methods, the hazards imposed by the acrylamide monomer are nonexistent, and its physical properties lend themselves to large size RNA separations.

As seen from the linear plots of mobility *vs.* log molecular weight (Figure 3) the molecular weight of an unknown RNA species can be estimated from its mobility if it is analytically coelectrophoresed with appropriate standards. This agarose gel system can be used on both the preparative and analytical scale with equal ease and resolution.

Acknowledgments

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Mechanism of Inactivation of L-Glutamate Dehydrogenase by Pyridoxal and Pyridoxal Phosphate†

Allister Brown, Johanna M. Culver, and Harvey F. Fisher*

ABSTRACT: We have studied the inactivation of glutamate dehydrogenase by pyridoxal and pyridoxal phosphate. Kinetic measurements on the sodium borohydride reduced adduct of pyridoxal phosphate and glutamate dehydrogenase show that the inactivation is reflected in the V_{max} parameter and not in the K_m parameter, indicating that the residual activity is due to unmodified enzyme. Ligand binding studies have established that allosteric activators form complexes with both the pyridoxal and pyridoxal phosphate modified enzymes, the stability and spectral properties of which closely resemble those of the native enzyme. In contrast, we found no evidence of complex formation of the pyridoxal phosphate modified enzyme with substrate acids or coenzymes. The pyridoxal modified enzyme did form a stable complex with NADPH which resembled that formed with the native enzyme. Formation of ternary complexes between the pyridoxal modified enzyme, NADPH, and the substrate acids was not observed, however, nor were complexes with glutamic and α-ketoglutaric acids detected with this protein. We conclude that the loss of catalytic activity which parallels the incorporation of pyridoxal (phosphate) into glutamate dehydrogenase occurs because the modified enzymes are unable to form complexes with the substrate acids.

Several group specific reagents have been applied to the modification of glutamate dehydrogenase (L-glutamic acid: NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3)). Pyridoxal phosphate (Anderson *et al.*, 1966) has been shown to be one of the most site-specific reagents (Piszkiewicz *et al.*, 1970; Moon *et al.*, 1972). Piszkiewicz *et al.* (1970) have shown that a single lysine residue per polypeptide reacts with pyridoxal phosphate, later established as *lys 126* in the revised amino acid sequence (Piszkiewicz *et al.*, 1970; Moon *et al.*, 1972).

Piszkiewicz and Smith (1971a) have shown that approximately 90% of the catalytic activity of glutamate dehydrogenase is reversibly lost when reacted with pyridoxal phosphate. The inactivation was attributed to imine formation between pyridoxal phosphate and the ϵ-amino group of *lysine 126*. The kinetics and equilibria of inactivation by pyridoxal and pyridoxal phosphate have also been investigated (Piszkiewicz and Smith, 1971a,b). The inactivation by these two aldehydes was shown to be consistent with imine formation with a group on the enzyme which has a $pK_{app} \approx$

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8.0. Reduced coenzyme was shown to competitively inhibit imine formation with pyridoxal phosphate (Piszkiewicz and Smith, 1971a) but it only decreased the rate of inactivation with pyridoxal (Piszkiewicz and Smith, 1971b). Goldin and Frieden (1972) have also studied the effects of the pyridoxal phosphate modification on some of the kinetic properties of the enzyme.

Several reasons for the change in activity often found to accompany modification by site-specific reagents may be postulated. In the case of the pyridoxal phosphate modification of glutamate dehydrogenase, Goldin and Frieden (1972) have postulated that *lys 126* is not essential for activity, implying that the modified protein is able to catalyze the reduction of coenzyme, but at a lower efficiency than the native enzyme. Piszkiewicz and Smith (1971a), on the other hand, have indicated that the residual activity resulted from a small concentration of unmodified enzyme catalyzing coenzyme oxidation or reduction at normal efficiency. We have supplemented kinetic studies with difference spectroscopy to study the ligand binding properties of glutamate dehydrogenase modified with either pyridoxal or pyridoxal phosphate. Cross and Fisher (1970) have shown that complex formation between glutamate dehydrogenase and many of the substrates and substrate analogs, as well as many activators and inhibitors, is accompanied by characteristic spectral perturbations. Many of the complexes detected by this technique have subsequently been shown to have spectra similar to those of intermediates observed during the oxidation or reduction of the coenzyme (Fisher *et al.*, 1970; Di Franco and Iwatsubo, 1971; Cross, 1972).

We report here our findings for the pyridoxal and pyridoxal phosphate modified proteins. The findings are shown to be consistent with the Cross and Fisher model (1970) for the subsites of glutamate dehydrogenase.

Experimental Section

Bovine liver glutamate dehydrogenase was obtained as an ammonium sulfate suspension from Sigma. The precipitate was dissolved in 0.1 M potassium phosphate buffer and dialyzed as described previously (Cross and Fisher, 1970). The nucleotides and coenzymes used in this study were purchased from Sigma and used without further purification. Pyridoxal hydrochloride and pyridoxal phosphate were purchased from Calbiochem. All other reagents were the highest quality available.

Pyridoxal Phosphate Modified Enzyme. To 20 ml of glutamate dehydrogenase (13.4 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.6) was added 20 mg of pyridoxal phosphate. The solution was allowed to stand at room temperature for 20 min in the dark. The solution was then cooled to 0° and sodium borohydride was added until the yellow color of excess pyridoxal phosphate was barely detectable. After standing for 15 min the solution was colorless. Excess sodium borohydride was consumed by addition of 1 drop of a solution of pyridoxal phosphate, followed by immediate application of the solution to a column of Sephadex G-25. The protein was eluted in the void volume of the column, using 0.1 M potassium phosphate (pH 7.6) as eluent. The recovered protein, 3–5 mg/ml, was stored at 4° until it was used.

Pyridoxal Modified Enzyme. The procedure which was used for the pyridoxal phosphate modified enzyme was followed using 10 ml of glutamate dehydrogenase (16.58 mg/ml) and 100 mg of pyridoxal hydrochloride and a reaction time at room temperature of 45–50 min. The recovered protein, 3–5 mg/ml, was stored in 0.1 M potassium phosphate buffer at 4° until it was used.

Kinetic Measurements. All kinetic measurements were carried out in 0.1 M potassium phosphate buffer (pH 7.6) at 25°. The activities of the native and modified protein were compared directly, using the standard assay procedure described previously (Cross and Fisher, 1970). The concentrations of unmodified enzymes were determined using the absorbance at 280 nm measured on a Zeiss PMQII. The molar absorptivity for glutamate dehydrogenase is ϵ_{280} 0.97 (cm mg/ml)$^{-1}$.

The molar absorptivity for GDH(P-Pxy)[1] was determined as follows; 5.0 mg of glutamate dehydrogenase was modified with pyridoxal phosphate (pH 7.6) filtered through Sephadex G-25 and all protein containing fractions were pooled. The pooled fractions were diluted to 10.0 ml with 0.1 M potassium phosphate buffer (pH 7.6) and the absorbance at 280 and 325 nm was determined. The molar absorptivity for the enzyme modified with pyridoxal phosphate, GDH(P-Pxy), was calculated, ϵ_{280} 1.25 (cm mg/ml)$^{-1}$, and using the same procedure for GDH(Pxy), was found to be ϵ_{280} 1.20 (cm mg/ml)$^{-1}$. The absorbance maximum at 325 nm for the modified enzymes was sensitive to pH and thus care should be exercised if this absorbance is to be used to determine modified protein concentration.

Difference Spectra. All difference spectral measurements were made on solutions in 0.1 M potassium phosphate buffer at pH 7.6 and 20° unless noted otherwise. The arrangement of cuvettes has been described previously (Cross and Fisher, 1970). Difference spectra were measured using a Cary Model 14 spectrophotometer interfaced to a Varian 620i spectral system. The output from the photomultiplier tubes was digitized and averages for each 0.5 nm were stored. A minimum of three scans were collected and averaged to produce the final spectrum.

Results

Pyridoxal Phosphate Modification. In order to study the modified enzyme conveniently, it was necessary to stabilize the pyridoxal phosphate–enzyme linkage. This was accomplished by sodium borohydride reduction of the imine (Piszkiewicz *et al.*, 1970; Goldin and Frieden, 1972), which we find to have no effect on the residual activity.

We have investigated the kinetic parameters for the oxidative deamination and the reductive amination reactions catalyzed by glutamate dehydrogenase, and compared them in Table I with the same parameters for GDH(P-Pxy). The uncertainty in the parameters pertaining to the conversions catalyzed by GDH(P-Pxy) are significantly greater than for those of the native enzyme because the assay procedure for GDH(P-Pxy) requires much higher protein concentrations and there is a greater scatter in initial velocities. This is particularly important in determining the parameters for glutamic acid where very small changes in the slope of the reciprocal plot produce large changes in the K_m value.

It can readily be seen from Table I that the Michaelis constant, K_m, for each substrate and coenzyme remains unchanged by the pyridoxal phosphate modification. The in-

[1] Abbreviations used are: GDH(P-Pxy), glutamate dehydrogenase modified with pyridoxal phosphate and reduced with sodium borohydride; GDH(Pxy), glutamate dehydrogenase modified with pyridoxal and reduced with sodium borohydride; 3-AcPyrA, 3-acetylpyridine adenine dinucleotide.

TABLE I: Comparison of Some of the Properties of Native and Pyridoxal Phosphate Modified Glutamate Dehydrogenase.[a]

Ligand	Native		Modified	
	$10^6 K_m^{app}$ (M)	V_{max}	$10^6 K_m^{app}$ (M)	V_{max}
NADPH	23 ± 1.0	116 ± 3.0	34 ± 3.5	2.5 ± 0.2
NADP[b]	125 ± 30	1.6 ± 0.1	153 ± 30	0.11 ± 0.01
L-Glutamic acid	467 ± 50	7.8 ± 0.5	1070 ± 400	0.16 ± 0.05

[a] All kinetic constants reported in this table are obtained by a weighted least-squares fit to the data. [b] Assay performed on modified protein which had lost 90–95% of its original activity. The remaining data were obtained on protein which had lost greater than 95% activity.

activation of glutamate dehydrogenase is reflected in a large decrease in the V_{max} values obtained. Changes in the V_{max} parameter may simply indicate a decrease in the concentration of the catalytically active species. Thus, these data tend to support the conclusions of Piszkiewicz and Smith (1971a) that a fraction of the enzyme remains unmodified and able to catalyze the oxidation and reduction of coenzymes with normal efficiency.

Kinetic studies of the type described above provide limited information about the changes in active site structure brought about by the modifying reagent. Additional information can be gained in the study of the pyridoxal phosphate modified glutamate dehydrogenase by using difference spectroscopy. We have sought evidence for complex formation between GDH(P-Pxy) and many of the compounds which give characteristic spectral perturbations with the native enzyme. No spectral perturbation could be detected when NADPH was added to a solution of GDH(P-Pxy).[2] The addition of excess L-glutamic acid did not produce a spectral perturbation although ternary complex formation between these two ligands and native enzyme occurs with a positive cooperativity. The concentrations of coenzyme and L-glutamic acid used in these experiments are more than 100 times greater than is necessary to detect spectral perturbations using the native enzyme. If complex formation between NADPH and GDH(P-Pxy) occurs, such a complex does not exhibit the spectral properties observed for complex formation with native enzyme. The gel filtration method of Fairclough and Fruton (1966) has been applied to determine the extent of complex formation between NADPH and GDH(P-Pxy). Thus, when GDH(P-Pxy), 5–10% residual activity, was eluted through a column of Sephadex G-25 at 20°, using 300 μM NADPH as eluent, less than 10% of the protein was involved in complex formation. This method has recently been successfully applied to studying the complex formation between NADPH and glutamate dehydrogenase (D. G. Cross and H. F. Fisher, unpublished data). These results clearly indicate that GDH(P-Pxy) and NADPH do not form a stable complex under conditions where complex formation between coenzyme and native enzyme is readily detectable. If the complexes detected spectrally with glutamate dehydrogenase are indeed significant to the catalytic process then the inability of GDH(P-Pxy) to form a complex with NADPH will account for the loss of catalytic activity of this protein.[3]

[2] A very small perturbation of the spectrum was observed, and was attributed to the residual native enzyme. The origin of the perturbation is uncertain but the perturbation is so slight that structural features could not be distinguished at this enzyme concentration.

[3] However, Goldin and Frieden (1972) have stated that NADPH binds equally well to the native and pyridoxal phosphate modified enzyme.

In contrast to the findings of NADPH, complex formation between GDH(P-Pxy) and ADP, a known "allosteric" activator of glutamate dehydrogenase, was readily detected. In Figure 1 the difference spectra for ADP complex formation with the native and pyridoxal phosphate modified proteins are compared. It is obvious that the spectra are indistinguishable and, thus, ADP must form a very similar complex with the two proteins. The concentration dependence of the spectral perturbations for ADP complex formation with GDH(P-Pxy) was examined and fitted to eq 1, where ΔA and $\Delta\epsilon$

$$\Delta A = \Delta\epsilon \times \frac{K_D + [E]_T + [L]_T - ((K_D + [E]_T + [L]_T)^2 - 4[E]_T[L]_T)^{1/2}}{2} \quad (1)$$

are respectively the observed absorbance and molar absorptivity for the spectral perturbation, K_D is the dissociation constant, and $[E]_T$ and $[L]_T$ are the total concentrations of enzyme and added ligand. The results of a typical experiment are shown in Figure 2 where the points are experimental and the line is theoretical. The values for K_D and $\Delta\epsilon$ are in good agreement with those obtained previously for ADP with the native enzyme (Fisher *et al.*, 1972) and are listed in Table II.

Several analogs and homologs of ADP were also examined for evidence of complex formation with the modified protein. The dissociation constant and molar absorptivity for the peak to trough absorbance change ($\epsilon_{283} - \epsilon_{254}$) for each analog examined is presented in Table II. In each case the difference spectra had the same shape as those observed for ADP; the

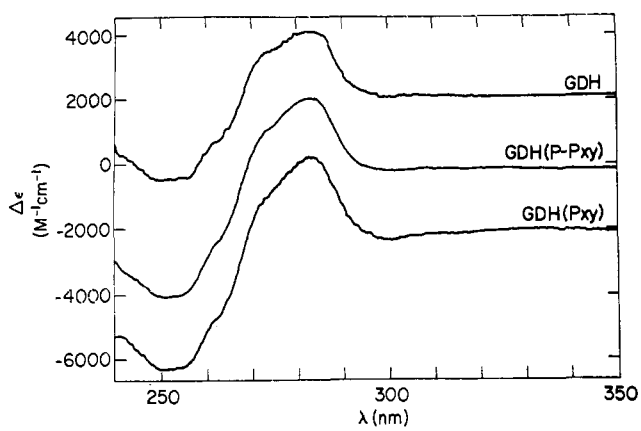

FIGURE 1: The difference spectra observed when ADP was incubated with glutamate dehydrogenase, GDH, pyridoxal phosphate modified glutamate dehydrogenase, GDH(P-Pxy), and pyridoxal modified glutamate dehydrogenase, GDH(Pxy).

TABLE II: Dissociation Constants and Molar Absorptivities for Complex Formation with L-Glutamate Dehydrogenase, GDH-(P-Pxy), GDH(Pxy).

Ligands	GDH		GDH(P-Pxy)		GDH(Pxy)	
	$10^6 K_D$ (M)	$\Delta\epsilon$ (M^{-1} cm^{-1})	$10^6 K_D$ (M)	$\Delta\epsilon$ (M^{-1} cm^{-1})	$10^6 K_D$ (M)	$\Delta\epsilon$ (M^{-1} cm^{-1})
ADP	3.6	5560[a]	3.37 ± 0.05	5980	2.31	6300
AMP	21.7	5900	13.8 ± 2.8	4050		
ADP-ribose	20.6[f]	4400	31.8	3460		
2′-Deoxy-ADP			15.8	5400		
NADH	65.4[f]	2890	41.4	3325		
3-AcPyrA			42.2	1510		
Leucine	270[b]	4800[b]	232 ± 4	3220		
α-Ketoglutaric acid	2800 ± 400[c]	800[c]	ND[d]		ND[d]	
NADPH	14.9[e]	2410[f]	ND		29.2 ± 0.2	3420
NADPH–Glu[g]	0.6 ± 0.1[h]	2410[e]	ND		16.0	1970
NADPH–GTP[g]	0.18[j]	2410[e]	ND		14.0	2590
NADPH–α-ketoglutaric acid[g]	0.5 ± 0.1[h]	2790	ND		ND	
$NADP^+$–α-ketoglutaric acid[i]	11.0 ± 5.3[c]	4800[c]	ND		ND	

[a] See Fisher *et al.* (1972). [b] See Prough *et al.* (1972). [c] See Cross *et al.* (1972). [d] ND = not detected. [e] D. G. Cross and H. F. Fisher, unpublished results. [f] See Cross and Fisher (1970). [g] The dissociation constants refer to an observed dissociation constant for NADPH at a specific concentration of second ligand. [h] See Cross (1972). [i] The dissociation constant refers to dissociation of α-ketoglutaric acid from the ternary complex. [j] Based on cooperativity of 85 for GTP inhibition.

differential molar absorptivities were smaller. Perhaps the most interesting ligands in this group are NADH and its 3-acetyl analog, reduced 3-acetylpyridine adenine dinucleotide, 3-AcPyrA. Both of these compounds formed stable complexes with GDH(P-Pxy), but the characteristic perturbation of the reduced nicotinamide chromophore near 340 nm observed on complex formation with native enzyme was not detected. The difference spectra which were obtained were identical with the ADP difference spectrum (Figure 3).

Several carboxylic and amino acids form stable complexes with glutamate dehydrogenase. Complex formation is accompanied by specific perturbations of certain of the chromophores on the enzyme surface (Cross *et al.*, 1972; Prough *et al.*, 1972; Prough and Fisher, 1972). In this study we have looked for these perturbations when GDH(P-Pxy) was incubated with leucine. At concentrations where leucine behaves as an activator and not as a substrate (Prough *et al.*, 1972; Prough and Fisher, 1972), the spectrum shown in Figure 4 was obtained. This difference spectrum was identical with that observed by Prough *et al.* (1972) for the same complex with glutamate dehydrogenase. The dissociation constant and molar absorptivity for the complex are compared with those for the same complex with unmodified enzyme in Table II. It is clear that the complexes formed with the two proteins are very similar. In contrast, when GDH(P-Pxy) was incubated with α-ketoglutaric acid, spectral perturbation of the enzyme chromophores seen with the native enzyme could not be detected. Cross *et al.* (1972) have shown that addition of NADP to solutions of glutamate dehydrogenase and α-ketoglutaric acid results in a marked enhancement of signal intensity for the 292–287-nm perturbation as well as generating a new feature between 283 and 263 nm. The addition of NADP to a solution of GDH(P-Pxy) and α-ketoglutaric acid did not produce a change detectable by difference spectroscopy.

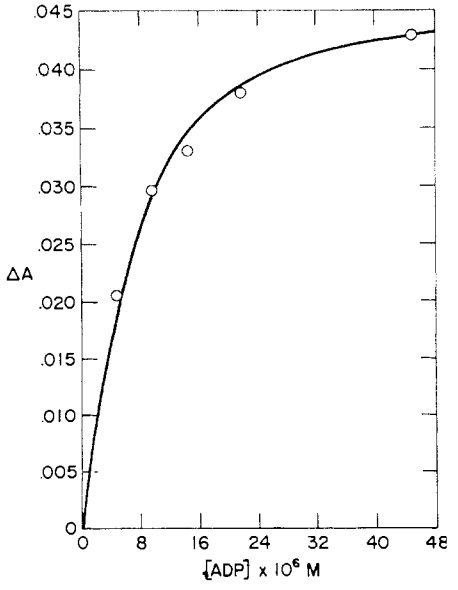

FIGURE 2: The concentration dependence of the spectral perturbation observed when ADP is incubated with pyridoxal phosphate modified glutamate dehydrogenase. The points are experimental and the curve is the theoretical fit of the data to eq 1.

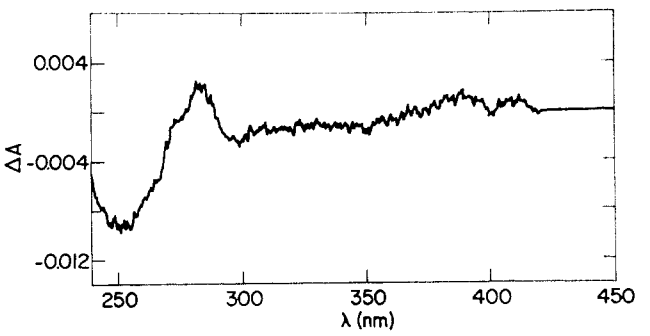

FIGURE 3: The difference spectrum observed when NADH was incubated with pyridoxal phosphate modified glutamate dehydrogenase.

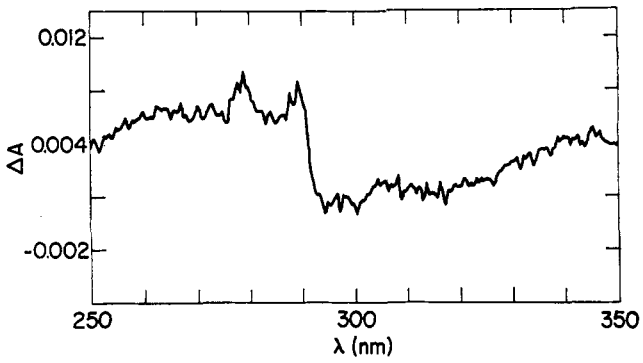

FIGURE 4: The difference spectrum observed when leucine is incubated with pyridoxal phosphate modified glutamate dehydrogenase.

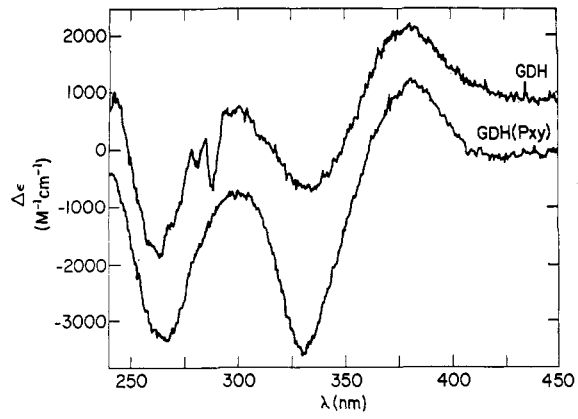

FIGURE 5: The difference spectrum obtained when NADPH is incubated with glutamate dehydrogenase, GDH, and pyridoxal modified glutamate dehydrogenase, GDH(Pxy).

Thus, it appears that α-ketoglutaric acid is unable to form either binary or ternary complexes with GDH(P-Pxy).

Pyridoxal Modified Enzyme. The reaction of pyridoxal with glutamate dehydrogenase is very similar to the reaction with pyridoxal phosphate (Piszkiewicz and Smith, 1971b). This led Piszkiewicz and Smith (1971b) to conclude that it is likely that the reaction with pyridoxal results in imine formation at *lys 126* (Piszkiewicz and Smith, 1971b). Those differences which were observed indicated that a comparison of the ligand binding properties of the two modified proteins would be instructive.

When we reacted glutamate dehydrogenase with pyridoxal using concentrations approximately ten times higher than described by Piszkiewicz and Smith (1971b), we obtained an enzyme which had lost approximately 90% of its catalytic ability. Reduction of the labile imine linkage did not alter this decreased catalytic activity. The sodium borohydride reduced product yielded the stable *N*-lysylpyridoxamine protein, GDH(Pxy),[1] which was used in the subsequent studies.

When ADP was mixed with GDH(Pxy) a difference spectrum shown in Figure 1 was obtained. The dissociation constant and molar absorptivities associated with this complex, presented in Table II, are similar to those obtained for native enzyme (Fisher *et al.*, 1972). Thus, none of the activator subsites for ADP are affected by the reaction of glutamate dehydrogenase with pyridoxal.

Incubation of GDH(Pxy) with NADPH gave rise to the difference spectrum in Figure 5. The main feature of the difference spectrum is the perturbation of the reduced nicotinamide absorption at 340 nm. The perturbation is characterized by a maximum at 380 nm, a minimum at 328 nm, and a slight hypochromicity of the absorption band. The minimum is shifted approximately 4 nm to shorter wavelength than in the corresponding complex with glutamate dehydrogenase and has a slightly lower minimum at 328 nm. In addition, the perturbation of enzyme chromophores, reported (Cross and Fisher, 1970) for the reduced coenzyme complex with native enzyme and characterized by a maximum at 293 nm and minimum at 287 nm, is not found in the difference spectrum for the GDH(Pxy)–NADPH complex. The dissociation constant and molar absorptivity for complex formation were determined using the nicotinamide perturbation signal and are compared with the values obtained for the native enzyme in Table II.

Several ternary complexes of glutamate dehydrogenase, NADPH, and a third ligand have been reported (Cross and Fisher, 1970; Cross, 1972; Di Franco and Iwatsubo, 1972). The dissociation constant for NADPH from these ternary complexes is often much smaller than for the binary complex, *i.e.*, a positive cooperativity is observed (Cross, 1972; Di Franco and Iwatsubo, 1972). The difference spectrum for the ternary complexes when either L-glutamic acid or guanosine triphosphate (GTP) is the third component is indistinguishable from that for the binary complex (Cross and Fisher, 1970; Cross, 1972). The difference spectrum obtained when either L-glutamic acid or GTP was added to GDH(Pxy) and NADPH was identical with the difference spectrum in Figure 5. However, as can be seen by the dissociation constants for NADPH (see Table II) determined in the presence of an excess of either L-glutamic acid or GTP, only marginal cooperativity can be claimed. The dissociation constant for NADPH is decreased only slightly in the presence of these additional compounds, indicating that if ternary complexes are formed with GDH(Pxy), these complexes are not the same highly cooperative complexes observed with the native enzyme (Cross and Fisher, 1970; Cross, 1972; Di Franco and Iwatsubo, 1972).

A third ternary complex of the native enzyme employs α-ketoglutaric acid as the additional component (Cross, 1972). In addition, α-ketoglutaric acid forms a binary complex with glutamate dehydrogenase (Cross *et al.*, 1972), as well as a ternary complex with NADP. Incubation of GDH(Pxy) with excess α-ketoglutaric acid or α-ketoglutaric acid in the presence of NADP produced no spectrally detectable changes. Furthermore, addition of NADPH to a solution of GDH(Pxy) and excess α-ketoglutaric acid gave rise to an absorbance centered at approximately 340 nm. After the initial loss of absorbance at 340 nm, due to the presence of contaminating ammonia (Cross, 1972), a difference spectrum could be obtained by subtracting the absorbance for the first addition of NADPH from the absorbance for subsequent additions. This difference spectrum was identical with the spectrum of Figure 5, *i.e.*, a shift in λ_{max} to longer wavelength. Cross (1972) has characterized the formation of the ternary complex of glutamate dehydrogenase, NADPH, and α-ketoglutaric acid as having λ_{max} at 332 nm, *i.e.*, a shift to shorter wavelength and a dissociation constant for NADPH of less than 10^{-6} M (see Table II). Although it was not possible to determine the dissociation constant accurately for the GDH(Pxy) system, it was possible to show that the dissociation constant for NADPH was greater than 15×10^{-6} M. These data clearly indicate that GDH(Pxy) does not form binary and ternary complexes with α-ketoglutaric acid, analogous to those re-

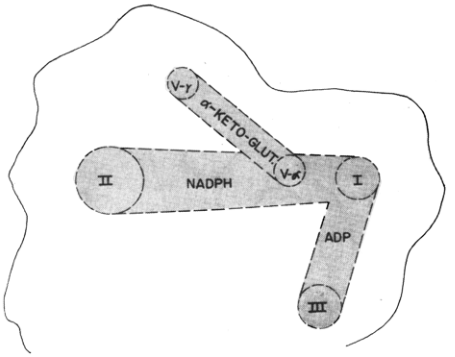

FIGURE 6: Schematic representation of the ligand binding subsite at the active site of glutamate dehydrogenase (from Cross and Fisher, 1970). The orientation and position of the ligand bindng sites do not necessarily correspond to the orientation on the enzyme surface. The diagram merely represents an interaction of the various ligands.

ported for native glutamate dehydrogenase (Cross, 1972; Di Franco and Iwatsubo, 1972).

Discussion

The data presented above for the pyridoxal phosphate modified enzyme indicate that this protein forms stable complexes with those species which act as activators for the unmodified enzyme, but does not form complexes with the substrate acids and coenzymes. It can be seen from the spectra in Figures 1, 3, and 4 and the data of Table II that the complexes of the activators with GDH(P-Pxy) are very similar to those with glutamate dehydrogenase. The coenzyme analog, NADH, is particularly interesting in this regard. The difference spectrum of Figure 3 indicates that NADH will bind to the enzyme surface at the ADP binding site, but not to the coenzyme binding site. Frieden was the first to suggest that NADH binds to the enzyme surface at two sites (Frieden, 1959), based on kinetic evidence and sedimentation data. Cross and Fisher (1970) observed that when NADH forms a complex with glutamate dehydrogenase a very complex difference spectrum is observed. In addition to the red shift of the nicotinamide absorption at 340 nm observed with NADPH, a red shift and hypochromicity of the adenine chromophore centered at 260 nm were observed. This perturbation was similar to that observed for ADP complex formation. Pantaloni and Dessen (1969) showed that the concentration dependence for the two perturbations differed, indicating complex formation at two separate sites. The difference spectrum obtained for the NADH–GDH(P-Pxy) complex is the first reported observation of NADH binding to the enzyme at a single site. In this regard it is noteworthy that Hucho *et al.* (1973) obtained a difference spectrum resembling that obtained for an ADP–glutamate dehydrogenase complex with NADH and the protein obtained by reaction of glutamate dehydrogenase and pyridoxal phosphate followed by intensive ultraviolet irradiation. These workers (Hucho *et al.*, 1973) concluded that NADH was unable to bind to the protein because the characteristic perturbation of the reduced nicotinamide chromophore was not detected.[4]

[4] The failure of these authors to recognize this second mode of complex formation with NADH has led to erroneous conclusions regarding the mode of coenzyme binding (Deppert *et al.*, 1973). We would like to emphasize that the binding of reduced coenzyme, as a catalyst only, should be studied using NADPH in order to avoid the confusion often encountered because of the dual mode of binding of NADH.

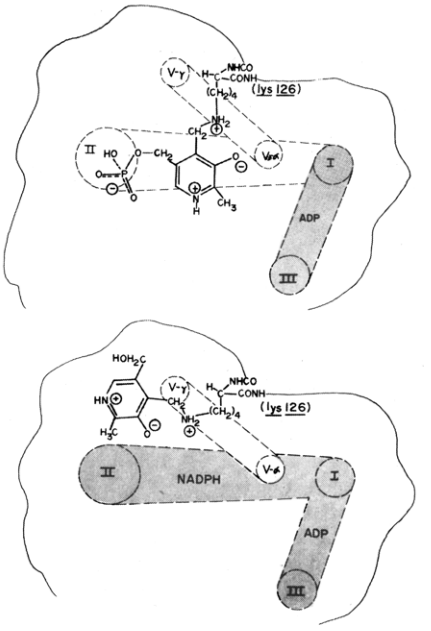

FIGURE 7: Schematic representation of the ligand binding sites at the active site of: (a) pyridoxal phosphate modified glutamate dehydrogenase, and (b) pyridoxal modified glutamate dehydrogenase.

The two-site binding of NADH to native glutamate dehydrogenase with a net negative cooperativity is analogous to the simultaneous binding of NADPH and ADP to the native enzyme (D. G. Cross and H. F. Fisher, unpublished data). Iwatsubo and Pantaloni (1967) have proposed that ADP activation of the steady state oxidation of glutamate is mediated by the increased rate of NADPH dissociation. Thus, it would appear that "self-activation" during substrate oxidation using NADH as coenzyme can be attributed to the ability of the reduced coenzyme to simultaneously form complexes at two distinct sites presumably with a negative cooperativity. The activation phenomenon would then occur in the same way as activation with ADP occurs, by increasing the rate of release of reduced coenzyme from the enzyme surface.

In contrast to the ability of GDH(P-Pxy) to bind ADP and monocarboxylic acid activators, we found no evidence for formation of stable complexes with the dicarboxylic acid substrates or with NADPH. This includes the ternary complexes between coenzyme and substrate which form with a positive cooperativity, and the ternary enzyme–NADPH–GTP complex.

The pyridoxal modified enzyme also forms a stable complex with ADP which has properties very similar to that of the native enzyme. In addition, a stable complex is formed with NADPH. This complex is very similar to the binary complex observed with the native enzyme except the minimum for the perturbation of the 340-nm absorbance occurs at shorter wavelength and is slightly more hypochromic. This may indicate a slight change in the microenvironment of the pyridoxamine substituent with an accompanying change in the absorbance centered at 325 nm. In addition, the tryptophan perturbation (287–292 nm) observed with the native enzyme has disappeared. The dissociation constant for the GDH-(Pxy)–NADPH complex is slightly larger than observed with the native enzyme. The higher dissociation constant may reflect the absence of the tryptophan perturbation, but we have no way to establish this at this time. We were unable to detect complex formation of GDH(Pxy) with the dicarboxylic acid substrates. Reduced coenzyme binding was observed in

the presence of excess α-ketoglutaric acid, glutamic acid, and excess GTP. However, the dissociation constants for these complexes indicated that only marginal cooperativity could be claimed for formation of ternary complexes. In addition, the blue-shifted, 340-nm absorbance band reported for the enzyme–NADPH–α-ketoglutaric acid (Cross, 1972) was not observed with GDH(Pxy), further supporting the conclusion that the modified enzymes are unable to form stable complexes with the dicarboxylic acid substrates.

A reasonably simple model which accounts for the differences observed for the pyridoxal and pyridoxal phosphate modified proteins can be proposed. This model will be constructed from the ligand binding site model proposed by Cross and Fisher (1970). Their model (Cross and Fisher, 1970) is a connectivity diagram which was derived to account for the large and complex set of facts which pertain to the mechanism of glutamate dehydrogenase catalysis. It is assumed by this model that all of the substrates under discussion may interact with the enzyme surface as bidentate ligands. The features which are pertinent to the present discussion are summarized below and illustrated schematically in Figure 6. The reduced coenzyme, NADPH, and allosteric activator, ADP, interact with the enzyme surface at three subsites, I, II, and III. The subsite common to both NADPH and ADP, subsite I, is the region which gives rise to the negative cooperativity when both ligands are bound simultaneously, *i.e.*, a net decrease in attractive forces occurs at this site. The ADP analogs bind to the enzyme surface at the same subsites as ADP, subsites I and III. Two subsites for the substrate carboxylate groups (Caughey *et al.*, 1957), V-α and V-γ, have also been postulated (Cross and Fisher, 1970). It should be possible to locate the subsites whose function is interfered with by reaction of glutamate dehydrogenase with pyridoxal or pyridoxal phosphate using this model and the data reported above.

Those compounds, which act as steady state activators for the reduction of NADP, form complexes with the modified and native enzymes with equal affinity. Thus, the subsites utilized in activator complex formation, subsites I and III, are not altered by modification with either pyridoxal or pyridoxal phosphate. Specific locations for interaction with the monocarboxylic amino acid activators has not been assigned but is thought to be near subsites I and III (R. A. Prough and H. F. Fisher, unpublished data). These sites are also unaltered by either modifier.

Neither of the modified proteins is able to form stable complexes with the substrate dicarboxylic acids. One very simple way to account for this requires that the pyridoxamine substituent occupy some portion of the substrate binding site, thereby preventing complex formation by steric exclusion (see Figure 7). Alternatively, *lys 126* may constitute a specific binding site for the substrates. Reaction with pyridoxal or pyridoxal phosphate at *lys 126* may alter the properties at that site sufficiently to prevent complex formation. A third, less likely possibility is that the pyridoxamine substituents prevent a conformational change from occurring, a process which may be necessary for substrate binding.

The modified proteins differ in their ability to form stable complexes with the reduced coenzyme, NADPH. The structures of the two modifiers differ only in the phosphate substituent on the C_5-methoxy group. The ability or inability of the two modified proteins to form a stable complex with NADPH must be related to this phosphate group. Thus, it appears that the pyridoxamine phosphate substituent must interact with the protein surface at two sites, *i.e.*, a bidentate binding (see Figure 7a). The bidentate binding restricts the conformations accessible to the pyridoxamine substituent. The resulting orientation of the pyridoxamine phosphate must prevent NADPH from interacting with the protein surface either by ligand exclusion or perhaps because the phosphate substituent is interacting at subsite II. The pyridoxamine substituent in GDH(Pxy) is not conformationally restricted by bidentate binding and can assume a conformation such that the NADPH binding site is accessible (see Figure 7b).

Many of the complexes which have been described above and which are detectable by difference spectroscopy have recently been shown to have the same properties as complexes detected during catalysis by the unmodified enzyme (Cross, 1972; Di Franco and Iwatsubo, 1972). The results obtained from difference spectral measurements on the modified enzymes can explain the loss of catalytic activity which parallels pyridoxal and pyridoxal phosphate incorporation. In any mechanism for the oxidation or reduction of coenzyme, enzyme complexes of the substrate acid are obligatory. Thus, the loss of catalytic activity occurs because the modified proteins are unable to form stable complexes with the substrate acid. In addition, GDH(P-Pxy) does not form a stable complex with NADPH and is inactivated for this reason also.

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