point is the average of two determinations; deviation of the experimental values from the mean was 5% or less.

**DISCUSSION**

Kinetic studies of pyridoxal kinase demonstrated that inhibition of the enzyme by GABA was pronounced at high concentrations of GABA and pyridoxal but was very slight at low concentrations of either of these two compounds. Similar results were obtained when p-alanine or 8-aminovaleric acid were substituted for GABA as the inhibitor, but glycine did not inhibit. The lack of correspondence between the inhibition data and conventional models of enzyme inhibition suggested that the enzyme and GABA did not interact directly. This conclusion was supported by the finding that GABA did not inhibit when pyridoxamine was substituted for glycinat. The similarity of the results for GABA, p-alanine, and 8-aminovaleric acid inhibition suggested that these compounds inhibited the enzyme by the same mode of action.

In view of these results, we considered two mechanisms for GABA inhibition of pyridoxal kinase which involve Schiff base formation between GABA and pyridoxal. The first was a mechanism in which pyridoxal kinase is inhibited by the pyridoxal-GABA adduct. The second mechanism was substrate depletion. According to the substrate depletion hypothesis, the reaction rate is slowed because of a substantial decrease in pyridoxal concentration due to the formation of an inert adduct between the substrate and the so-called "inhibitor." This inhibition is only apparent since there is no direct interaction of the enzyme with either the so-called inhibitor or the adduct that forms. For the present system, calculation of the adduct concentration as a function of the pyridoxal and GABA concentration showed that only small amounts of adduct actually form at pH 6.0. The amount of pyridoxal available to bind to the enzyme is not greatly different with or without GABA present at any of the concentrations used in these experiments. This was confirmed by the failure of glycine to inhibit pyridoxal kinase. Comparison of the association constants measured under the same conditions showed that glycine and pyridoxal react to a greater extent than do GABA and pyridoxal. Since glycine did not inhibit the enzyme, it appears that the effective concentration of pyridoxal did not change to an extent that could significantly affect the forward rate of reaction when glycine was added to the reaction mixture.

Our evidence provides direct support for the mechanism in which the pyridoxal-GABA imine is the true inhibitor of pyridoxal kinase. In this mechanism, the adduct that forms from the reaction of the substrate and the so-called inhibitor binds to the enzyme to produce a catalytically inactive form of the enzyme. By this scheme, the low degree of inhibition at 2.0 mM GABA and 100 pM pyridoxal can be explained by the low concentration of adduct formed under these conditions; the calculated concentration was 0.04 pM. The greater amount of inhibition observed at 2 mM GABA and 500 pM pyridoxal can be attributed to the larger concentration of adduct under these conditions (0.20 pM). Thus, the apparent inhibition of pyridoxal kinase by GABA can be explained more accurately on the basis of an indirect effect of GABA on the enzyme. GABA must first react with pyridoxal to form an imine adduct which then interacts directly with the enzyme as a noncompetitive inhibitor with respect to pyridoxal.

Since glycine failed to inhibit pyridoxal kinase, we investigated the inhibitory potential of p-alanine and 8-aminovaleric acid, two compounds that are structural homologues of GABA and glycine. Both of these compounds inhibited pyridoxal kinase, and since it is expected that they also react with pyridoxal to form a Schiff base adduct, we concluded that they probably inhibited by the same mode of action as GABA. Thus, it seems contradictory that glycine lacked any inhibitory potential for pyridoxal kinase. However, because glycine is an a-amino acid and p-alanine, GABA, and 8-aminovaleric acid are o-amino acids, glycine probably forms an imine adduct that differs from the imines of pyridoxal and p-alanine, GABA, or 8-aminovaleric acid in its ability to interact with the enzyme site responsible for inhibition of activity. This difference may be rationalized on the basis that Schiff base adducts formed from the condensation of a-amino acids and pyridoxal are known to chelate divalent cations with very large affinities (19, 21–23). The equilibrium constant of formation of a 1:1:1 complex (pyridoxal:glycine:Zn(II)) is 2.7 x 10^{10} M^{-1}; the value for the 2:2:1 complex is 7.3 x 10^{16} M^{-1} (19). The
interaction of adducts formed between pyridoxal and \(\omega\)-amino acids with divalent metal cations has not been reported, but, for structural reasons, these imines probably cannot tightly bind Zn\(^{2+}\). Thus, glycine might not inhibit pyridoxal kinase because the pyridoxal-glycine imine, once formed, is immediately converted to an irreversible Zn\(^{2+}\) complex. In contrast, the pyridoxal-GABA imine probably does not bind Zn\(^{2+}\) tightly and therefore could readily inhibit the enzyme. Spectrophotometric studies of solutions containing pyridoxal, glycine, or GABA, and graded concentrations of Zn\(^{2+}\) (pH 6.0, 37°C, tetracarboxylase) showed that complexes of pyridoxal:glycine:Zn\(^{2+}\) readily form under the conditions of the pyridoxal kinase assay, whereas similar complexes of pyridoxal, GABA, and Zn\(^{2+}\) form to a much lesser extent, if at all, under the same conditions.

It has previously been suggested that GABA inhibition of pyridoxal kinase represents a feedback control point in the synthesis of GABA, since pyridoxal-P is the cofactor for glutamate decarboxylase (the enzyme that catalyzes the formation of GABA) (7). Such inhibition of pyridoxal kinase by GABA would be expected to reduce the amount of pyridoxal-P available to bind to glutamate decarboxylase and thereby reduce the amount of catalytically active glutamate decarboxylase. As a result, GABA production would decrease. Although pyridoxal kinase is inhibited by GABA, two lines of evidence suggest that this inhibition is of limited importance in the regulation of GABA synthesis. First, GABA, and probably other inhibitory metabolites, does not affect pyridoxal kinase directly. In view of the mechanism of GABA inhibition, it appears that the metabolic capable of inhibiting pyridoxal kinase does so by interacting with pyridoxal first. The state of inhibition of pyridoxal kinase is determined not only by the concentration of the inhibitory metabolite, but also by the concentration of pyridoxal. Since the concentration of pyridoxal in brain is estimated to be about 1 nmol/g wet weight (28), it seems unlikely that the imines formed between pyridoxal and GABA would be present in sufficient concentration to substantially inhibit the enzyme. Secondly, the inhibition of pyridoxal kinase is not specific for one metabolite such as GABA. Several laboratories have shown that a large number of neurochemically important metabolites inhibit pyridoxal kinase (7–10). Since the inhibition of pyridoxal kinase should affect all pyridoxal-P-requiring enzymes, it is unlikely that any one of the metabolites specifically controls its own synthesis by feedback inhibition of pyridoxal-P synthesis.

REFERENCES

\(^{2}\) D. M. Abercrombie and D. L. Martin, unpublished observations.