Inhibition of Glutamic Dehydrogenase by Pyridoxal 5′-Phosphate*

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ABSTRACT: Bovine liver glutamic dehydrogenase is inactivated by incubation at 34° with pyridoxal 5′-phosphate. Both the glutamic and alanine dehydrogenase activities of the enzyme are lost simultaneously. The presence of pyridoxal 5′-phosphate prevents aggregation of the enzyme at high protein concentrations. Restoration of catalytic activity and the ability to aggregate can be accomplished by dialysis of the inactivated enzyme. Spectral evidence is presented to indicate that the inactivation proceeds through Schiff base formation with amino groups of the enzyme Sodium borohydride reduction of the pyridoxal 5′-phosphate inactivated enzyme produces a stable pyridoxyl–enzyme derivative that cannot be reactivated by dialysis. The pyridoxyl–enzyme was studied through fluorescence spectroscopy. ε-Pyridoxyllysine was identified in acid hydrolysates of the pyridoxyl–enzyme. Glutamic dehydrogenase was also demonstrated to be inactivated by substituted benzaldehydes. Substituent effects on the inactivation process were investigated.

The molecular weight of bovine liver glutamic dehydrogenase [L-glutamate:NAD$^+$ (P) oxidoreductase (deaminating), EC 1.4.1.3] was first determined by Olson and Anfinsen (1952) to be 1×10^6 at concentrations of the enzyme greater than 5 mg/ml. On dilution, the molecular weight of the enzyme decreases. Similar dilution effects on molecular weight were observed in a variety of studies of this enzyme (Kubo *et al.*, 1959; Fischer *et al.*, 1962; Greville and Horne, 1963; Frieden, 1963a). Recently, Rogers *et al.* (1965) reported a molecular weight of 270,000 for glutamic dehydrogenase which was determined at low concentrations of the enzyme comparable to those normally used in kinetic studies. This is in agreement with previous proposals that the smallest active molecular unit of the enzyme lies in the molecular weight range of 250,000–350,000 (Frieden, 1962; Churchich and Wold, 1963; Frieden, 1963b; Tomkins *et al.*, 1964). Further irreversible subdivision of the enzyme can be accomplished in the presence of denaturants. Marler and Tanford (1964), for example, have reported subunits of approximately 50,000 mol wt on treatment of the enzyme with guanidine hydrochloride.

The quaternary structure as well as the catalytic activity of bovine liver glutamic dehydrogenase can be altered reversibly, by a variety of small molecular weight compounds (see review by Reithel, 1963). In most cases, those compounds that inhibit glutamic dehydrogenase activity interfere with the ability of a monomeric form of the enzyme to aggregate at higher concentrations and these effects are normally accompanied by an enhancement of the alanine dehydrogenase activity of the protein (Bitensky *et al.*, 1965a; Anderson and Reynolds, 1966). Many of the compounds that function in this manner occur naturally in biological materials, which raises many interesting questions concerning the role that these compounds might play in the *in vivo* functioning of the enzyme. In this respect, the primary observation described in the present study, the inhibition of glutamic dehydrogenase by pyridoxal 5′-phosphate, prompted a more detailed study of the effects of this and related compounds on the chemical and physical properties of this enzyme.

Experimental Section

Materials. NAD$^+$, NADH, pyridoxal, pyridoxal 5′-phosphate, and crystalline bovine liver glutamic dehydrogenase (sodium phosphate–glycerol solution) were obtained from the Sigma Chemical Co. Substituted benzaldehydes were obtained from the Eastman Kodak Co. Sodium borohydride was purchased from Metal Hydrides, Inc. Polylysine, av mol wt 100,000, was obtained from the Pilot Chemical Co., Boston, Mass.

ε-Pyridoxyllysine was prepared using a procedure described by Dempsey and Snell (1963). The α-acetyl derivative required for this preparation was synthesized according to Dempsey and Christensen (1962).

Methods. Glutamic dehydrogenase activity was measured in 10 ml of reaction mixtures containing 0.01 M Tris–acetate buffer, pH 8.03, 6.7×10^{-4} M NAD$^+$, and 0.05 M glutamic acid. Reactions were initiated by the addition of 30 μg of enzyme. Initial velocities were obtained at 25° by recording the reduction of NAD$^+$ at 340 mμ in a temperature-controlled cell compartment of a Gilford Model 2000 recording spectrophotometer. Preincubation of the enzyme with inhibitors was carried out at 34° in 0.4 ml of reaction mixtures containing 0.05 M sodium phosphate buffer, pH 7.2, inhibitor, and 4×10^{-6} M glutamic

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[1] Abbreviations used in this work: PPal, pyridoxal 5′-phosphate; GDH, glutamic dehydrogenase; NAD$^+$, nicotinamide-adenine dinucleotide; NADH, reduced NAD$^+$.

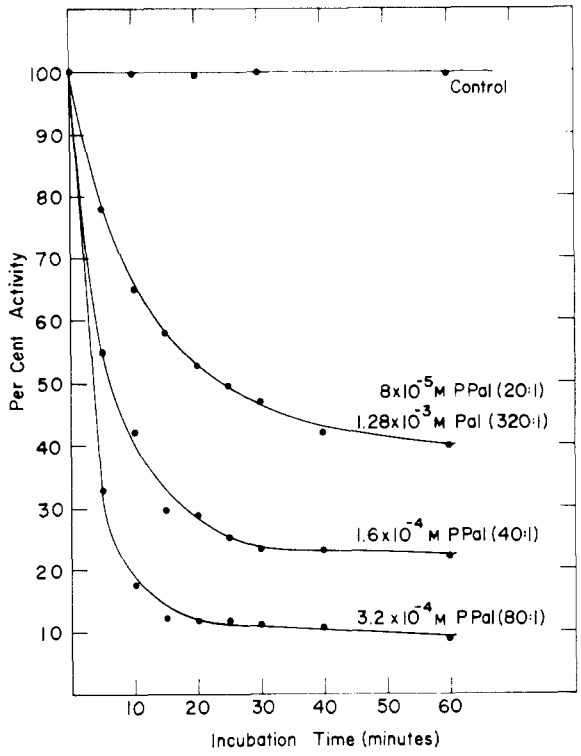

FIGURE 1: Inactivation of glutamic dehydrogenase activity by pyridoxal 5′-phosphate and pyridoxal. Incubations were carried out at 34° in 0.05 M sodium phosphate buffer, pH 7.2. The enzyme concentration was 4×10^{-6} M. Ratios shown in parentheses are molar ratios of inhibitor to enzyme.

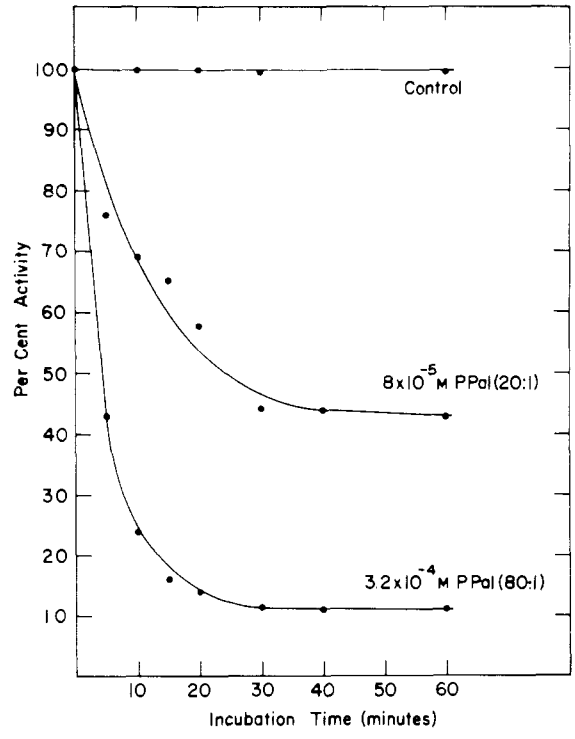

FIGURE 2: Inactivation of alanine dehydrogenase activity by pyridoxal 5′-phosphate. Incubations were carried out at 34° in 0.05 M sodium phosphate buffer, pH 7.2. The enzyme concentration was 4×10^{-6} M. Ratios shown in parentheses are molar ratios of inhibitor to enzyme.

dehydrogenase. A molecular weight of 250,000 was used for the calculation of molar concentrations of the enzyme.

Alanine dehydrogenase activity was measured in 3.0 ml of reaction mixtures containing 0.1 M Tris–HCl buffer, pH 9.06, 0.15 M NH_4Cl, 0.04 M sodium pyruvate, 0.16 M NaCl, and 8.46×10^{-5} M NADH. Reactions were initiated by the addition of 200 μg of glutamic dehydrogenase. Initial velocities were obtained at 25° by recording the decrease in optical density at 340 mμ in a temperature-controlled cell compartment of a Gilford Model 2000 recording spectrophotometer. Preincubation of the enzyme with inhibitors was carried out at 34° in 2.0-ml reaction mixtures containing 0.05 M sodium phosphate buffer, pH 7.2, inhibitor, and 4×10^{-6} M glutamic dehydrogenase. Unless stated otherwise, reduction of glutamic dehydrogenase derivatives was carried out in 0.05 M sodium phosphate buffer, pH 7.2, at 4° by the addition of solid $NaBH_4$.

Sedimentation velocity experiments were conducted in the Spinco Model E analytical ultracentrifuge at constant temperatures in the range of 6–8°. For sedimentation velocity studies, the ultracentrifuge was operated at 42,040 rpm and the sedimentation constants reported were corrected for the density and viscosity of water at 20°.

Fluorescence spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromators were set to give a band width of 3 mμ. The ultraviolet emission spectra of protein samples were recorded over the wavelength range of 275–430 mμ with excitation at 280 mμ. The fluorescence of the pyridoxyl–enzyme chromophore was obtained by exciting at 325–327 mμ and recording the emission spectrum over the wavelength range of 330–440 mμ.

Spectrophotometric measurements were carried out in either a Cary Model 11 or a Bausch and Lomb 505 recording spectrophotometer. Measurements of pH were made at 25° with a Radiometer pH meter, type PHM 4b with a G-200-B glass electrode.

Results

The effect of a variety of aromatic aldehydes on the activity of crystalline bovine liver glutamic dehydrogenase (GDH) was investigated by preincubating the enzyme with the aldehydes in 0.05 M sodium phosphate buffer, pH 7.2, at 34°. Aliquots of the preincubation mixtures were removed and transferred to assay mixtures in which initial velocities of NAD^+ reduction were obtained at 25°. In the absence of aldehydes,

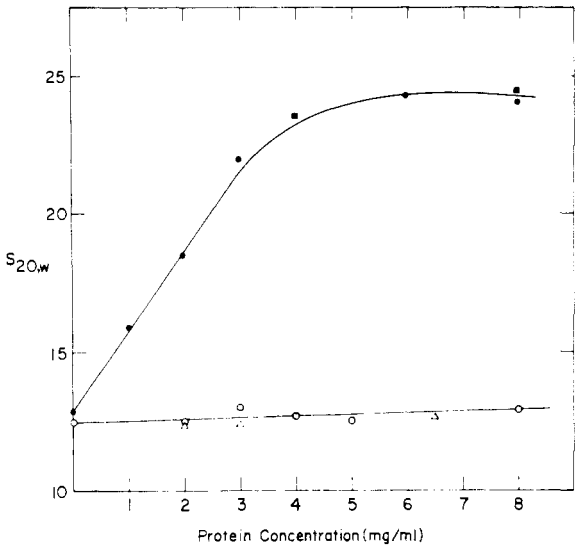

FIGURE 3: Sedimentation velocity studies of glutamic dehydrogenase derivatives. Ultracentrifugation was carried out at 42,040 rpm in 0.05 M sodium phosphate buffer, pH 7.2. *Closed circles*, native glutamic dehydrogenase; *open circles*, glutamic dehydrogenase plus PPal incubated at 34° for 20 min. The molar ratio PPal:GDH was 50 for each sample studied. *Triangles*, pyridoxyl–enzyme prepared by $NaBH_4$ reduction of the pyridoxal-inactivated enzyme; *squares*, $NaBH_4$-reduced native glutamic dehydrogenase.

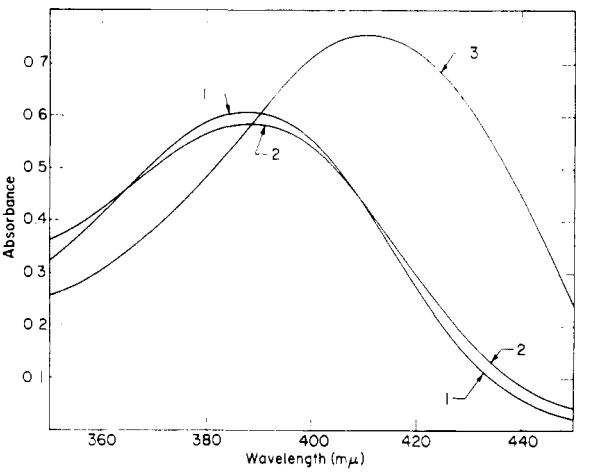

FIGURE 4: The effect of glutamic dehydrogenase and polylysine on the spectrum of pyridoxal 5′-phosphate. Curve 1, 1.3×10^{-4} M PPal in 0.05 M sodium phosphate buffer, pH 7.2; curve 2, 1.3×10^{-4} M PPal plus 2.6×10^{-6} M glutamic dehydrogenase in 0.05 M sodium phosphate buffer, pH 7.2, after incubation at 34° for 20 min; curve 3, 1.3×10^{-4} M PPal plus 2×10^{-4} M polylysine in 0.05 M sodium phosphate buffer, pH 7.2, after incubation at 34° for 15 min (same spectrum obtained after 45 min of incubation).

the activity of glutamic dehydrogenase remained essentially constant over a 60-min preincubation period (Figure 1). In most cases, less than 10% inactivation occurred over a 120-min incubation period. During the 60-min incubation period, the presence of small amounts of pyridoxal 5′-phosphate (PPal) in the incubation mixture caused extensive inactivation of the enzyme. The inactivation of the enzyme by PPal is not reversed by the dilution process of transferring the aliquot to the assay mixture. Concentrations of PPal identical with those resulting from the dilution process, when added directly to the assay mixture, do not significantly affect the activity of the enzyme. If the enzyme after incubation with PPal is dialyzed against 0.05 M sodium phosphate buffer, pH 7.2, complete activity is restored. Inactivation of the enzyme resulting from incubation in the presence of 1.6 and 3.2×10^{-4} M PPal is shown in Figure 1. These concentrations of PPal represent molar ratios (PPal:GDH) of 40:1 and 80:1, respectively. The enzyme is also inactivated by pyridoxal; however considerably higher concentrations of the unphosphorylated derivative are required to bring about inactivation (Figure 1). Identical inactivation curves were obtained with 1.28×10^{-3} M pyridoxal and 8×10^{-5} M PPal indicating that PPal is approximately 16 times as effective as pyridoxal in inactivating the enzyme. The degree of inactivation of the enzyme during the preincubation period increases with increasing concentrations of PPal (Figure 1). PPal:GDH ratios higher than 80:1 caused only slight increases in the inactivation of the enzyme. Ratios of 125:1 and 250:1 both resulted in 93% inactivation in a 20-min incubation period. The inhibition of glutamic dehydrogenase activity by PPal is accompanied by a comparable inhibition of the alanine dehydrogenase activity of this enzyme. The effect of two concentrations of PPal on the alanine dehydrogenase activity is shown in Figure 2. Approximately 86% inhibition of alanine dehydrogenase activity was observed in 20 min with 3.2×10^{-4} M PPal. This can be compared to 91% inhibition of glutamic dehydrogenase activity of the enzyme obtained with this concentration of PPal.

In order to obtain further information on the mechanism of inactivation, it was of interest to examine the physical and chemical properties of the PPal-modified enzyme. For this purpose the sedimentation properties as well as the fluorescence properties of the native and modified glutamic dehydrogenase were studied. As the concentration of glutamic dehydrogenase is increased from 1 to 5 mg/ml, aggregation of the enzyme occurs as indicated by the increase in sedimentation coefficient from approximately 13 to 26 (Figure 3). Under conditions where the enzyme is 90% inhibited by PPal, the sedimentation coefficient of the sample over a wide range of concentrations remains identical with the limiting value (Figure 3).

The reaction of glutamic dehydrogenase with PPal is accompanied by characteristic changes in the absorption spectrum of PPal. As illustrated in Figure 4, the

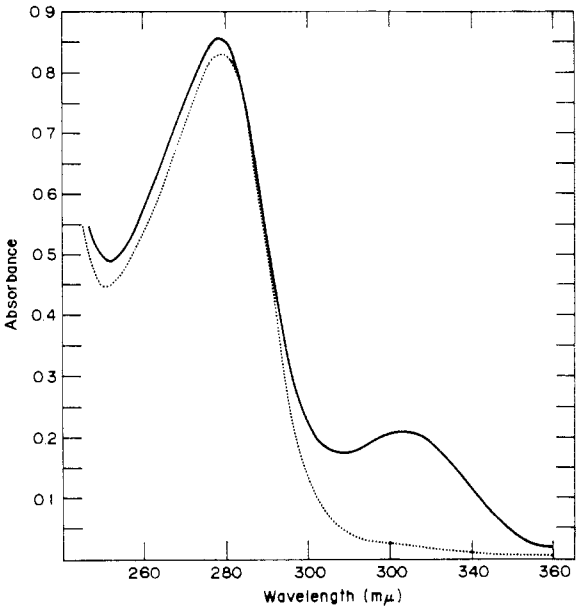

FIGURE 5: Spectrum of pyridoxyl–enzyme. Pyridoxyl–enzyme was prepared by incubating glutamic dehydrogenase with PPal at 34° for 20 min in 0.05 M sodium phosphate buffer, pH 7.2, at a PPal:GDH of 50. The sample was reduced with $NaBH_4$ in the cold and dialyzed exhaustively against 0.05 M sodium phosphate buffer, pH 7.2. The concentration of pyridoxyl–enzyme was 4×10^{-6} M for the spectral studies. The dashed line represents the spectrum of 4×10^{-6} M native glutamic dehydrogenase.

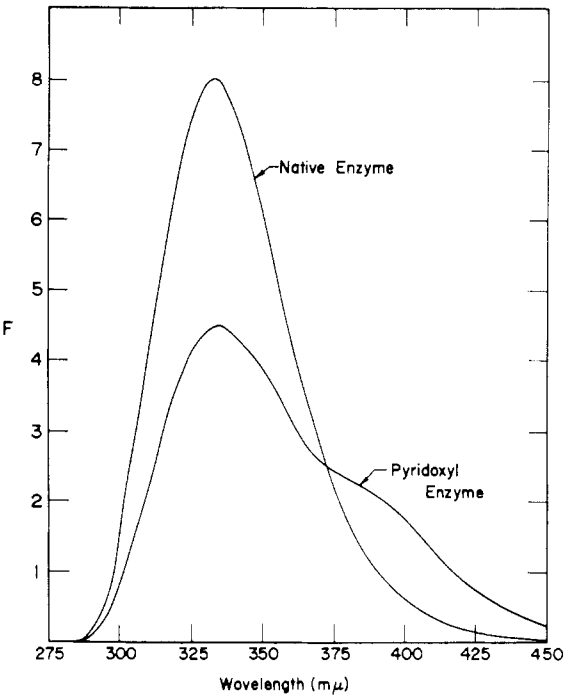

FIGURE 6: Fluorescence emission spectra of glutamic dehydrogenase derivatives. Spectra were obtained in 0.05 M sodium phosphate buffer, pH 7.2, with excitation at 280 mμ. Both samples had an optical density of 0.940 at 280 mμ. Pyridoxyl–enzyme for this stduy was prepared as described in Figure 5. F = fluorescence intensity (arbitrary units).

optical density at 390 mμ decreases simultaneously with an increase at 450 mμ. Similar changes have been reported for the reaction of PPal with free amino acids and have been attributed to the formation of Schiff bases. The rather small spectral change observed on the addition of glutamic dehydrogenase to PPal would indicate that relatively few molecules of PPal are utilized in the inactivation of the enzyme. To demonstrate that larger spectral changes would be observed if the involvement of PPal was more extensive, a study was made of the addition of polylysine to PPal (Figure 4). On the assumption that Schiff base formation is involved in the inactivation of glutamic dehydrogenase by PPal, attempts were made to stabilize the inactive form of the enzyme by $NaBH_4$ reduction. Inactive glutamic dehydrogenase was prepared by incubation with PPal at a PPal:GDH ratio of 50, reduced with $NaBH_4$ at 4° and dialyzed 24 hr against three changes of 0.05 M sodium phosphate buffer, pH 7.2. No restoration of enzyme activity was accomplished by dialysis at this point. This is in contrast to the complete restoration of activity observed by dialysis of the PPal-inactivated enzyme which was carried out prior to $NaBH_4$ reduction. It should be noted that reduction by $NaBH_4$ at 4° followed by dialysis against sodium phosphate buffer has no effect on the activity of native glutamic dehydrogenase.

The spectrum of the reduced PPal-inactivated glutamic dehydrogenase (pyridoxyl–enzyme) is shown in Figure 5. The second absorption maximum in the region of 325 mμ which is absent in the spectrum of the native enzyme is characteristic of pyridoxamine derivatives. Using a molar extinction coefficient of 9710 for ε-pyridoxyllysine (Fischer *et al.*, 1963) and assuming that the pyridoxyl–enzyme should exhibit a comparable absorption, one can calculate from Figure 5 that approximately 4–5 moles of PPal have combined with the enzyme per 250,000 mol wt. The pyridoxyl–enzyme was also studied by means of fluorescence spectroscopy and it was found that excitation of the pyridoxyl–enzyme at 325 mμ resulted in one major emission band at 390 mμ, which is characteristic of pyridoxamine 5′-phosphate residues (Churchich, 1965b). The fluorescence spectrum of the pyridoxyl–enzyme differs completely from that of the native enzyme when excited at 280 mμ. While the native enzyme exhibits a maximum emission at 331–333 mμ, the pyridoxyl–enzyme is characterized by two fluorescence bands at 333 mμ and 390 mμ, respectively (Figure 6). The fluorescence band at 390 mμ presumably arises through energy transfer from the aromatic residues to the bound pyridoxyl groups (Churchich, 1965a), and this interpretation is consistent with the finding that the presence

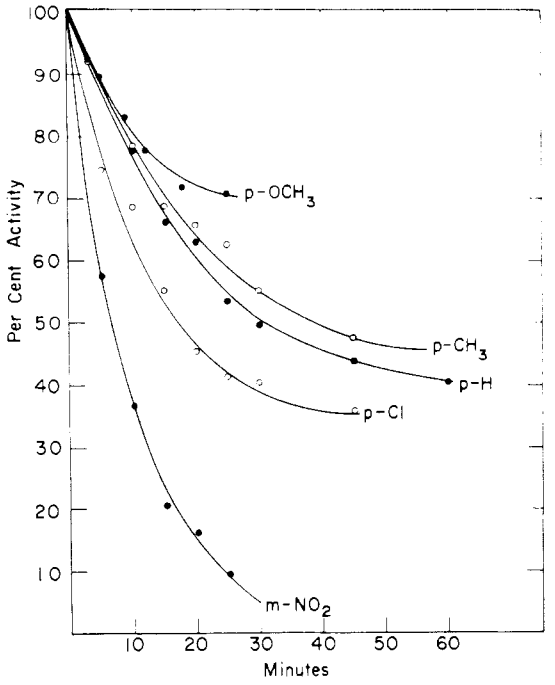

FIGURE 7: Inactivation of glutamic dehydrogenase by substituted benzaldehydes. Incubation of glutamic dehydrogenase with substituted benzaldehydes was carried out in 0.05 M sodium phosphate buffer, pH 7.2, at 34°. The concentration of benzaldehydes used was 1.25×10^{-3} M in all cases.

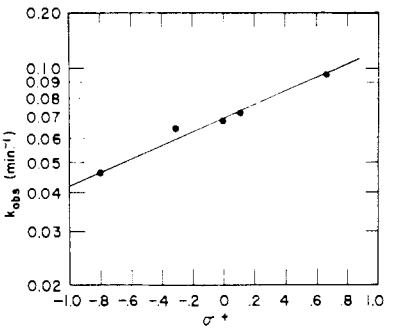

FIGURE 8: Substituent effects on glutamic dehydrogenase inactivation by substituted benzaldehydes.

of pyridoxyl groups causes a dramatic quenching of the native enzyme fluorescence (Figure 6).

Attempts were made to further characterize the nature of the pyridoxyl–enzyme derivative. Crystalline glutamic dehydrogenase (10 mg) was incubated at 34° in 0.05 M sodium phosphate buffer, pH 7.2, with PPal at a PPal:GDH of 80. After 30 min of incubation, the pH of the mixture was adjusted to 4.2 with 4.5 N acetic acid and the inactivated enzyme was reduced by the addition of approximately 5 mg of $NaBH_4$ according to the procedure of Dempsey and Christensen (1962). The mixture was dialyzed 4 hr against three changes of 0.05 M acetic acid, pH 4.2, and then 24 hr against two changes of distilled water. The dialyzed pyridoxyl–enzyme was then hydrolyzed 18 hr in 6 N HCl at 105° in a sealed vial. The hydrolysate was evaporated to dryness, redissolved in a small amount of distilled water, and spotted on Whatman 3MM chromatography paper. Ascending chromatography in the dark was used for development with a solvent mixture composed of water–methanol–ethanol–benzene–pyridine–dioxane, 25:25:10:10:10:10 (Dempsey and Christensen, 1962). For ease of comparison, samples of ϵ pyridoxyllysine were spotted on either side of the hydrolysate spot. After development, a diffuse fluorescent spot was located in the region immediately adjacent to the pyridoxyllysine spots (R_F 0.35). The sample spot was eluted from the paper with water, concentrated, and rechromatographed on Whatman 3MM paper with known pyridoxyllysine samples. Ascending chromatography in the dark was again used for development with a solvent mixture composed of 1-butanol–pyridine–acetic acid–water, 30:20:6:24 (Fischer *et al.*, 1963). After development, a well-defined fluorescent spot was located having an R_F of 0.22. The R_F for pyridoxyllysine under these conditions was 0.20.

The specificity of the reaction between PPal and native glutamic dehydrogenase which leads to inactivation of the enzyme was examined by studying the effects of substituted benzaldehydes on the activity of the enzyme during a preincubation period. All five substituted benzaldehydes studied were shown to inactivate the enzyme (Figure 7). The rate of inactivation as well as the extent of inactivation was similarly related to the nature of the substituent groups of the benzaldehydes employed. Pseudo-first-order rate constants were calculated for the inactivation of the enzyme by the various benzaldehydes. A plot of the logarithm of the rate constants against σ^+-substituent constants (Okamoto and Brown, 1958) gives a straight line with a ρ^+ value of 0.21 (Figure 8).

It was of interest to investigate the possibility that sulfhydryl groups were altered during the inactivation of glutamic dehydrogenase by PPal. The number of sulfhydryl groups of the native enzyme and the pyridoxyl–enzyme were determined by amperometric titration with $AgNO_3$ as described by Grisolia *et al.* (1962). In agreement with these authors, the native enzyme was found to contain 8 moles of sulfhydryl groups/250,000 mol wt. Under these same conditions, the pyridoxyl–enzyme was found to contain an average of 8.5 moles of sulfhydryl groups/250,000 mol wt. This latter value is comparable, within the experimental error of the method, to that obtained with the native enzyme.

Discussion

Bovine liver glutamic dehydrogenase has been demonstrated to be inactivated by incubation with a variety of aldehydes. Of special interest is the inactiva-

tion of the enzyme by the coenzyme, PPal. Incubation of glutamic dehydrogenase at 34° with PPal causes a time-dependent loss in catalytic activity (Figure 1). This inactivation is irreversible in that dilution of the enzyme–inhibitor mixture for the purpose of rate measurement does not result in the reversal of the inactivation process. Inactivation does not therefore arise from the formation of a freely dissociable enzyme–inhibitor complex. The restoration of activity that can be accomplished at this point by dialysis would indicate a more covalentlike interaction between the enzyme and PPal. The loss of glutamic dehydrogenase activity of the enzyme was accompanied by a concomitant loss in the alanine dehydrogenase activity of the enzyme (Figure 2). In this respect, inactivation by PPal differs from the inhibition of glutamic dehydrogenase by many so-called effector molecules since the latter case is normally accompanied by an enhancement of the alanine dehydrogenase activity of the enzyme (Bitensky *et al.*, 1965b).

The degree of inactivation of glutamic dehydrogenase by PPal during a given period of incubation increases as the ratio of PPal to the enzyme increases (Figure 1). Considerable inhibition of the enzyme was observed at very low concentrations of PPal. The inhibition of both glutamic dehydrogenase and alanine dehydrogenase activity of the enzyme is accompanied by a profound effect on the ability of the enzyme to aggregate. A 20-min incubation of the enzyme with PPal at a PPal:GDH ratio of 50 results in a 90% inactivation of the enzyme and the prevention of aggregation at higher protein concentrations (Figure 3). Over the protein concentration range where aggregation of native glutamic dehydrogenase occurs, the sedimentation coefficient of the PPal-inactivated enzyme remains essentially constant at approximately 12.5, the sedimentation coefficient normally attributed to the catalytically active monomeric unit of the enzyme. Therefore, PPal interferes with the polymerization process responsible for the formation of higher molecular weight material from the monomeric form but does not promote further dissociation of the monomeric form to smaller molecular weight subunits.

The nature of the interaction between PPal and glutamic dehydrogenase is an interesting question since the formation of the derivative simultaneously alters several properties of the enzyme. The interaction of PPal with many enzymes has been demonstrated to involve the formation of a Schiff base between the coenzyme and an amino group of the protein, in most cases an ϵ-amino group of a lysine residue. Fischer (1964) has recently reviewed the procedures used in the stabilization of protein Schiff bases through the $NaBH_4$ reduction technique. The spectral changes observed on the addition of glutamic dehydrogenase to PPal (Figure 4) are consistent with those observed on the formation of PPal Schiff bases (Blakely, 1955; Matsuo, 1957). Although more extensive, similar spectral changes were observed on the addition of polylysine to PPal. The fact that larger changes were not observed in the interaction of glutamic dehydrogenase with PPal would indicate that relatively few PPal molecules are needed to inactivate the enzyme. Studies on the $NaBH_4$ reduction of the pyridoxal-inactivated enzyme affords supporting evidence for a primary Schiff base formation in the inactivation process. After $NaBH_4$ reduction of the pyridoxal-inactivated enzyme, dialysis of the enzyme derivative formed (pyridoxyl–enzyme) does not result in the restoration of catalytic activity. The spectrum of the dialyzed pyridoxyl–enzyme (Figure 5) is characteristic of pyridoxamine derivatives (Snell, 1963). The fluorescence emission band at 390 mμ observed when the pyridoxyl–enzyme is excited at 325–327 mμ is likewise characteristic of pyridoxamine derivatives (Churchich, 1965b). Sedimentation studies of the catalytically inactive pyridoxyl–enzyme demonstrated it to have sedimentation properties identical with those of the pyridoxal-inactivated enzyme (before reduction) (Figure 3). As in the case of the pyridoxal-inactivated enzyme, no tendency to aggregate was observed at higher concentrations indicating that a monomeric unit is still involved. $NaBH_4$ reduction of native glutamic dehydrogenase has no significant effect on the ability of the enzyme to aggregate at high concentrations. From the 325-mμ maximum of the absorption spectrum of the pyridoxyl–enzyme produced at a PPal:GDH ratio of 50, it was calculated that 4–5 moles of PPal was bound/250,000 mol wt of the enzyme. This is an estimated value based on a molar extinction coefficient of 9710 for ϵ-pyridoxyllysine (Fischer *et al.*, 1963) and the assumption that the pyridoxyl–enzyme should exhibit an absorption comparable to that of free pyridoxyllysine.

After acid hydrolysis of the pyridoxyl–enzyme, pyridoxyllysine was identified by paper chromatography of the hydrolysate in two different solvent systems. It is possible that smaller amounts of other pyridoxyl derivatives may be present in the acid hydrolysate; however, pyridoxyllysine appears to account for the major part of the fluorescent material obtained.

The sulfhydryl groups of glutamic dehydrogenase appear not to be affected by the reaction of the enzyme with PPal since the same number of sulfhydryl groups were determined by amperometric titration of the native and the pyridoxyl–enzyme. However, an involvement of sulfhydryl groups would not be expected on the basis of the manner in which the properties of the enzyme are affected, since Bitensky *et al.* (1965a) demonstrated that mercurials enhance the glutamic dehydrogenase activity and inhibit the alanine dehydrogenase activity of the enzyme.

The inactivation of glutamic dehydrogenase with PPal appears then to result from an interaction of PPal with amino groups of the enzyme. Frieden (1965) recently reported alteration of sedimentation properties of this enzyme by acetylation; however, catalytic activity was not affected by this process. Presumably, simple alteration of amino groups would not then explain the more extensive changes in properties of the enzyme observed in interactions with PPal. The pyridoxyl group would represent a much larger blocking group than the acetyl group and would be negatively

charged. These properties could be responsible for the effect observed on catalytic activity through direct interaction with catalytically important functional groups of the enzyme or through the promotion of an unfavorable conformational change.

Inactivation of glutamic dehydrogenase occurs at relatively low concentrations of PPal (Figure 1). Pyridoxal likewise inactivates glutamic dehydrogenase (Figure 1); however, much higher concentrations of the unphosphorylated compound are required. One could suggest that the phosphate group of PPal confers a greater specificity on the interaction of this compound with the enzyme; however, it has been pointed out by Cordes and Jencks (1962a) that the existence of pyridoxal in the unreactive internal hemiacetal form could help explain the greater reactivity of PPal with nucleophilic reagents. PPal was demonstrated to be approximately 16 times more effective than pyridoxal in the inactivation of glutamic dehydrogenase. Very similar relative reactivities were observed in the reactions of these compounds with semicarbazide (Cordes and Jencks, 1962a).

Other aldehydes, such as substituted benzaldehydes, will also inactivate glutamic dehydrogenase (Figure 7) although in most cases these compounds are even less reactive than pyridoxal. Substituent effects on the reaction of benzaldehydes with the enzyme are shown in Figure 8. The ρ^+ value of 0.21 compares favorably with the ρ^+ value of 0.39 obtained in the studies of the condensation of aniline with substituted benzaldehydes (Cordes and Jencks, 1962b).

The importance of the PPal inactivation of glutamic dehydrogenase to the normal functioning of the enzyme is not immediately obvious. Many compounds, such as purine nucleotides, pyridine nucleotides, and nonpolar inhibitors, in addition to affecting the catalytic activities and sedimentation properties of the isolated enzyme, have also been demonstrated to affect the oxidation of glutamic acid in intact mitochondria (Kielley, 1957; Quagliariello *et al.*, 1965a,b; Balazs, 1965; Christian and Anderson, 1965). In several of these cases, the importance of the effects observed were discussed relative to control mechanisms of the mitochondrial dehydrogenase. In this respect, it is of interest that the mitochondrial oxidation of glutamic acid can occur through a PPal-dependent transaminase pathway as well as the glutamic dehydrogenase catalyzed reaction (Muller and Leuthardt, 1950; Krebs and Bellamy, 1960; Borst and Slater, 1961; Jones and Gutfreund, 1961; Borst, 1962; Balazs, 1965; Quagliariello *et al.*, 1965a,b). The diverse effects of PPal on these enzymes that are related through the utilization of a common substrate raises interesting questions concerning the role of PPal in mitochondrial processes.

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Glutamic–Aspartic Transaminase. XI. Reactivity toward Thiosemicarbazide*

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ABSTRACT: The second-order rate constants for the reaction of thiosemicarbazide with the phosphopyridoxal form of the pig heart extramitochondrial glutamic–aspartic transaminase were compared with those for its glutarate complex. The greater reactivity of the acidic form of the enzyme was confirmed. Glutarate prevented this increase in reactivity as the pH was lowered. Increasing the concentration of glutarate, at pH 7.9, reduces the rates of both combination and dissociation to the same extent so that the thiosemicarbazide dissociation constant is unaffected. These studies suggest the possibility that there is an obligatory sequence in the formation and dissolution of enzyme–substrate multiple ligands.

The extramitochondrial glutamic–aspartic transaminase (EC 2.6.1.1.) of pig heart is a pH indicator in the visible range due to the fact that it contains, as a chromophore, firmly bound pyridoxal phosphate (Jenkins *et al.*, 1959b). This phosphopyridoxal form may be readily shown to react not only with protons but also with both carbonyl reagents (Jenkins *et al.*, 1959a; Sizer and Jenkins, 1962) and certain dicarboxylic acids (Jenkins *et al.*, 1959b). The derivatives formed with dicarboxylic acids and carbonyl reagents are also pH indicators (Sizer and Jenkins, 1963).

The reaction with glutaric acid has recently been reinvestigated in some detail to find out how the carboxyl groups were reacting with the protein. Since the glutarate dissociation constant increased markedly with increasing pH, it was concluded that at high pH values only one carboxyl group is bound whereas both are bound at low pH values. It was found that the carboxyl group which binds at both high and low pH values does so by displacing a buffer anion from a positive site on the protein ($K_{eq} \cong 1$). The other carboxyl group binds to the acidic form of the pH-indicating chromophore.

In preliminary studies it was noted that, among carbonyl reagents, thiosemicarbazide ($NH_2CSNHNH_2$) was experimentally advantageous because it reacts slowly, absorbs only at wavelengths shorter than 310 mμ, is stable, and has pK values (1.5 and 10.3) out of the range of investigation (Sizer and Jenkins, 1963). In a series of imidazole–chloride buffers it was found that there was an increased affinity of the enzyme for the carbonyl reagent at low pH values. This was due solely to a greater reactivity of the acidic form of the enzyme, not to a faster rate of dissociation of thiosemicarbazide from the complex.

Unfortunately, these studies were made with an enzyme preparation now known to be heterogeneous by the criteria of starch gel electrophoresis and CM-Sephadex chromatography. We have, however, confirmed the earlier work with a single isozyme and compared the reactivities of the enzyme with its glutarate complexes. It was hoped that such an investigation would throw light on a possible function of one of the substrate carboxyl groups in the initial formation of a Schiff base between the substrate amino group and the bound pyridoxal phosphate.

Materials and Methods

Glutaric acid was prepared from Matheson glutaric anhydride by three recrystallizations from water. Thiosemicarbazide was from Eastman. Solutions which had been kept at 4° for 2 months were found to give results identical with those obtained with fresh solutions. Tris and pyrophosphate buffers were prepared from Tris base and sodium pyrophosphate by

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