Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. Roy. Soc., Ser. B 167*, 378.
Edsall, J. T. (1943), *in* Proteins, Amino Acids, and Peptides, Cohn, E. J., and Edsall, J. T., Ed., New York, N. Y., Hafner Publishing Co., p 445.
Eisenberg, H., and Tomkins, G. M. (1968), *J. Mol. Biol. 31*, 37.
French, T. C., Auld, D. S., and Bruice, T. C. (1965), *Biochemistry 4*, 77.
Frieden, C. (1963a), *Enzymes 7*, 3.
Frieden, C. (1963b), *J. Biol. Chem. 238*, 3286.
Hill, R. J., and Davis, R. W. (1967), *J. Biol. Chem. 242*, 2005.
Holbrook, J. J., and Jeckel, R. (1969), *Biochem. J. 111*, 689.
Holbrook, J. J., Roberts, P. A., Robson, B., and Stinson, R. A. (1970), *Proc. Int. Congr. Biochem., 8th*, 83.
Meister, A. (1965), *in* Biochemistry of the Amino Acids, Vol. 1, 2nd ed, New York, N. Y., Academic Press, p 310.
Metzler, D. E. (1957), *J. Amer. Chem. Soc. 79*, 485.
Murdock, A. L., Grist, K. L., and Hirs, C. H. W. (1966), *Arch. Biochem. Biophys. 114*, 375.
Piszkiewicz, D., Landon, M., and Smith, E. L. (1970), *J. Biol. Chem. 245*, 2622.
Piszkiewicz, D., Landon, M., and Smith, E. L. (1971), *J. Biol. Chem. 246*, 1324.
Piszkiewicz, D., and Smith, E. L. (1971), *Biochemistry 10*, 4544.
Rand-Meir, T., Dahlquist, F. W., and Raftery, M. A. (1969), *Biochemistry 8*, 4206.
Rupley, J. A. (1967), *Proc. Roy. Soc., Ser. B 167*, 416.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.

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

Dennis Piszkiewicz and Emil L. Smith†

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).

* From the Department of Biological Chemistry, UCLA School of Medicine, and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received June 5, 1971.* This investigation was supported by Grant GM 11061 from the National Institute of General Medical Sciences, U. S. Public Health Service.

† To whom inquiries and reprint requests should be sent.

Equilibria Calculations. Equilibrium constants for the formation of enzyme inactivated by pyridoxal 5′-phosphate at constant pH (K_{pH}) were calculated as described in the preceding report (Piszkiewicz and Smith, 1971).

Kinetic Calculations. If the inactivation reaction is carried out with the concentration of pyridoxal 5′-phosphate 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.

In kinetic studies involving enzymes, it is advisable to consider the possibility of noncovalent complex formation prior to reaction. Thus, we should consider the possibility of the reaction of the enzyme (E) with pyridoxal 5′-phosphate (P) as

$$E + P \underset{K_I}{\rightleftharpoons} E\cdot P \xrightarrow{k_0} EP \quad (1)$$

By applying a Michaelis–Menten- (1913) type treatment, we may derive

$$k_{obsd} = \frac{k_0[P]}{K_I + [P]} \quad (2)$$

and in a manner analogous to that of Lineweaver and Burk (1934) we may obtain the following linear transformation

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_0}\left(\frac{1}{[P]}\right) + \frac{1}{k_0} \quad (3)$$

Thus, a plot of $1/k_{obsd}$ *vs.* $1/[P]$ will demonstrate if complex formation occurs prior to reaction, and, if this is the case, provide a means of evaluating the constants K_I and k_0.

Results

Equilibrium Studies with Pyridoxal 5′-Phosphate. The equilibrium constants for the formation of pyridoxal 5′-phosphate inactivated enzyme (K_{pH}) were calculated at various pH values from 5.05 to 8.35. Values of K_{pH} calculated at constant pH were found not to vary significantly with the concentration of pyridoxal 5′-phosphate used or the resulting degree of inactivation of enzyme; the six reactive amino groups (of the six identical subunits) of the active enzyme appeared to react equivalently. The K_{pH} values calculated from the degree of enzyme inactivation have been plotted as a function of pH in Figure 1.

The dependence of K_{pH} on pH may be related to the dissociation constants of the various ionic forms of reactive amino group, pyridoxal 5′-phosphate, and the imine product and a single overall equilibrium constant for imine formation. The equilibria to be considered are shown in Figure 2.

Defining K_{pH} as the determined equilibrium constant at any pH

$$K_{pH} = \frac{([EP_1] + [EP_2] + [EP_3] + [EP_4])}{([P_1] + [P_2] + [P_3] + [P_4])([E_1] + [E_2])} \quad (4)$$

and defining

$$K = \frac{[EP_4]}{[P_4][E_2]} \quad (5)$$

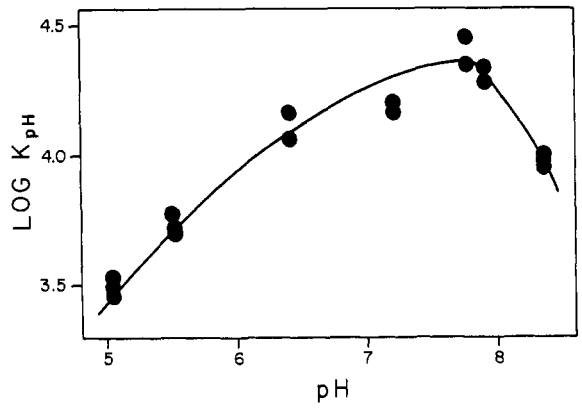


FIGURE 1: Variation of the logarithm of the equilibrium constant (K_{pH}) for the formation of pyridoxal 5′-phosphate inactivated enzyme with pH. The points were determined experimentally, and the line was calculated from eq 6.

it can be shown (Metzler, 1957; Auld and Bruice, 1967a) that

$$\log K_{pH} = \log K + \log\left(\frac{a_H{}^3}{K_{1EP}K_{2EP}K_{3EP}} + \frac{a_H{}^2}{K_{2EP}K_{3EP}} + \frac{a_H}{K_{3EP}} + 1\right) - \log\left(\frac{a_H{}^3}{K_{1P}K_{2P}K_{3P}} + \frac{a_H{}^2}{K_{2P}K_{3P}} + \frac{a_H}{K_{3P}} + 1\right) - \log\left(\frac{a_H}{K_E} + 1\right) \quad (6)$$

The pK_a of the reactive amino group (the ϵ-amino group of lysine-97) (Figure 2) has been determined by equilibrium and kinetic studies with pyridoxal to be 8.0 ± 0.3 (Piszkiewicz and Smith, 1971). The dissociation constants of pyridoxal 5′-phosphate have been estimated by Williams and Nielands (1954). The pK_a of the protonated phenolic oxygen of the imine (K_{3EP}) was assumed to be identical with that of the imines formed by pyridoxal with valine and glycine (Metzler, 1957). The dissociation constants of protonated pyridinium nitrogen of the imine (K_{1EP}) and the second dissociation of the phosphate of the imine (K_{2EP}) were determined from the best fit of eq 6 to the experimental values of K_{pH} (Figure 1). Since K_{1EP} and K_{2EP} have their greatest influence on K_{pH} at the lower and higher pH values employed respectively, they could be varied independently to give the best fit.

The curve drawn through the data in Figure 1 was calculated from eq 6 by employing the pK values shown in Figure 2 with values of K, K_{1EP}, and K_{2EP} which gave the best fit to the experimentally determined values of K_{pH}. By this method, it was determined that $\log K = 2.50 \pm 0.1$ with $K = 3.16 \times 10^2\ M^{-1}$, $pK_{1EP} = 5.2 \pm 0.2$ with $K_{1EP} = 6.31 \times 10^{-6}$ M, and $pK_{2EP} = 8.0 \pm 0.2$ with $K_{2EP} = 10^{-8}$ M.

Kinetic Studies with Pyridoxal 5′-Phosphate. Rates of inactivation of glutamate dehydrogenase at constant concentration (1.8×10^{-5} M) were determined at several pH values and at varying concentrations of pyridoxal 5′-phosphate. Representative plots of $\log[(Act_\infty - Act_0)/(Act_\infty - Act_t)]$ *vs.* time are shown in Figure 3. Such plots were generally found to be linear for over two half-lives of the reaction; hence, pseudo-first-order rate constants (k_{obsd}) of enzyme inactivation at any constant pH and pyridoxal 5′-phosphate concentration were obtained by multiplying by 2.303 the slope of these plots.

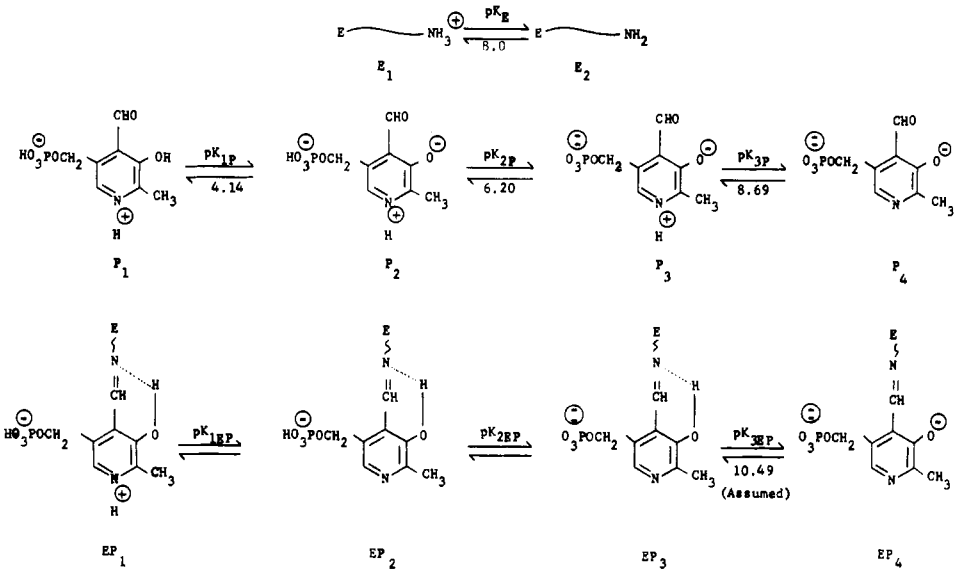


FIGURE 2: Acidic dissociation constants of amino group, pyridoxal 5′-phosphate, and imine. The sources of the various pK values (under double arrows) is given in the text.

Lineweaver–Burk- (1934) type plots of $1/k_{obsd}$ *vs.* 1/[pyridoxal 5′-phosphate] were determined at seven pH values (Figure 4) and can be fitted best at all pH values by a line intersecting the abscissa at 1/[pyridoxal 5′-phosphate] = -400 M^{-1}. Therefore, noncovalent complex formation prior to imine formation was indicated, and the dissociation constant for this complex, which showed no significant variation with pH, was found to be $K_I = 2.5 \pm 1.0 \times 10^{-3}$ M.

One may question the interpretation that the kinetically determined constant K_I actually represents Michaelis–Menten-type complex formation between pyridoxal 5′-phosphate and dehydrogenase rather than carbinolamine formation. This alternate interpretation of K_I is unlikely since it was observed only in the reaction with pyridoxal 5′-phosphate. Pyridoxal,

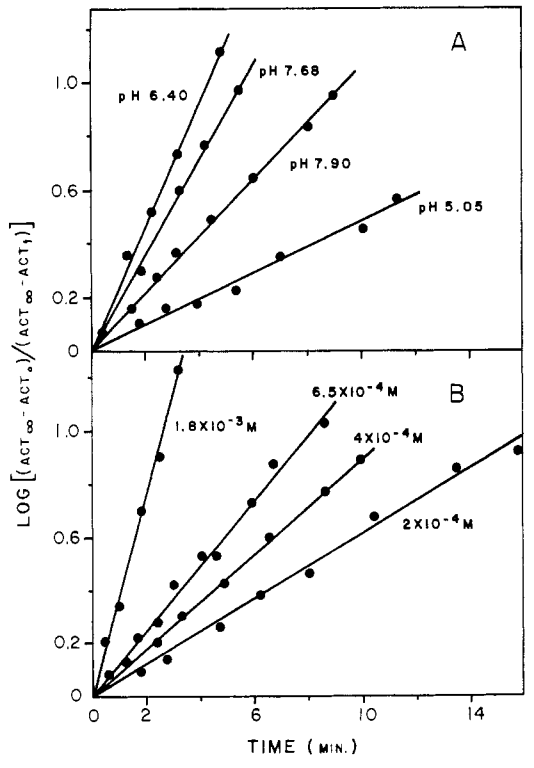


FIGURE 3: (A) Pseudo-first-order plots for the inactiavtion of enzyme (at 1.8×10^{-5} M) by pyridoxal 5′-phosphate (9×10^{-4} M) at the pH values indicated. (B) Pseudo-first-order plots for the inactivation of enzyme (at 1.8×10^{-5} M) at pH 7.20 ± 0.02 by pyridoxal 5′-phosphate at the concentrations indicated.

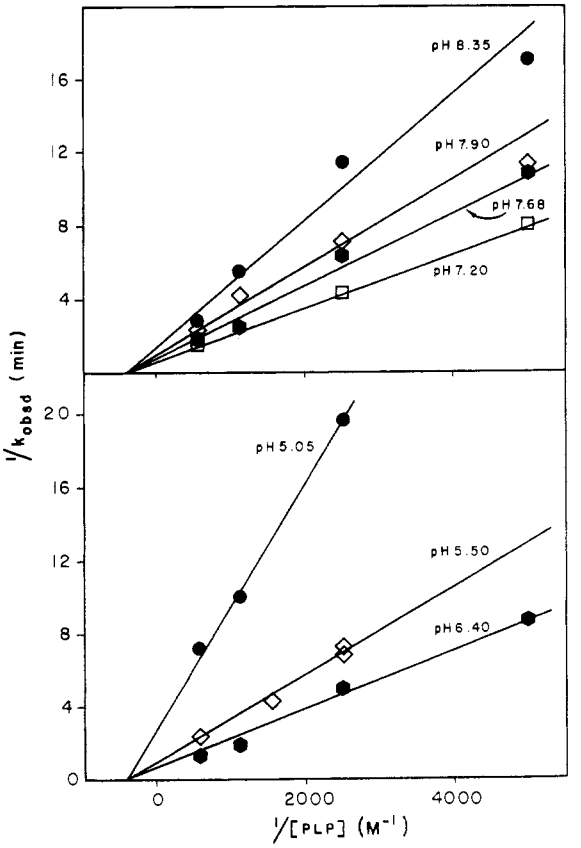
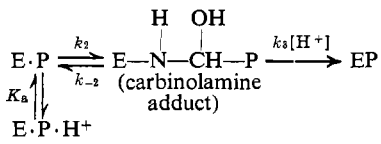


FIGURE 4: Lineweaver–Burk-type plots of $1/k_{obsd}$ *vs.* 1/[pyridoxal 5′-phosphate] (abbreviated as 1/[PLP]) for the inactivation of glutamate dehydrogenase (at 1.8×10^{-5} M).

which differs only in the absence of the 5'-phosphate, would be expected to exhibit complex formation with a very similar value of K_I if carbinolamine formation was involved. Model studies (Auld and Bruice, 1967a; French *et al.*, 1965) have shown that the rate of imine formation and the position of the overall equilibrium vary greatly with pH at values near the pK_a of the reacting amine. Therefore, it is reasonable to conclude that a rapid equilibrium formation of carbinolamine would also vary with pH; its position at any pH would be expected to be a function of the mole fraction of reacting amino group. Since no significant variation of K_I with pH was observed, it is unlikely that K_I describes carbinolamine formation.

The maximum rate constant of imine formation (k_0) at any pH may be calculated as the reciprocal of the intercept of the ordinate (Figure 4). When these values of k_0 are plotted as a function of pH (Figure 5), a bell-shaped pH dependence is observed. Four kinetically equivalent mechanisms may be considered which can accommodate the kinetic data.

MECHANISM ONE. The bell-shaped pH dependence (Figure 5) of enzyme inactivation may be the result of the fact that imine formation is a two-step process: nucleophilic addition of the free amino group to the aldehyde is followed by hydrogen ion catalyzed dehydration of the carbinolamine adduct. The reaction for the inactivation of the dehydrogenase after the initial noncovalent complex formation may be expressed as



(7)

The mechanism given by eq 7 which results in the bell-shaped pH dependence of k_0 (Figure 5) is analogous to that proposed by Jencks (1959) to describe a similar pH dependence of the rate of imine formation by hydroxylamine with acetone or furfural. When viewed qualitatively, the reaction to form carbinolamine adduct *via* k_2 would be rate determining at low pH; the acid-catalyzed dehydration of carbinolamine adduct *via* $k_3[H^+]$ would be rate limiting at high pH. For this interpretation to be valid when applied to the inactivation of glutamate dehydrogenase, the assumption must be made that k_{-2} is very large, and that when the inactivation mixture is diluted in the assay of activity, all carbinolamine rapidly reverts to free enzyme.

By assuming that all enzyme is in the form of the noncovalent intermediate PLP·GDH, and by involving the steady-state hypothesis for the carbinolamine adduct, it can be demonstrated that

$$k_0 = \frac{k_2 K_{app} a_H}{K_{app} k_{-2}/k_3 + K_{app} a_H + a_H^2} \quad (8)$$

where a_H is the hydrogen ion activity as determined with the glass electrode. The best fit of eq 8 to the experimental points of Figure 5 was obtained by using the values, $k_2 = 1.8 \pm 0.2$ min^{-1}, $k_{-2}/k_3 = 10^{-8}$ M, and $pK_{app} = 5.5 \pm 0.2$ with $K_{app} = 3.32 \times 10^{-6}$. In the mechanism described by eq 7 and 8, pK_{app} is most easily interpreted as that of the ϵ-amino group of lysine-97 within the noncovalent enzyme–inactivator complex.

Several criticisms of mechanism one may be made.

First, the 3-hydroxyl group has been found to catalyze carbinolamine dehydration in model studies of structural analogs of pyridoxal 5'-phosphate (Auld and Bruice, 1967b): the rate of hydronium ion catalyzed dehydration is of a significantly lower magnitude, and consequently it is not observed. Thus, rate-limiting hydronium ion catalyzed dehydration of carbinolamine at high pH would not be anticipated on the basis of model studies.

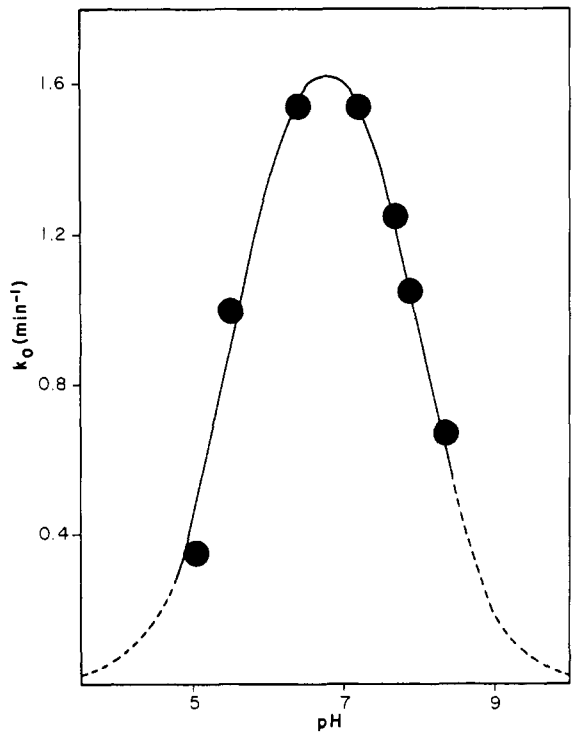


FIGURE 5: First-order rate constants for the inactivation of enzyme by pyridoxal 5'-phosphate (k_0) plotted as a function of pH. The points are experimental, and the curve was calculated from either eq 8, 10, or 14.

Second, one may question the abnormally low pK_a of 5.5 which is ascribed to the ϵ-amino group of lysine-97. This apparent pK_a is approximately 3.9–5.1 units lower than would be expected for an ϵ-amino group in a protein (Edsall, 1943) and approximately 2.5 units lower than the value for this group in free enzyme as determined in the study with pyridoxal (Piszkiewicz and Smith, 1971). The lowest pK_a ascribed to an ϵ-amino group to date is 5.9 for the active-site lysine of acetoacetate decarboxylase (Schmidt and Westheimer, 1971). Thus, the abnormally low pK_a of 5.5 ± 0.2 for the ϵ-amino group in mechanism one should be viewed with caution.

A third criticism of the mechanism described by eq 7 and 8 may be based on the magnitude of k_2. Noncovalent complex formation has been shown to precede covalent-bond formation, and such complex formation would be anticipated to accelerate significantly the second-order rate of reaction (Bruice, 1970). Thus, if eq 7 and 8 accurately describe the reaction mechanism, the second-order rate constant for the reaction of pyridoxal 5'-phosphate with enzyme ($k_2/K_m = 7.2 \times 10^2$ M^{-1} min^{-1}) should be significantly greater than the second-order rate constant of inactivation by pyridoxal ($k_{2(PAL)} = 1.7 \times 10^2$ M^{-1} min^{-1}) (Piszkiewicz and Smith, 1971). Since k_2/K_m for the inactivation by pyridoxal 5'-phosphate is not very much larger than $k_{2(PAL)}$ for the inactivation by pyridoxal, one may question that eq 7 and 8 accurately describe the mechanism of inactivation. Furthermore, it has been estimated (Metzler and Snell, 1955) that approximately one-

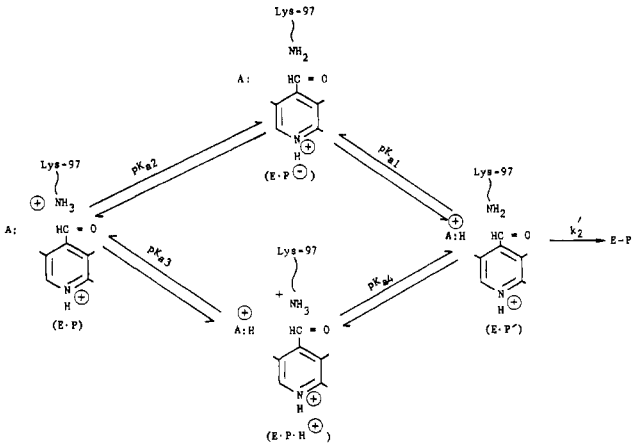
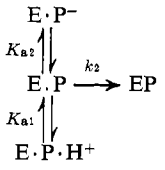


FIGURE 6: Various ionic forms of reactive amino group and pyridoxal 5′-phosphate within the noncovalent complex with the dissociation equilibria through which they are related.

fourth of the pyridoxal exists in the unreactive internal hemiacetal form at neutral pH. If the value of $k_{2(PAL)}$ were corrected for the fraction in the unreactive form, it would be approximately the same as k_2/K_m, the second-order rate constant for the reaction of pyridoxal 5′-phosphate with enzyme. Thus, on the basis of the rate constants of inactivation, it is unlikely that mechanism one accurately describes the mechanism of imine formation by pyridoxal 5′-phosphate with enzyme.

MECHANISM TWO. The reactive complex is the monoacid form of a diacid reactive species. The inactivation reaction after the initial noncovalent complex formation may be expressed as



(9)

where P is pyridoxal 5′-phosphate, E is enzyme, E·P is the reactive form of the noncovalent complex, K_{a1} and K_{a2} are the two ionization constants, and k_2 the first-order rate constant for imine formation. If it is assumed that all enzyme is in the form of the noncovalent intermediate, E·P (*i.e.*, saturating concentrations of pyridoxal 5′-phosphate are used), it may be shown that

$$k_0 = \frac{k_2 K_{app1} a_H}{K_{app1} K_{app2} + K_{app1} a_H + a_H^2} \quad (10)$$

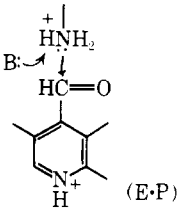
The best fit of eq 10 to the points on Figure 4 is obtained when the constants employed are, $k_2 = 1.8 \pm 0.2$ min^{-1}, $pK_{app1} = 5.5 \pm 0.2$ with $K_{app1} = 3.32 \times 10^{-6}$ M, and $pK_{app2} = 8.0 \pm 0.2$ with $K_{app2} = 10^{-8}$ M.

In this mechanism, K_{app1} would describe the ionization of the ε-amino group, while K_{app2} would describe the ionization of an acidic group within the noncovalent enzyme–inactivator complex. Thus, at low pH the rate-limiting step would be nucleophilic attack of the amino group on the aldehyde, and at high pH the rate-determining step would be dehydration of the carbinolamine which is catalyzed by the acidic group of $pK_{app2} = 8.0 \pm 0.2$. It should be noted that in the equilibrium study described above, the second ionization of the phosphate group of the imine product has been found to have a pK_a of 8.0 ± 0.2. Thus, in mechanism two it is conceivable that the phosphate group could catalyze carbinolamine dehydration. This possibility is considered further in the Discussion below.

Mechanism two may be questioned for two reasons. First, as in the case of mechanism one the ε-amino group would have an abnormally low pK_a of 5.5 ± 0.2. Second, this mechanism proceeds with a second-order rate constant for inactivation of $k_2/K_m = 7.2 \times 10^2$ M^{-1} min^{-1}. Again, as in the case of mechanism one this value is not much larger than the second-order rate constant for inactivation by pyridoxal (Piszkiewicz and Smith, 1971), and it appears to be unreasonably low for this unimolecular process.

MECHANISM THREE. As in the mechanism just discussed, the reactive complex would be the monoacid form of a diacid reactive species. The inactivation reaction would be expressed by eq 9 and the rate would be described by eq 10. Mechanism three differs from mechanism two in the identity of the groups whose ionizations are described by pK_{app1} and pK_{app2}.

The value of pK_{app2} is identical with the pK_a of the ε-amino group of lysine-97 as determined by the equilibria and kinetic studies with pyridoxal (Piszkiewicz and Smith, 1971), and it might be related to this group. However, this interpretation of pK_{app2} would require that the amino group be in the protonated form, a situation incompatible with a mechanism involving nucleophilic attack by this group on the aldehyde. The ionization described by pK_{app1} could be related to a basic group within the noncovalent complex. It might function in the rate-limiting step as a general base which abstracts the proton from the reacting amino group and thereby assists the



(11)

nucleophilic attack. This interpretation leaves much to be desired, however. It fails to explain why rates of inactivation equal to or greater than found at the pH optimum (Figure 5) were not observed at higher pH values where the reactive amino group is in the form of its conjugate base. Also, as in the mechanism described by eq 7 and 8, it requires a second-order rate constant for inactivation ($k_2/K_m = 7.2 \times 10^2$ M^{-1} min^{-1}) which appears to be unreasonably small.

MECHANISM FOUR. The reactive form of the noncovalent complex would require the conjugate base of the amino group and an acidic group (A) of low pK_a. This reactive species (E·P′) may be related to the form which predominates at neutral pH (E·P in eq 11) by the equilibria shown in Figure 6. From the dissociation equilibria, and a material balance of the species shown in Figure 5, it may be derived that

$$k_0 = \frac{k_2' K_{a2} K_{a4} a_H}{K_{a1} K_{a2} K_{a4} + K_{a1} K_{a4} a_H + K_{a2} K_{a4} a_H + K_{a2} a_H^2} \quad (12)$$

Since $K_{a2} = K_{a4}$, eq 12 reduces to

$$k_0 = \frac{k_2' K_{a2} a_H}{K_{a1} K_{a2} + K_{a1} a_H + K_{a2} a_H + a_H^2} \quad (13)$$

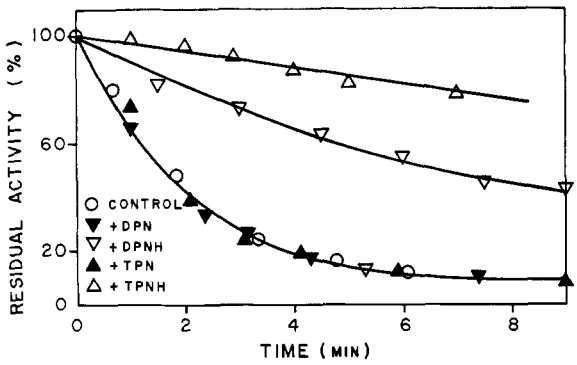


FIGURE 7: Time course of the reactions of pyridoxal 5'-phosphate (1.8×10^{-3} M) with glutamate dehydrogenase (1.8×10^{-5} M) at pH 7.13 ± 0.02 in the presence of DPN, DPNH, TPN, or TPNH (each at a concentration of 5×10^{-3} M).

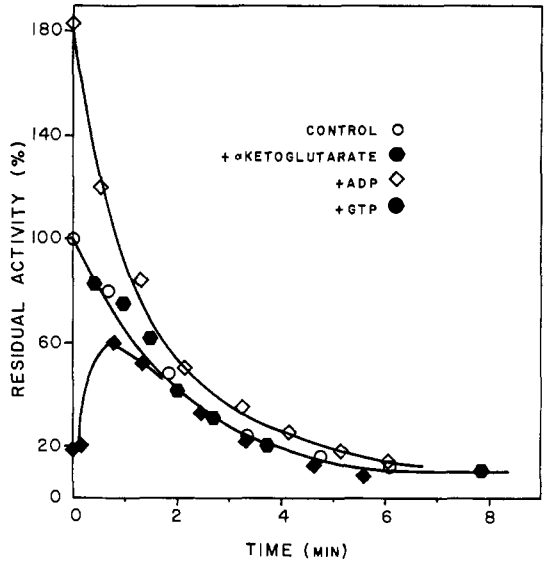


FIGURE 8: Time course of the reactions of pyridoxal 5'-phosphate (1.8×10^{-3} M) with glutamate dehydrogenase (1.8×10^{-5} M) at pH 7.13 ± 0.02 in the presence of α-ketoglutarate, ADP, or GTP (each at 5×10^{-3} M).

And since K_{a1} is much larger than K_{a2}, eq 13 reduces to

$$k_0 = \frac{k'_2 K_{a2} a_H}{K_{a1} K_{a2} + K_{a1} a_H + a_H^2} \qquad (14)$$

The identical forms of eq 10 and 14 is obvious. Equation 10 is related to eq 14 by $k'_2 = k_2 K_{a1}/K_{a2}$. Thus, eq 14 may be fitted to the data of Figure 4 by employing the constants, $k'_2 = 600 \pm 60$ min^{-1} p$K_{app1} = 5.5 \pm 0.2$ with $K_{app1} = 3.3 \times 10^{-6}$ M, and p$K_{app2} = 8.0 \pm 0.2$ with $K_{app2} = 10^{-8}$ M.

In mechanism four p$K_{app2} = 8.0 \pm 0.2$ is equal to the pK_a of the ε-amino group of lysine-97 as determined in the kinetic and equilibrium studies with pyridoxal (Piszkiewicz and Smith, 1971), and it obviously describes the dissociation of this group. In addition, p$K_{app1} = 5.5 \pm 0.2$ would describe the ionization of an acidic group within the noncovalent enzyme–inactivator complex. In this interpretation, the rate-determining step at low pH would be nucleophilic attack by the amino group on the aldehyde, and the rate-limiting step at high pH could be dehydration of the carbinolamine which is catalyzed by the acidic group of p$K_{app1} = 5.5 \pm 0.2$. In the case of mechanism four, as in mechanism two, the possibility that the acidic group described by pK_{app1} is the monoprotonated phosphate of pyridoxal 5'-phosphate should be considered. This group has p$K_a = 6.20$ in the free form of the molecule (Williams and Nielands, 1954) and p$K_a = 8.0 \pm 0.2$ in the product imine (from the equilibrium study, above). Thus, while the actual pK_a of this group in the noncovalent complex is unknown, its pK_a value in the free molecule is not radically different from pK_{app1} which was determined kinetically.

The validity of mechanism four may be evaluated by comparing the second-order rate constant of imine formation by pyridoxal ($k_{2(PAL)} = 1.7 \times 10^2$ M^{-1} min^{-1}) (Piszkiewicz and Smith, 1971) with the second-order constant for the reaction of enzyme with pyridoxal 5'-phosphate ($k'_2/K_m = 2.4 \times 10^5$ M^{-1} min^{-1}). The value of k'_2/K_m (2.4×10^5 M^{-1} min^{-1}) is more than three orders of magnitude greater than the value of $k_{2(PAL)}$ (Piszkiewicz and Smith, 1971). Thus, the magnitude of k'_2 appears to be reasonable.

One may also calculate the ratio of k'_2 for the first-order rate of imine formation with pyridoxal 5'-phosphate to k_2 for the second-order rate of imine formation with pyridoxal. This ratio of $k'_{2(PLP)}/k_{2(PAL)}$ has the units of molarity and gives the concentration of pyridoxal required to inactivate the dehydrogenase at a rate equal to the maximum rate of inactivation by pyridoxal 5'-phosphate. This ratio of a first-order rate to the second-order rate of an analogous reaction has been defined as the "effective concentration" (Jencks, 1969). Such effective concentrations may be calculated from model studies of intra- and intermolecular reactions; these range from 30 to 3×10^7 M (Bruice and Pandit, 1960a,b). The effective concentration for the reaction of pyridoxal with dehydrogenase necessary to give the maximum rate observed with pyridoxal 5'-phosphate may be calculated to be $k'_{2(PLP)}/k_{2(PAL)} = 6.0 \times 10^2$ min^{-1}/1.7×10^2 M^{-1} min^{-1} = 3.5 M. Although this value is somewhat lower than might have been anticipated on the basis of model studies (Bruice and Pandit, 1960a,b), it does suggest that the value of $k'_{2(PLP)}$ employed in the calculations is a reasonable value for the actual rate constant of imine formation with pyridoxal 5'-phosphate, and that the mechanism given in eq 11 (and Figure 6) is also reasonable.

Effects of Substrate, Coenzyme, and Allosteric Modifiers on Rates of Inactivation. The time course of the inactivation of enzyme by pyridoxal 5'-phosphate in the presence of DPN, DPNH, TPN, or TPNH is presented in Figure 7. 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 the control value (Figure 7) 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 ($k_{obsd} = 0.51$ min^{-1}) measured in the absence of added ligand. Thus, DPN and TPN offered no detectable protection from the inactivation of pyridoxal 5'-phosphate. The rates of enzyme inactivation in the presence of DPNH and TPNH were significantly lower than those of the control, and, therefore, afford some protection of the reactive amino group of the enzyme. A detailed investigation of this protection is presented below.

The time course of the inactivation of enzyme by pyridoxal 5'-phosphate in the presence of α-ketoglutarate, ADP, and GTP is given in Figure 8. As in the experiments performed in the presence of coenzymes (Figure 7) 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

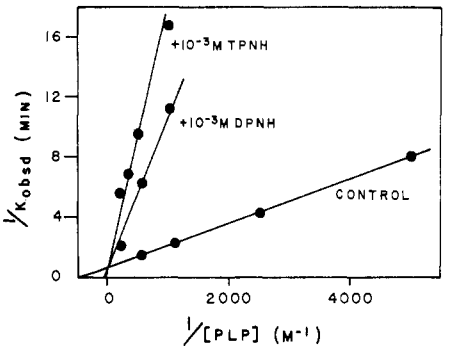


FIGURE 9: Lineweaver–Burk-type plots of $1/k_{obsd}$ *vs.* 1/[pyridoxal 5′-phosphate] (abbreviated as 1/[PLP]) for the inactivation of glutamate dehydrogenase (at 1.8×10^{-5} M) at pH 7.20 ± 0.02 in the presence of 10^{-3} M DPNH or 10^{-3} M TPNH or in the absence of these substances.

activities significantly higher and lower than the control, respectively, due to a carry-over of 10^{-5} M ADP or GTP from the reaction mixture to the assay solution and were expected. Thus, these data have been plotted relative to the control (Figure 8). The rates of inactivation of enzyme by pyridoxal 5′-phosphate in the presence of α-ketoglutarate or ADP did not differ significantly from the control.

When the enzyme was reacted with pyridoxal 5′-phosphate in the presence of GTP a rapid initial apparent stimulation of activity was followed by a gradual loss of activity similar to that of the control (Figure 8). Since activity at zero time was depressed below the control due to carry-over of 10^{-5} M GTP, the apparent stimulation may be interpreted as a loss of inhibition by GTP. As in the case of the inactivation of enzyme by pyridoxal (Piszkiewicz and Smith, 1971) one may conclude that as inactivation of enzyme by pyridoxal 5′-phosphate proceeds, a "reverse allosteric effect" is observed in which covalent binding of pyridoxal 5′-phosphate 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 observed first-order rate constants (k_{obsd}) for the inactivation of glutamate dehydrogenase in the presence of 10^{-3} M DPNH, 10^{-3} M TPNH, and in the absence of these substances were determined over a range of pyridoxal 5′-phosphate concentrations. A Lineweaver–Burk- (1934) type plot of $1/k_{obsd}$ *vs.* the reciprocal of inactivator concentration for each of these three cases is shown in Figure 9. The addition of DPNH or TPNH to the reaction mixture was found to increase the slope of the resulting line on the double-reciprocal plot (Figure 9) without changing the intercept of the ordinate; this behavior is characteristic of competitive inhibition. Thus, if the inactivation of enzyme (E) by pyridoxal 5′-phosphate (P) in the presence of DPNH or TPNH proceeds by the mechanism

$$\begin{array}{ccc} E + P \rightleftharpoons E\cdot P \xrightarrow{k_0} EP & & \\ K_d \uparrow\downarrow + \text{DPNH (+ TPNH)} & & (15) \\ E\cdot\text{DPNH (E·TPNH)} & & \end{array}$$

we may derive (where TPNH may replace DPNH)

$$k_{obsd} = \frac{k_0[P]K_d}{K_I K_d + K_I[\text{DPNH}] + K_d[P]} \qquad (16)$$

This expression is analogous to one which describes competitive inhibition of enzyme-catalyzed reactions, and it may be similarly transformed to give

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_0}\left(1 + \frac{[\text{DPNH}]}{K_d}\right)\left(\frac{1}{[P]}\right) + \frac{1}{k_0} \qquad (17)$$

Thus, in the plots of $1/k_{obsd}$ *vs.* 1/[P] corresponding to inactivations performed in the presence of DPNH or TPNH (Figure 8), the intercepts of the ordinate were $1/k_0$, and the slopes were $K_I/k_0(1 + [\text{DPNH}]/K_d)$. The dissociation constants (K_d) for the complexes formed by enzyme (E) with DPNH and enzyme with TPNH have been calculated from the slopes of the double-reciprocal plots (Figure 9), the previously determined values of K_I and k_0, and the concentration of protector as 1.8×10^{-4} and 9.7×10^{-5} M, respectively. It would be desirable to compare the calculated values of K_d for DPNH and TPNH with the K_m values for these substances. Unfortunately, such a comparison is not straightforward because of the complex dependence of rate on coenzyme concentration and overall mechanism (Yielding *et al.*, 1964; Engel and Dalziel, 1970).

Discussion

Pyridoxal 5′-phosphate inactivates bovine liver glutamate dehydrogenase by forming an imine with the ε-amino group of lysine-97 of the subunit polypeptide chain (Piszkiewicz *et al.*, 1970). While this reaction results in a loss of catalytic activity, it also has additional effects on the physical characteristics of the enzyme. Anderson *et al.* (1966) had previously demonstrated by sedimentation–equilibrium studies that the enzyme, after reaction with pyridoxal 5′-phosphate, loses its ability to aggregate at high protein concentrations. In this study we have shown that partial reaction (*i.e.*, at least one but not all six of the reactive groups in the active enzyme unit) results in a loss of the ability of GTP to inhibit the enzyme (see Figure 8). Although this "reverse allosteric effect" was observed for GTP, no similar effect was observed for the allosteric activation by ADP. In addition, it is noteworthy that in the equilibrium and kinetic studies reported here no positive or negative cooperativity was observed among the six reacting amino groups; these all appeared to react at the same rate and reach the same equilibrium. Clearly, the effects of pyridoxal 5′-phosphate on glutamate dehydrogenase are many and complex.

From the equilibrium study of the inactivation of glutamate dehydrogenase by pyridoxal 5′-phosphate it has been possible to determine the pK_{app} value of the protonated pyridinium nitrogen of the imine product (EP_1 in Figure 2) as $pK_{1EP} = 5.2 \pm 0.2$. This value is approximately 3.5 units lower than that of the identical group of the free inactivator ($pK_a = 8.69$) (Williams and Nielands, 1954). In the reaction of pyridoxal with valine (Metzler, 1957), the pK_a of the pyridinium nitrogen was found to decrease from 8.66 to 5.88 in the imine. Thus, the value of $pK_{1EP} = 5.2 \pm 0.2$, which was determined by the equilibrium study described above, does not differ significantly from one which might be predicted on the basis of the model study (Metzler, 1957).

The equilibrium study has also allowed the determination of the second dissociation of the phosphate of the imine product (EP_2 in Figure 2) as $pK_{2EP} = 8.0 \pm 0.2$. This value is almost two units higher than that of the same group in the unbound compound (Williams and Nielands, 1954). Two possible causes of this phenomenon may be considered. First,

the phosphate group may reside in a hydrophobic region on the surface of the enzyme which would inhibit the formation of a greater charge. Second, the proton of the second dissociation may be involved in a hydrogen bond which could conceivably lessen its availability to solvent.

Our kinetic study has shown that the reaction of pyridoxal 5'-phosphate with the ϵ-amino group of lysine-97 proceeds through formation of an apparent noncovalent complex prior to imine formation. The dissociation constant for this complex has been determined by kinetic analysis to be $K_I = 2.5 \pm 1.0 \times 10^{-3}$ M, and found to be invariant with pH. Recently, this behavior has been shown to have a precedent in the binding of pyridoxal 5'-phosphate to the essential lysyl ϵ-amino group of glutamate decarboxylase (O'Leary and Malik, 1971). Such complex formation between pyridoxal 5'-phosphate and glutamate decarboxylase was not unexpected since imine formation is part of its physiological mechanism; however, noncovalent complex formation in the case of glutamate dehydrogenase could not have been predicted since no physiological role for this reaction had been previously demonstrated.

The bell-shaped pH dependence of the rate of imine formation (Figure 5) was accommodated most easily by mechanism four (Figure 6) which required as the reactive form of the noncovalent complex the conjugate base of the amino group with $pK_{app2} = 8.0 \pm 0.2$ and an acidic group (A) with $pK_{app1} = 5.5 \pm 0.2$ (eq 14). Inasmuch as we have found a dependence on only one ionizable group, the reactive amino group, in the similar study with pyridoxal (Piszkiewicz and Smith, 1971) it seems likely that the acidic ionization can be related to the phosphate group of pyridoxal 5'-phosphate. This group, which has $pK_a = 6.20$ in the free form of the molecule (Williams and Nielands, 1954), could conceivably catalyze carbinolamine dehydration in an intramolecular manner. Thus, at low pH the phosphate group might perform the same function as has been observed for the 3-hydroxyl group at high pH (Auld and Bruice, 1967b).

The significance of this mechanism in relation to other reactions involving pyridoxal 5'-phosphate should be noted. Model studies (such as those by Auld and Bruice, 1967a,b; French *et al.*, 1965) have concentrated on compounds lacking the phosphate group and not possessing the hydroxymethyl substituent ortho to the 4-aldehyde group. The rationale was that of simplification of the kinetics and equilibria by the removal of the ionizations of the phosphate group and elimination of internal hemiacetal formation by the hydroxyl group. It was further assumed (French *et al.*, 1965) that in most cases the phosphate would be electrostatically bound to the enzyme surface and not likely to participate in imine formation. While this assumption may be correct for models of some pyridoxal 5'-phosphate reactions, it has not been shown to be universally valid. No evidence has been presented which indicates that the phosphate is always involved in binding to apoenzyme. Furthermore, since the phosphate group is multifunctional, it could function both in binding to protein and catalysis. This appears to be the case in the inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate. Indeed, since the evidence presented above strongly suggests that the phosphate may participate in imine formation, a reevaluation of model mechanistic studies is in order. It is possible that this group may catalyze carbinolamine dehydration and other reactions of pyridoxal 5'-phosphate in a significant number of physiological processes.

The catalytic activity of the enzyme is protected from inactivation by pyridoxal 5'-phosphate by both TPNH and DPNH (Figure 7). This protection is analogous to competitive inhibition (Figure 9), and suggests that the inactivator binds at a binding site of reduced coenzyme. Similarly, the catalytic activity of the enzyme is partially protected by TPNH and DPNH from inactivation by pyridoxal (Piszkiewicz and Smith, 1971). Since protection was not total in the case of inactivation by pyridoxal, reduced coenzyme reduced the rate of imine formation but did not completely block access to the reactive amino group. Since pyridoxal 5'-phosphate and the reduced coenzymes, DPNH and TPNH, possess a pyridinium ring, one may ask if the ring of the inactivator binds to the site on the enzyme surface normally occupied by the ring of the reduced coenzyme. The significance of such binding is uncertain inasmuch as pyridoxal which also contains this structure, inactivates the enzyme at a significant rate even when the enzyme is saturated with DPNH or TPNH. It is presently unknown why the reduced coenzymes protect the enzyme from inactivation whereas DPN and TPN have no such effect.

The phosphate group of the inactivator is essential for binding since pyridoxal 5'-phosphate undergoes noncovalent binding to the enzyme but pyridoxal does not. Since α-ketoglutarate does not protect the enzyme from inactivation by pyridoxal 5'-phosphate the phosphate group would not appear to bind at either of the carboxyl group binding sites of the substrate. It is more likely that the phosphate group of the inactivator occupies a site at or near the position occupied by the pyrophosphate portion of coenzyme.

Glutamate dehydrogenase is one of the few enzymes involved in mammalian amino acid metabolism which does not require pyridoxal 5'-phosphate as a cofactor, yet this compound is required by other enzymes in pathways involving the metabolism of glutamic acid, *e.g.*, transaminases and glutamate decarboxylase. The possible physiological significance of the inactivation of this enzyme by pyridoxal 5'-phosphate as a form of metabolic regulation has been noted previously (Anderson *et al.*, 1966; Piszkiewicz *et al.*, 1970). Our data may contribute to a solution of this problem. The dissociation constant for the reaction of pyridoxal 5'-phosphate with glutamate dehydrogenase has a minimal value of 4.4×10^{-5} M at pH 7.7 (Figure 1). The concentration of pyridoxal 5'-phosphate in rat liver has been reported to be 0.86 ± 0.1 μg/g of whole tissue or 3.3×10^{-6} M (Wachstein and Moore, 1958); the concentration of this compound in bovine liver is likely to be very similar. If there is a local concentration of this compound in the mitochondria, the intracellular location of the glutamate dehydrogenase, it could inactivate a significant fraction of the enzyme, and function as a metabolic regulator. Conversely, if the concentration of pyridoxal 5'-phosphate in the mitochondria were lower than that of the whole tissue, or if a sufficient fraction of this compound was bound to protein, the possible significance of the inactivation reaction would be less.

One may question the physiological significance of the inactivation reaction on the basis of the relatively slow rates of inactivation as compared to rates of enzyme-catalyzed reactions. Recently, Frieden (1970) has discussed the significance of a large number of enzymes which exhibit a slow response in some kinetic characteristic to a rapid change in ligand (either substrate or modifier) concentration. The inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate clearly fits into this category of enzyme behavior which Frieden has termed hysteretic. Frieden (1970) postulated that such hysteretic processes may play a significant role in the regulation of complex metabolic processes. Thus, it is possible that the relatively slow inactivation of glutamate dehydrogenase by

pyridoxal 5′-phosphate represents such a form of metabolic regulation.

Acknowledgments

We are indebted to Mrs. Treasure Gallo and Mrs. Maria Tamasi for their excellent technical assistance. We also thank Dr. David Sigman for his many criticisms and suggestions.

References

Anderson, B. M., Anderson, C. D., and Churchich, J. E. (1966), *Biochemistry 5*, 2893.
Auld, D. S., and Bruice, T. C. (1967a), *J. Amer. Chem. Soc. 89*, 2083.
Auld, D. S., and Bruice, T. C. (1967b), *J. Amer. Chem. Soc. 89*, 2098.
Bruice, T. C. (1970), *Enzymes 2*, 217.
Bruice, T. C., and Pandit, V. K. (1960a), *J. Amer. Chem. Soc. 82*, 5858.
Bruice, T. C., and Pandit, V. K. (1960b), *Proc. Nat. Acad. Sci. U. S. 46*, 402.
Edsall, J. T. (1943), *in* Proteins, Amino Acids and Peptides, Cohn, E. J., and Edsall, J. T., Ed., New York, N. Y., Hafner Publishing Co., p 445.
Engel, P. C., and Dalziel, K. (1970), *Biochem. J. 118*, 409.
Frieden, C. (1970), *J. Biol. Chem. 245*, 5788.
French, T. C., Auld, D. S., and Bruice, T. C. (1965), *Biochemistry 4*, 77.
Jencks, W. P. (1959), *J. Amer. Chem. Soc. 81*, 475.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill Book Co., p 10.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Metzler, D. E. (1957), *J. Amer. Chem. Soc. 79*, 485.
Metzler, D. E., and Snell, E. E. (1955), *J. Amer. Chem. Soc. 77*, 2431.
Michaelis, L., and Menten, M. L. (1913), *Biochem. Z. 49*, 333.
O'Leary, M. H., and Malik, J. M. (1971), *J. Biol. Chem. 246*, 544.
Piszkiewicz, D., Landon, M., and Smith, E. L. (1970), *J. Biol. Chem. 245*, 2622.
Piszkiewicz, D., and Smith, E. L. (1971), *Biochemistry 10*, 4538.
Schmidt, Jr., D. E., and Westheimer, F. A. (1971), *Biochemistry 10*, 1249.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Wachstein, M., and Moore, C. (1958), *Proc. Soc. Exp. Biol. 97*, 905.
Williams, V. R., and Nielands, J. B. (1954), *Arch. Biochem. Biophys. 53*, 56.
Yielding, K. L., Tomkins, G. M., and Trundle, D. S. (1964), *Biochim. Biophys. Acta 85*, 342.

Studies on the Role of Calcium in Thermolysin*

Joseph Feder,† Linda R. Garrett, and Bernard S. Wildi

ABSTRACT: It has been found that the thermostable neutral protease from *Bacillus thermoproteolyticus*, thermolysin, contains four rather tightly bound calcium atoms per molecule. Removal of three to four of these calcium atoms has been achieved without effect on the catalytic properties of the enzyme but with marked reversible loss of thermostability. Addition of calcium to the low calcium enzyme results in restabilization of the molecule. The use of gel filtration over Sephadex G-25 and G-75 equilibrated with EDTA and 1,10-phenanthroline for simultaneous removal of calcium and zinc to yield low calcium inactive apoenzyme has permitted these manipulations which under other circumstances result in rapid autolysis of the enzyme. The stabilizing effect of additional calcium on the thermolysin was also studied.

The role of calcium in maintaining stability has been reported for a number of different enzymes. The stabilization of trypsin solutions by calcium has been reported (Bier and Nord, 1951; Gorini, 1951; Green *et al.*, 1952). Various α-amylases have been shown to be stabilized by calcium (Stein and Fischer, 1958; Stein *et al.*, 1964; Hsiu *et al.*, 1964) and Vallee *et al.* (1959) demonstrated the presence of at least one atom of calcium per molecule for certain of these enzymes. The maintenance of a taut enzyme molecule by calcium also has been reported for the *Escherichia coli* glutamine synthetase (Shapiro and Ginsburg, 1968). The bacterial metallo neutral proteases are particularly sensitive to the presence of calcium in solution for stability. The *Bacillus subtilis* neutral protease (McConn *et al.*, 1964; Tsuru *et al.*, 1966a,b) as well as the alkaline protease (Tsuru *et al.*, 1966a,b; Matsubara *et al.*, 1958) is stabilized by calcium. Similarly, calcium and other divalent cations stabilize the neutral proteases from *Bacillus megaterium* (Millet, 1969), *Streptomyces griseus* (Nomoto *et al.*, 1960), *Pseudomonas aeruginosa* (Morihara, 1963), and *Bacillus cereus* (J. Feder, 1971, unpublished data). The thermostable thermolysin also requires calcium ions for stability (Endo, 1962; Ohta *et al.*, 1965, 1966; Matsubara, 1967; Ohta, 1967). Removal of the calcium from the thermolysin molecule results in loss of the thermal stability of the enzyme. Since thermolysin and the other neutral proteases from the bacilli such as the *Bacillus subtilis* enzyme are very similar, with respect to specificity, zinc content and general catalytic properties as well as a dependence on the presence of calcium for

* From the New Enterprise Division, Monsanto Company, St. Louis, Missouri 63166. *Received July 8, 1971.*