Acknowledgment

We are indebted to Dr. Oscar Bodansky for his continued support and valuable advice and discussion during the course of these studies.

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Bovine Liver Glutamate Dehydrogenase. Equilibria and Kinetics of Inactivation by Pyridoxal*

Dennis Piszkiewicz and Emil L. Smith†

ABSTRACT: Pyridoxal inactivates glutamate dehydrogenase presumably by forming an imine with the ϵ-amino group of lysine-97, as in the case of pyridoxal 5′-phosphate. The equilibrium constants for imine formation at varying pH values (K_{pH}) have been calculated from the initial concentrations of enzyme and pyridoxal and the final degree of inactivation. The variation of K_{pH} with pH has been related to the dissociation constants of the reactive ϵ-amino group, pyridoxal, and the product imine, and a single equilibrium constant for imine formation. When this treatment was applied to the inactivation of glutamate dehydrogenase by pyridoxal with the known K_a values of pyridoxal and reasonable, assumed values for the K_a values of the ionizating groups of the imine product, the reactive ϵ-amino group was found to have $pK_a = 7.9 \pm 0.2$. Inactivation of glutamate dehydrogenase by pyridoxal was found to be a kinetically second-order process for which the rate constant of inactivation was dependent upon the mole fraction of a conjugate base having $pK_{app} = 8.1 \pm 0.2$; this group was concluded to be the ϵ-amino group of lysine-97. The enzyme was protected from pyridoxal inactivation by DPNH and TPNH.

Glutamate dehydrogenase (L-glutamate:DPN(TPN) oxidoreductase (deaminating), EC 1.4.1.3) occupies an important position in the nitrogen metabolism of mammals since it catalyzes a reaction which is the major pathway for the interconversion of α-amino group nitrogen and ammonia (Meister, 1965; Frieden, 1963a). Although other important enzymes of amino acid metabolism generally employ pyridoxal 5′-phosphate as a cofactor in transamination or decarboxylation reactions, glutamate dehydrogenase is inhibited by this compound through the formation of an imine with the ϵ-amino group of a lysyl residue (Anderson *et al.*, 1966). In addition, this enzyme is subject to allosteric regulation by a variety of nucleoside polyphosphates (Frieden, 1963a,b); for example, GTP acts as an inhibitor and ADP as an activator. In its active form the dehydrogenase is composed of six-subunit polypeptide chains (Eisenberg and Tomkins, 1968).

A recent report from this laboratory has presented a tentative but almost complete amino acid sequence of the subunit polypeptide chain of the enzyme from bovine liver which indicated that the six subunits are identical, each with a molecular weight of 56,000 (Smith *et al.*, 1970). We have also described the specific nitration of a tyrosyl residue which is accompanied by a loss of allosteric inhibition by GTP (Piszkiewicz *et al.*, 1971), and we have identified lysine-97 as the residue which is labeled during the reversible inhibition of the enzyme by pyridoxal 5′-phosphate (Piszkiewicz *et al.*, 1970).

Pyridoxal has also been shown to inactivate glutamate dehydrogenase (Anderson *et al.*, 1966), and it is likely that this is also the result of imine formation with the ϵ-amino group of lysine-97. The present study describes the equilibria and kinetics of inactivation of the dehydrogenase by pyridoxal, and the effects of substrate, cofactors, and allosteric modifiers on the rates of enzyme inactivation. Its purpose was to determine the mechanism of inactivation and to probe the

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physical character of the site of reaction by equilibria and kinetic methods. The accompanying report (Piszkiewicz and Smith, 1971) describes the equilibria and kinetics of imine formation by pyridoxal 5'-phosphate with lysine-97.

Experimental Section

Materials. A crystalline suspension of bovine liver glutamate dehydrogenase in 2 M $(NH_4)_2SO_4$ was purchased from Boehringer (Mannheim, Germany). Pyridoxal, DPN, DPNH, TPN, TPNH, ADP, and GTP were obtained from Calbiochem. All other reagents were of the highest purity available.

Enzyme Assay. All enzyme activity measurements were made with a Beckman Kintrac VII recording spectrophotometer with the cell compartment maintained at 30°. Glutamate dehydrogenase activity was measured spectrophotometrically by following the formation of reduced DPN at 340 nm. In preliminary studies of dialyzed enzyme 2×10^{-2} M ammonium ion had no effect on rates of inactivation of enzyme. Therefore, to simplify the routine, crystalline enzyme was removed from $(NH_4)_2SO_4$ suspension by centrifugation and dissolved in buffer to give the stock solutions at 1.8×10^{-5} M (1 mg/ml). Assays were performed by the addition of 25 μl of stock enzyme solution to 2.5 ml of stock substrate solution containing 10^{-4} M DPN and 5×10^{-3} M glutamate in 0.1 ionic strength phosphate buffer (pH 8.00). The buffer employed was formed by mixing appropriate volumes of 0.1 ionic strength and KH_2PO_4 and 0.1 ionic strength K_2HPO_4.

Equilibria and Kinetic Measurements. The inactivation of glutamate dehydrogenase (1 mg/ml) by pyridoxal was studied at 30° in 0.1 ionic strength phosphate buffers. Buffers at the desired pH values were formed as described above. Enzyme concentration was 1.8×10^{-5} M (1 mg/ml) based on a molecular weight of the subunit polypeptide chain of 56,000 (Smith *et al.*, 1970). The concentrations of pyridoxal employed ranged from 1.2×10^{-3} to 5×10^{-3} M. Aliquots of 25 μl were removed at appropriate intervals after addition of the inhibitor and assayed for residual activity. It should be noted that in those cases where inactivation was very rapid, the cooperation of two experimenters was required in order to obtain sufficient data for the determination of accurate rates of inactivation.

Equilibrium Calculations. If it is assumed that the reactive ϵ-amino group of lysine-97 of the enzyme (E) reacts with pyridoxal (P) to yield the corresponding Schiff base (EP) with no significant concentration of the intermediate carbinolamine present, the equilibrium may be expressed as

$$E + P \underset{k_{-0}}{\overset{K_{pH}}{\underset{k_0}{\rightleftharpoons}}} EP \qquad (1)$$

At any constant pH, then, $K_{pH} = [EP]/[E][P]$. If the inactivation of enzyme (E) is studied at a concentration of pyridoxal (P) in great excess over enzyme, the concentrations of E and EP may be calculated from a knowledge of total enzyme concentration (E_{total}), the enzyme activity at the inception of the inactivation reaction (Act_0), and the residual activity after completion of reaction (Act_∞). Thus, K_{pH} may be calculated as

$$K_{pH} = \frac{[E_{total}] - [E_{total}]Act_\infty/Act_0}{[E_{total}]Act_\infty/Act_0[P]} \qquad (2)$$

Kinetic Calculations. If the inactivation reaction is carried out with the concentration of pyridoxal in great excess over enzyme, pseudo-first-order rate constants (k_{obsd}) may be obtained by multiplying by 2.303 the slope of a plot of $\log[(Act_\infty - Act_0)/(Act_\infty - Act_t)]$ *vs.* time.

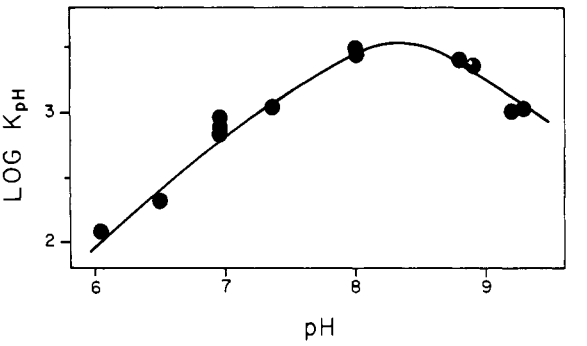

FIGURE 1: Variation of the logarithm of the equilibrium constant (K_{pH}) for the formation of pyridoxal-inactivated enzyme with pH. The points were determined experimentally, and the line was calculated from eq 6.

Since imine formation is a reversible process the observed rate may be expressed by

$$-d[E]/dt = k_0[E][P] - k_{-0}[EP] \qquad (3)$$

At high concentrations of pyridoxal (P) the forward rate $k_0[E][P]$ is much greater than that of the reverse reaction, $k_{-0}[EP]$. Thus, eq 3 reduces to $-d[E]/dt = k_0[E][P]$ and $k_{obsd} = k_0[P]$. Most of the experiments were performed with high concentrations of pyridoxal which resulted in more than 90% inactivation. Therefore, the observed pseudo-first-order rate constants (k_{obsd}) were reasonable approximations of the actual forward rate constants ($k_0[P]$). Observed second-order rate constants of inactivation (k_0) were calculated from $k_0 = k_{obsd}/[P]$.

Results

In a previous report (Piszkiewicz *et al.*, 1970) we have described experiments which demonstrated that pyridoxal 5'-phosphate inactivates glutamate dehydrogenase by forming an imine with the ϵ-amino group of lysine-97 of the subunit polypeptide chain. In view of the structural similarities between pyridoxal and pyridoxal 5'-phosphate and the similarities in the equilibria and kinetics of enzyme inactivation by these two substances (which are described in this and the following report), it is reasonable to conclude that both of these substances inactivate the enzyme by reacting with the same functional group of the enzyme. Thus, in the studies described below, the inactivation of glutamate dehydrogenase by pyridoxal has been interpreted as the result of imine formation with the ϵ-amino group of lysine-97.

Equilibrium Studies with Pyridoxal. The equilibrium constants for the formation of pyridoxal-inactivated enzyme at constant pH (K_{pH}) were calculated for varying pyridoxal concentrations over the pH range from 6.05 to 9.28 by means of eq 2. Values of K_{pH} were found not to vary significantly with the concentration of pyridoxal employed or the resulting degree of inactivation of enzyme; thus, the six reactive amino groups of the six-subunit chains of the catalytically active enzyme appeared to be equivalent in these experiments. The values of log K_{pH} determined from the degree of enzyme inactivation have been plotted as a function of pH in Figure 1.

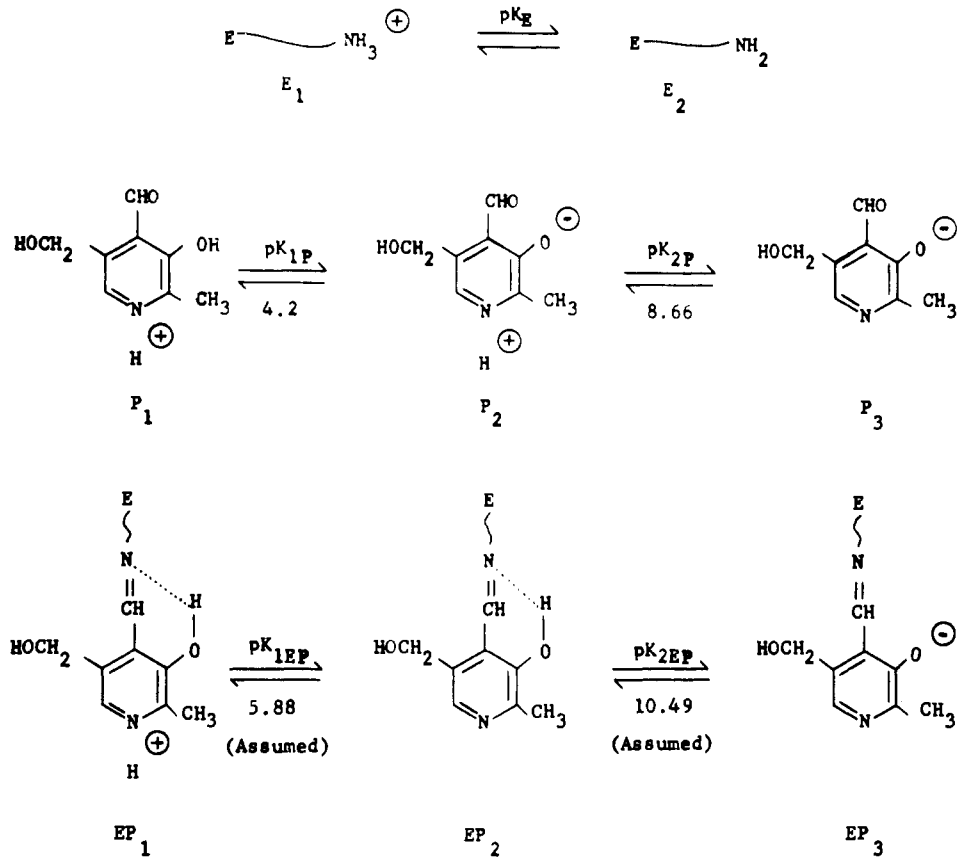

FIGURE 2: Acidic dissociations of amino group, pyridoxal, and imine. The source of the various pK values (under double arrows) is given in the text.

The variation of K_{pH} as a function of pH may be related to the dissociation constants of the various ionic forms of reactive amine (*i.e.*, the ϵ-amino group of lysine-97), pyridoxal, the product imine, and a single overall equilibrium constant for imine formation. This treatment was initially developed by Metzler (1957) to describe the pH dependence for imine formation by pyridoxal with various amino acids, and specific details of the derivation have been provided by Auld and Bruice (1967).

The various equilibria to be considered are given in Figure 2. The dissociation constants for pyridoxal and imine have been assumed to be identical with those of the corresponding species described by Metzler (1957). The dissociation constant of the reactive amine (the ϵ-amino group of lysine-97) is to be derived from the best fit of the theoretical treatment to the experimental data. Since K_{pH} has been defined as the overall equilibrium constant at any given pH

$$K_{pH} = \frac{([EP_1] + [EP_2] + [EP_3])}{([P_1] + [P_2] + [P_3])([E_1] + [E_2])} \quad (4)$$

If we define K as the apparent equilibrium constant for the reaction of the pyridoxal anion (P_3) with the free amino group (E_2) to yield the anion of the imine (EP_3), we may write the equilibrium likely to predominate at pH values above 11 as

$$K = \frac{[EP_3]}{[P_3][E_2]} \quad (5)$$

We may then incorporate eq 5 into eq 4, as described by Metzler (1957) and Auld and Bruice (1967), to obtain

$$\log K_{pH} = \log K + \log\left(\frac{a_H^2}{K_{1EP}K_{2EP}} + \frac{a_H}{K_{2EP}} + 1\right) - \log\left(\frac{a_H^2}{K_{1P}K_{2P}} + \frac{a_H}{K_{2P}} + 1\right) - \log\left(\frac{a_H}{K_E} + 1\right) \quad (6)$$

In eq 6, a_H is hydrogen ion activity as measured by the glass electrode.

The curve drawn through the data in Figure 1 was calculated from eq 6 by employing the dissociation constants given in Figure 2 and values of K and K_E which gave the best fit to the experimentally determined values of K_{pH}. Thus, it was determined that log K = 1.95 ± 0.1 with K = 89 M^{-1} and pK_E = 7.9 ± 0.2 with $K_E = 1.58 \times 10^{-8}$ M. The value of K is comparable in magnitude to that determined for numerous primary amines and amino acids in a similar equilibrium with pyridoxal (Metzler, 1957). The value of pK_E by definition represents the apparent dissociation constant of the protonated ϵ-amino group of lysine-97.

It should be noted that the assumed values for the dissociation constants of the imine product, pK_{1EP} and pK_{2EP} (Figure 2), fall outside the pH range over which data could be obtained. In the pH range studied, pK_{1EP} and pK_{2EP} would make a minimal contribution to the shape of the curve in Figure 1 as calculated from eq 6. Thus, the actual values of pK_{1EP} and pK_{2EP} could vary from the assumed values by 0.5

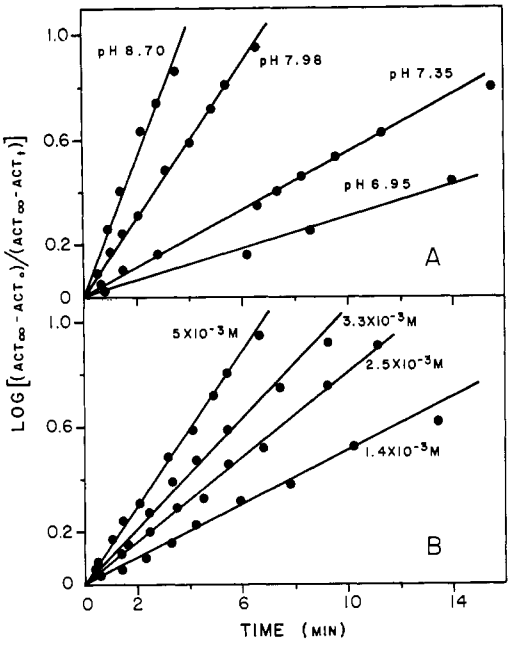

FIGURE 3: (A) Pseudo-first-order plots for the inactivation of enzyme (at 1.8×10^{-5} M) by pyridoxal (5×10^{-3} M) at the pH values indicated. (B) Pseudo-first-order plots for the inactivation of enzyme (at 1.8×10^{-5} M) at pH 7.98 ± 0.02 by pyridoxal at the concentrations indicated.

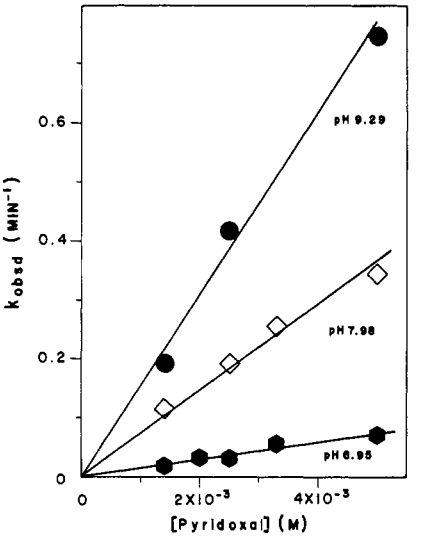

FIGURE 4: Plots of pseudo-first-order rate constants (k_{obsd}) for the inactivation of glutamate dehydrogenase (at 1.8×10^{-5} M) as a function of pyridoxal concentration.

pK unit or more and not cause a significant change in the theoretical fit of eq 6 to the data of Figure 1.

Kinetic Studies with Pyridoxal. Rates of inactivation of glutamate dehydrogenase at constant concentration (1.8×10^{-5} M) were determined under conditions of varying pH and pyridoxal concentration. Representative plots of $\log[(Act_\infty - Act_0)/(Act_\infty - Act_t)]$ *vs.* time were determined under conditions of constant pyridoxal concentration (5×10^{-3} M) at several pH values; these data are presented in Figure 3A. Similarly, plots derived from data obtained at constant pH at several concentrations of pyridoxal are presented in Figure 3B. Such plots were generally found to be linear for over two half-times of the reaction. Thus, the ε-amino groups of lysine-97 of the six-subunit polypeptide chains of the active enzyme reacted at identical rates. Pseudo-first-order rate constants (k_{obsd}) of enzyme inactivation at any pH (as in Figure 3A) and any pyridoxal concentration (as in Figure 3B) were obtained by multiplying the slopes of these plots by 2.303.

Plots of k_{obsd} *vs.* pyridoxal concentration (Figure 4) were found to be linear. Thus, there is no apparent saturation effect and hence no evidence for a rate-limiting step involving noncovalent complex formation prior to the inactivation reaction. The inactivation reaction is apparently a second-order process, as expected.

The second-order rate constants (k_0) of enzyme inactivation by pyridoxal determined at varying pH values have been plotted in Figure 5. The observed curve may be interpreted as a dependence of the rate constant (k_0) on the mole fraction of the conjugate base form of a functional group on the enzyme (E). Thus, for the reaction

$$\begin{array}{ccc} E + P & \xrightarrow{k_2} & EP \\ \Big\downarrow\uparrow K_a & & \\ E{\cdot}H^+ & & \end{array} \qquad (7)$$

it can be shown that

$$k_0 = k_2\left[\frac{K_{app}}{K_{app} + a_H}\right] \qquad (8)$$

A theoretical curve calculated from eq 8 has been fitted to the experimental values (Figure 4) by employing the values of $k_2 = 1.7 \times 10^2 \pm 0.2$ M^{-1} min^{-1} and $pK_a = pK_{app} = 8.1 \pm 0.2$ with $K_{app} = 7.94 \times 10^{-9}$ M. The most reasonable interpretation of pK_{app} is that it describes the ionization of the ε-amino group of lysine-97.

In view of the various ionizations of the reacting species (Figure 2) the interpretation of the kinetic data of Figure 5 by eq 8 may be an oversimplification. One might consider

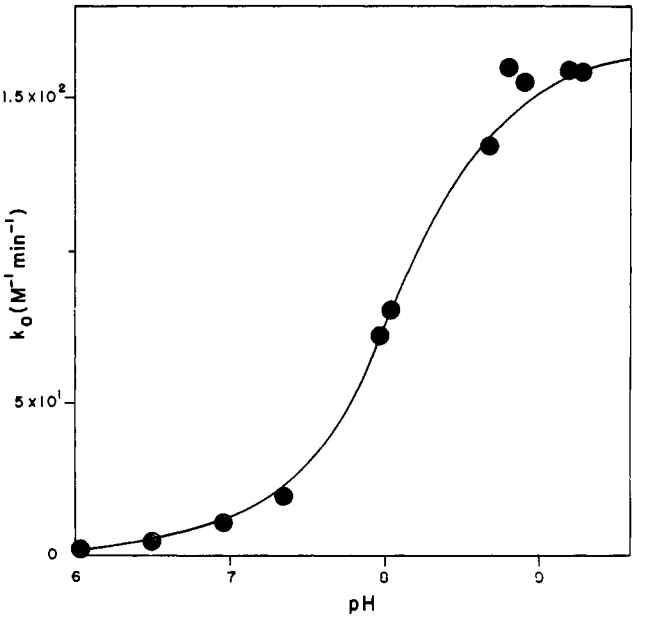

FIGURE 5: Second-order rates of inactivation of enzyme by pyridoxal (k_0) plotted as a function of pH. The points were determined experimentally, and the curve was calculated from eq 8.

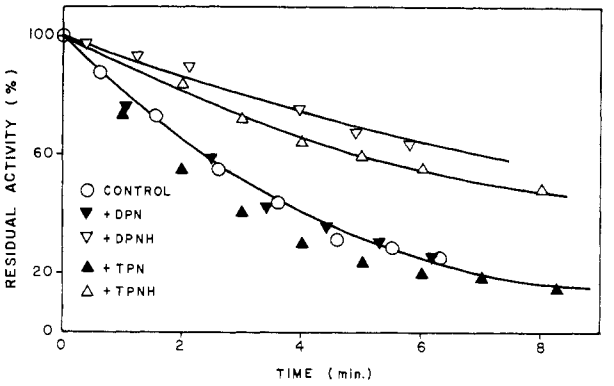

FIGURE 6: Time course of the reactions of pyridoxal (5×10^{-3} M) with glutamate dehydrogenase (at 1.8×10^{-5} M) at pH 7.98 ± 0.02 in the presence of DPN, DPNH, TPN, or TPNH (each at a concentration of 5×10^{-3} M).

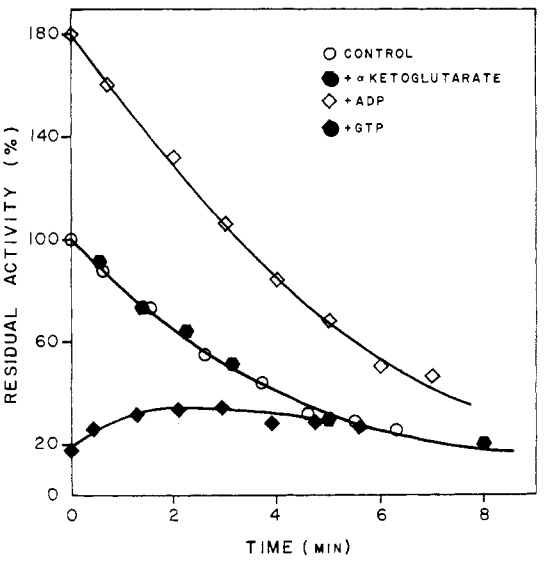

FIGURE 7: Time course of the reactions of pyridoxal (5×10^{-3} M) with glutamate dehydrogenase (at 1.8×10^{-5} M) at pH 7.98 ± 0.02 in the presence of α-ketoglutarate, ADP, or GTP (each at 5×10^{-3} M).

the three ionic forms of pyridoxal (Figure 2) and anticipate each to react with lysine-97 at a different rate due to a differing electronic effects of the ionizing groups. To illustrate, the reactions of valine, glycine, glutamate (French *et al.*, 1965), and alanine (Auld and Bruice, 1967) with 3-hydroxypyridine-4-aldehyde, a structural analog of pyridoxal, exhibit an approximately sigmoid pH dependence similar to that of Figure 5, with inflection points at the approximate pK_a's of the reacting amino groups. Slight deviations from an exactly sigmoid pH dependence were observed. These were accommodated by writing the overall rate of imine formation as the sum of the rates of formation from the three ionic forms of the aldehyde with a different value of k_2 for each ionic form. For the reaction of glutamate dehydrogenase with pyridoxal, an identical treatment might also be valid; however, it would not give a better fit of the theoretical curve to the experimental points (Figure 5) than was obtained from eq 8.

Effects of Substrate, Coenzymes, and Allosteric Modifiers on Rates of Inactivation. The time course of the inactivation of enzyme by pyridoxal in the presence of DPN, DPNH, TPN, or TPNH is presented in Figure 6. Although activities at zero time varied somewhat as a result of coenzyme being carried over from the incubation mixture to the assay mixture, all have been corrected to 100% of control (Figure 6) to simplify the presentation. The rates of enzyme inactivation in the presence of DPN and TPN did not differ significantly from that of the control experiment ($k_{obsd} = k_0[P] = 0.34$ min^{-1}) performed in the absence of added ligand. Thus, these substances offered no protection from the inactivation by pyridoxal. The rates of enzyme inactivation in the presence of DPNH ($k_{obsd} = 0.084$ min^{-1}) and TPNH ($k_{obsd} = 0.12$ min^{-1}) were significantly lower than the control, and, therefore, protection of the reactive amino group of the enzyme is afforded by these substances. A detailed investigation of this protection is presented below.

The time course of the inactivation of enzyme by pyridoxal in the presence of α-ketoglutarate, ADP, and GTP is given in Figure 7. As in the experiments performed in the presence of coenzymes (Figure 6) the experiment carried out in the presence of α-ketoglutarate has been corrected to 100% of control. The experiments performed in the presence of the activator ADP and the inhibitor GTP had zero-time activities significantly higher and lower than the control, respectively, because of the carry-over of 10^{-5} M ADP or GTP from the reaction mixture to the assay solution. These differences in activities at zero time were the anticipated result of the carry-over of ligand from reaction mixture to assay solution, and they have been plotted relative to the control (Figure 7). The rates of inactivation of enzyme by pyridoxal in the presence of α-ketoglutarate and ADP were found not to differ significantly from the control. Therefore, these substances offered no protection to the enzyme from inactivation by pyridoxal.

When glutamate dehydrogenase was reacted with pyridoxal in the presence of GTP an initial apparent stimulation of activity was followed by a gradual loss of activity similar to that of the control (Figure 7). Since activity at zero time was depressed below the control due to carry-over of 10^{-5} M GTP, the apparent stimulation may more reasonably be interpreted as a loss of inhibition by GTP. Two possible causes of this phenomenon may be considered. First, it is possible that pyridoxal has reacted with the amino group of the guanosine moiety of GTP, thereby destroying the allosteric inhibitor. This possibility may be discounted since other nucleoside derivatives with amino groups, DPNH, TPNH, and ADP, exert their influence throughout the entire course of the inactivation reaction (Figures 6 and 7), and clearly are not removed from the reaction mixture by reaction with pyridoxal. A second, more reasonable interpretation is that as inactivation of enzyme by pyridoxal proceeds, a reversed allosteric effect is observed in which covalent binding of pyridoxal at the catalytic site modifies a function on the site of the enzyme where GTP binds to produce allosteric inhibition.

Effect of DPNH and TPNH on Rate of Enzyme Inactivation. The rate constants for inactivation of glutamate dehydrogenase at a constant pH of 7.98 and a pyridoxal concentration of 5×10^{-3} M were determined in the presence of varying concentrations of DPNH and TPNH. The decrease in the observed pseudo-first-order rate constants (k_{obsd}) for the inactivation of enzyme with increasing concentration of protector, either DPNH or TPNH, is given in Figure 8. Although significant protection was afforded by these substances, complete protection from inactivation was not obtained even at very high concentrations of DPNH or TPNH. This phenomenon may be explained if it is assumed that the enzyme (E) when

complexed with DPNH (or TPNH) may also react with pyridoxal (P), but at a lower rate than the free enzyme. Thus, if the overall reaction is expressed as

$$\begin{array}{ccc} E + P & \xrightarrow{k_0} & EP \\ K_d \uparrow\downarrow & & \\ +DPNH & & \\ E\cdot DPNH + P & \xrightarrow{k'_0} & EP\cdot DPNH \end{array} \qquad (9)$$

the rate of inactivation is given by

$$-d[E_{total}]/dt = k_0[E][P] + k'_0[E\cdot DPNH][P] \qquad (10)$$

One may then calculate that

$$k_{obsd} = k_0\left[\frac{K_d}{K_d + [DPNH]}\right] + k'_0\left[\frac{[DPNH]}{K_d + [DPNH]}\right] \qquad (11)$$

The theoretical curves in Figure 8 have been generated from eq 11 by using values of $k_0 = 0.34$ min^{-1}, $k'_0 = 0.11$ min^{-1}, $K_d = 5 \times 10^{-5}$ M for DPNH, and $K_d = 2 \times 10^{-5}$ M for TPNH. Thus, glutamate dehydrogenase when complexed with reduced coenzyme is inactivated at approximately a third the rate observed for the reduced coenzyme.

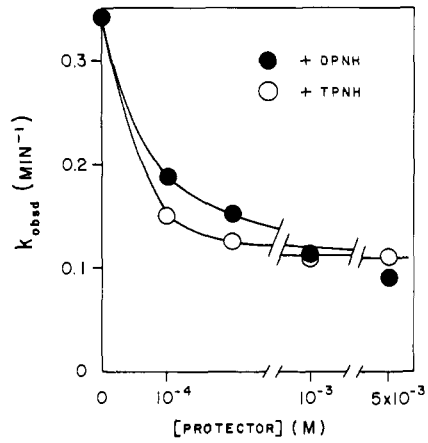

FIGURE 8: Variation of pseudo-first-order rate constants (k_{obsd}) for inactivation of enzyme (at 1.8×10^{-5} M) by pyridoxal (at 5×10^{-3} M) with increasing concentrations of DPNH and TPNH at pH 7.98 ± 0.03. The points were determined experimentally, and the curves were generated from eq 11.

Discussion

From the equilibrium study of the inactivation of glutamate dehydrogenase by pyridoxal it has been possible to determine the pK_{app} of the reacting amino group as 7.9 ± 0.2. This value of pK_E represents the apparent dissociation constant of the protonated ε-amino group of lysine-97.

The kinetic study of the inactivation of dehydrogenase by pyridoxal has shown that the reaction is apparently a second-order process with the rate of reaction being proportional to the mole fraction of a basic group with pK_{app} of 8.1 ± 0.2 (eq 7 and 8). The most reasonable interpretation of pK_{app} is that it also describes the ionization of the ε-amino group of lysine-97, and the rate-limiting step is nucleophilic addition of this group to the aldehyde. Thus, for the inactivation of glutamate dehydrogenase by pyridoxal the kinetically determined pK_{app} = 8.1 ± 0.2 is in excellent accord with the value of pK_E = 7.9 ± 0.2 for the identical ionization, as determined from the equilibrium study.

Since the reaction with pyridoxal is kinetically second order, it is reasonable to conclude that the pK_a of the reacting amino group on the free enzyme is in this range (*i.e.*, pK_E = pK_a = 8.0 ± 0.3). This pK_a value is 1.4–2.6 units lower than might be expected for a similar group in a protein (Edsall, 1943). The abnormally low pK_a of the amino group is analogous to the abnormally high pK_a of a carboxyl group found at the catalytic site of lysozyme. The γ-carboxyl of glutamic acid-35 of lysozyme has been assigned pK_a values ranging from 5.8 (Rand-Meir *et al.*, 1969) to 6.3 (Rupley, 1967), almost two units higher than would be anticipated for this group on the surface of a protein (Edsall, 1943). The abnormally high pK_a of glutamic acid-35 lysozyme has been ascribed to its location in a hydrophobic region of the molecule (Blake *et al.*, 1967). It is possible that an analogous location of lysine-97 of glutamate dehydrogenase in a hydrophobic environment is partially responsible for its abnormally low pK_a. A second factor which may be involved in the low pK_a value of the ε-amino group of lysine-97 of glutamate dehydrogenase is the possible presence of additional cationic groups in the vicinity of lysine-97 which would retard the formation of an additional positive charge. It is a reasonable assumption that such groups are present in this region to explain both the substrate specificity for the dicarboxylic acids, glutamate and α-ketoglutarate, and the reaction properties of lysine-97 with pyridoxal 5′-phosphate (Piszkiewicz and Smith, 1971). Thus, the presence of other amino groups, guanidinium groups, or imidazole groups near the ε-amino group of lysine-97 in the native enzyme would tend to lower the pK_a of the latter.

The abnormally low pK_a of lysine-97 would result in a greater mole fraction in the reactive unprotonated form at neutral pH values, and consequently a greater rate of reaction than any of the other amino groups of the protein. Precedents for increased reactivity resulting from abnormally low pK_a values have been ascribed to the specific reactions of 1-fluoro-2,4-dinitrobenzene with the ε-amino group of lysine-41 in ribonuclease A (Murdock *et al.*, 1966) and to the amino-terminal valyl residue of the α chain of human hemoglobin (Hill and Davis, 1967).

In conclusion, four reagents have been found to inactivate glutamate dehydrogenase by reacting with the ε-amino group of lysine-97 of the subunit chain. These compounds are pyridoxal 5′-phosphate (Piszkiewicz *et al.*, 1970), *N*-(*N*′-acetyl-4-sulfamoylphenyl)maleimide (Holbrook and Jeckel, 1969), 4-iodoacetamidosalicylic acid (Holbrook *et al.*, 1970), and cyanate (E. L. Smith and F. Veronese, 1971, unpublished data). It is likely that most of these specific labeling reactions are due to the inherent reactivity of lysine-97 because of its low pK_a.

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Bovine Liver Glutamate Dehydrogenase. Equilibria and Kinetics of Imine Formation by Lysine-97 with Pyridoxal 5′-Phosphate*

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ABSTRACT: Pyridoxal 5′-phosphate inactivates glutamate dehydrogenase by forming an imine with the ϵ-amino group of lysine-97. The equilibrium constants for imine formation at varying pH values (K_{pH}) have been calculated from the initial concentrations of enzyme and pyridoxal 5′-phosphate and the final degree of inactivation. The variation of K_{pH} with pH has been related to the dissociation constants of reactive ϵ-amino group, pyridoxal 5′-phosphate, and the product imine, and a single equilibrium constant for imine formation. Application of this treatment with a reasonable, assumed pK_a value for the protonated phenolic oxygen of the imine product, the pK_a's of the protonated pyridinium nitrogen of the imine and the second dissociation of the phosphate of the imine were calculated to be 5.2 ± 0.2 and 8.0 ± 0.2, respectively. Rate studies showed that inactivation of enzyme by pyridoxal 5′-phosphate proceeded through formation of an apparent noncovalent complex prior to imine formation. The K_d for this complex was evaluated as 0.0025 M, and was independent of pH. The first-order rate constants of imine formation gave a bell-shaped curve with pK_{app1} = 5.5 ± 0.2 and pK_{app2} = 8.0 ± 0.2. Four possible mechanisms which can describe this bell-shaped pH dependence are considered; one of these appears to represent the most probable interpretation. DPNH and TPNH competitively inhibited the enzyme inactivation by pyridoxal 5′-phosphate.

Recent reports from this laboratory have described the determination of the nearly complete amino acid sequence of bovine liver glutamate dehydrogenase [L-glutamate:DPN (TPN) oxidoreductase (deaminating), EC 1.4.1.3) (Smith *et al.*, 1970), and the identification of lysine-97 as the site of reaction during inactivation by pyrodoxal 5′-phosphate (Piszkiewicz *et al.*, 1970; see also Anderson *et al.*, 1966). The preceding paper (Piszkiewicz and Smith, 1971) has described a study of the equilibria and kinetics of the inactivation of the enzyme by pyridoxal, presumably by imine formation with the ϵ-amino group with lysine-97.

The present study describes the equilibria and kinetics of inactivation by pyridoxal 5′-phosphate, and the effects of substrate, cofactors, and allosteric modifiers on the rates of enzyme inactivation. Our purpose was to determine the mechanism of inactivation, to probe the physical character of the site of reaction, and to explore the possible physiological significance of the reversible inactivation of glutamate dehydrogenase by pyridoxal 5′-phosphate.

Experimental Section

Materials. Bovine liver glutamate dehydrogenase was purchased from Boehringer (Mannheim, Germany). Pyridoxal 5′-phosphate, DPN, DPNH, TPN, TPNH, ADP, and GTP were obtained from Calbiochem.

Equilibria and Kinetic Measurements. All measurements of enzyme activity and all reactions of pyridoxal 5′-phosphate with enzyme were performed at 30° in 0.1 ionic strength phosphate buffers as described in the preceding report (Piszkiewicz and Smith, 1971).

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