The Effect of Pyridoxal Phosphate Modification on the Catalytic and Regulatory Properties of Bovine Liver Glutamate Dehydrogenase*

BARRY R. GOLDIN AND CARL FRIEDEN
From the Department of Biological Chemistry, Washington University, St. Louis, Missouri 63110

SUMMARY

The kinetic properties of bovine liver glutamate dehydrogenase have been examined as a function of pyridoxal phosphate modification, this reagent having been previously shown to react specifically with lysine-97 of the tentative sequence proposed by Smith et al. (1970) Proc. Nat. Acad. Sci. U. S. A. 67, 724. It is found that partial modification (two to three groups per six chains in the active enzyme) leads to almost complete loss of excess NADH inhibition. Modification also leads to an increase in the inhibition constant for GTP as well as a progressive loss of activity. The nature of these changes suggest strong subunit-subunit interaction with respect to some of the regulatory properties of the enzyme. The altered inhibition constant for GTP is apparently a consequence of a change in the rate of a coenzyme-GTP-induced conformational change. Loss of activity after pyridoxal phosphate modification may not be complete since some residual activity always remains. Furthermore, modification has a slight activating effect on activity when the enzyme is assayed in the presence of excess GTP. It is concluded that, although lysine-97 is an important residue in the catalytic reaction, it may not be essential for activity.

Prior modification of lysine-428 with trinitrobenzenesulfonate, which also results in loss of excess NADH inhibition, does not affect the rate or extent of pyridoxal phosphate modification.

EXPERIMENTAL PROCEDURE

Materials—Bovine liver glutamate dehydrogenase was obtained as a crystalline suspension in ammonium sulfate from Sigma Chemical Co. The crystals were centrifuged and dissolved in 0.05 M potassium phosphate buffer at pH 8.0 and recentrifuged to remove any undissolved protein. The enzyme solution was then subjected to gel filtration on a Sephadex G-25 column equilibrated with the phosphate buffer. The resulting enzyme solution, which was free of ammonium sulfate, was stable to freezing.

Chemical modification has been extensively used as a tool to study the relation of specific amino acid residues to the kinetic and molecular properties of enzymes (1-3). In previous papers from this laboratory, it was shown that modification of specific lysyl residues of bovine liver glutamate dehydrogenase by acetic anhydride (4) or trinitrobenzenesulfonate (5, 6) leads to the alteration of such properties. Rather thorough studies with trinitrobenzenesulfonate (TNBS)† have shown that this reagent reacts most rapidly with lysine-428 of the tentative sequence proposed by Smith et al. (7). Reaction occurs with only 3 of the 6 possible residues at this position within the monomeric protein (composed of six identical subunits) and leads to changes in some of the kinetic and molecular characteristics. Thus, the trinitrophenylated enzyme retains full catalytic activity, but inhibition by excess NADH is lost and the modified enzyme is unable to polymerize (5, 6).

Several years ago, Anderson et al. (8) observed that pyridoxal phosphate and other substituted benzaldehydes reacted with a lysyl residue of the enzyme (one per subunit) and that such modification led to a loss in activity as well as depolymerization of the enzyme. Subsequently, Smith et al. (7) and Piszkiewicz et al. (9) found that this reaction was specific, that the particular residue reacted was lysine-97 and that this group has an apparent pK value of about 8 (10). There appears to be little or no overlap in the reactivities of the different lysyl residues in that TNBS does not react with lysine-97 (5) and pyridoxal phosphate does not react with lysine-428 (9).

In the present paper, we examine in greater detail the consequences of the pyridoxal phosphate modification. As with TNBS, it appears that only partial modification of a specific lysyl residue leads to complete loss of excess NADH inhibition. On the other hand, there are distinct differences between the enzyme modified with TNBS compared to that modified with pyridoxal phosphate. Furthermore, pyridoxal phosphate modification appears to proceed normally when the enzyme has been previously modified with TNBS.

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Coenzymes, purine nucleotides, and the pyridoxal phosphate were obtained from Sigma Chemical Co. These compounds were used without further purification. Stock solutions of pyridoxal phosphate were prepared immediately before use.

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† The abbreviation used is: TNBS, trinitrobenzenesulfonate.
Procedure for Pyridoxal Phosphate Modification—Pyridoxal phosphate was incubated with glutamate dehydrogenase in 0.05 M phosphate buffer at pH 8.0 at 25° except as noted. Further details regarding concentrations and temperatures are given in the figure legends. When determining the number of residues modified, the reaction was stopped by the addition of 0.05 ml of sodium borohydride (1.5 mg per ml) per ml of reaction mixture. The solution was then dialyzed extensively and the number of groups reacted calculated from the absorbance at 325 nm with a molar extinction coefficient of 9,700 (8). Protein concentrations were determined both by absorbance at 280 nm and the micro-biuret method (11). The number of groups modified was based on the polypeptide chain molecular weight of 56,100 (7). After a calibration curve had been obtained (see Fig. 1), the number of groups modified was based on the specific activity of the enzyme with NADPH as coenzyme. Modification at different temperatures does not appear to affect the results.

Kinetic Studies—Unless otherwise noted all assays were performed in 0.01 M Tris-acetate (0.01 M with respect to acetate) containing 10 μM EDTA at pH 8.0 and 25°. The α-ketoglutarate and NH₄Cl concentrations were 5 × 10⁻³ and 5 × 10⁻² M, respectively. Cells of 1-cm path length were used for measuring NADH and NADPH oxidation at low coenzyme concentration. Shorter light path cells (0.2 cm) were used when the reduced coenzyme concentration was above 200 μM. Initial velocities were measured spectrophotometrically at 340 nm with an expanded scale recorder (0 to 0.1 absorbance full scale). When kinetic studies were to be performed on modified enzyme (Figs. 1 to 5), the pyridoxal phosphate was reduced onto the enzyme with sodium borohydride (see above) at various time intervals after initiation of the reaction. The reaction mixture was then diluted into cold 0.1 M Tris-acetate buffer, pH 7.15, containing 1 mM phosphate. Enzyme from this dilution was then diluted further into the assay mixture.

When the rate of activity loss in the presence of ligands was compared to that in the absence of ligands (see Fig. 6), the modification reaction was stopped by dilution into cold 0.1 M Tris-acetate buffer, pH 7.15, containing 1 mM phosphate, and the activity measured by using an aliquot of the diluted solution. Under these conditions, the activity of the diluted enzyme is stable over several hours indicating that the modification has ceased and that there is not an appreciable dissociation of the pyridoxal phosphate from the enzyme.

The presence of coenzymes or nucleotides in the modification reaction mixture did not affect initial velocity measurements after dilution into the assay mixture because their final concentrations were too low.

Stopped Flow Experiments—Stopped flow experiments were performed with a Durrum stopped flow spectrophotometer (2-cm light path) under conditions described in the legend to Fig. 5.

RESULTS

Enzymatic Activity as Function of Extent of Modification

Anderson et al. (8) and Piszkiewicz et al. (9) have shown that with NAD as coenzyme, enzymatic activity is decreased by at least 90% when approximately 1 lysyl residue in each polypeptide chain of glutamate dehydrogenase is modified with pyridoxal phosphate. Our experiments confirm this result regardless of the assay method, but we find that the shape of the activity curve as a function of the extent of modification is dependent on which coenzyme is used. Fig. 1 shows that the activity decreases linearly as a function of the number of pyridoxal phosphate groups incorporated per polypeptide chain with NADPH as coenzyme in the assay. The same curve is obtained regardless of the NADPH concentration in the assay.

Using these results, it was possible, in subsequent experiments, to determine the number of groups modified on the basis of enzymatic activity by assaying with NADPH as coenzyme. This procedure was used for the remaining data presented here except as noted.

It was observed, during the course of these experiments, that enzymatic activity by pyridoxal phosphate modification was never complete even under conditions where total loss might have been expected. For example, with 500 μM pyridoxal phosphate, a pseudo-first order rate constant of 0.11 min⁻¹ may be calculated from the data between 100 and 15% activity (see Fig. 6). Accordingly one would expect that the activity would fall below 1% after 40 min. However, after 180 min, approximately 10% of the activity still remained. Further inactivation may be obtained by reacting this enzyme with sodium borohydride, removing the excess sodium borohydride by dialysis and readdition of high levels of pyridoxal phosphate. It is found that over two lysine groups are modified per polypeptide chain, but even under these conditions, some enzymatic activity (~5%) is still present. These results suggest that pyridoxal phosphate modification of the rapidly reacting lysyl residue does not lead to complete loss of enzymatic activity. This conclusion is also
Fig. 2. Glutamate dehydrogenase activity as a function of number of groups modified with pyridoxal phosphate assayed with NADH as coenzyme. Glutamate dehydrogenase (1 mg per ml) was reacted with 1.2 mM pyridoxal phosphate in 0.05 M potassium phosphate buffer, pH 8.0 at 6°C. The reaction was stopped by the addition of 0.05 ml of sodium borohydride (1.5 mg per ml) per ml of reaction mixture. Aliquots were diluted 100-fold into Tris-acetate buffer, pH 7.15 and 0°C. The activity was measured with either 100 μM or 1 mM NADH. The number of groups was determined by assaying the same aliquots with NADPH and correlating activity with groups according to the data of Fig. 1. The explanation of the dashed line is given in the text.

implied by the data of Fig. 1 which indicate some residual activity after extrapolation of the points to one group modified per subunit, although the extrapolation may be uncertain because of some nonspecificity in the modification reaction or a slight error in the extinction coefficient of the pyridoxyl enzyme (8). We will return to this point in the “Discussion” in conjunction with other data.

Fig. 2 shows the activity versus extent of the modification when activity is assayed at two different NADH concentrations. At high NADH levels, there is an initial rapid rise followed by a loss in enzymatic activity as modification proceeds. At lower levels of NADH (100 μM) an increase in activity is not seen, but the relation between activity and extent of modification is not linear. Because we had observed a similar result with modification by TNBS (6), it was considered likely that the results observed with the NADH assay were a consequence of the loss of excess NADH inhibition. That this is the case is illustrated by the double reciprocal plot presented in Fig. 3. As shown here, loss of excess NADH inhibition is essentially complete when an average of 0.35 group per polypeptide chain is modified (i.e. between two and three groups per monomer are reacted). These results indicate that loss of NADH inhibition occurs well before all the pyridoxole chains in the monomer are modified. On this basis, we have attempted to fit the data of Fig. 2 (at 1 mM NADH) to a model which assumes complete loss of excess NADH inhibition after partial loss in enzymatic activity. The dashed line drawn in Fig. 2 has been calculated from the assumption that loss of excess NADH inhibition is complete after random modification of two or more chains of the six-chain monomer and that those chains which remain unmodified have 2.75 times as much activity as normal while those modified are inactive. The factor of 2.75 is larger than the experimentally determined value as derived from the data of Fig. 3 at 1 mM NADH. However, one would expect this latter value to be too low since activation and loss of activity occur simultaneously. Of the several models tried, this one gave the best fit of the experimental data.

Changes in Purine Nucleotides Effects as Function of Modification with Pyridoxal Phosphate

GTP—As is well known, purine nucleotides markedly affect glutamate dehydrogenase activity. GTP, in particular, is a strong inhibitor with an inhibition constant of less than 10^-4 M regardless of which coenzyme is used. Fig. 4 is a log plot of the
The inhibition constant for GTP as a function of pyridoxal phosphate modification with either NADH or NADPH as coenzyme. In both cases, the inhibition constant increases continually, although for the NADH case, the initial rise is slightly larger. The difference at low extents of modification is probably related to the loss of excess NADH inhibition since, as has been shown previously (6), loss of such inhibition does result in a slight increase of the GTP inhibition constant. It is interesting to note that the inhibition constant for GTP increases throughout the modification, in distinct contrast to the loss of excess NADH inhibition which is complete after partial modification (see Figs. 2 and 3).

A mechanism for the change in the inhibition constant for GTP resulting from pyridoxal phosphate modification can be deduced from recent observations made on the rates of the conformational changes of glutamate dehydrogenase (12). It has been found that the addition of NADPH and GTP to native enzyme results in changes which may be described by assuming two conformational changes, the first of which is induced by the coenzyme itself and precedes a second conformational change which is induced by GTP in the presence of coenzyme. The second change is reflected in the depolymerization of enzyme. Because the inhibition constant for GTP is increased by pyridoxal phosphate modification, it was of interest to see whether the rate of depolymerization was affected by such modification. Fig. 5 shows the difference in rate of depolymerization observed for native enzyme and for enzyme in which the inhibition constant is increased 4-fold when NADPH is used as coenzyme (i.e. enzyme modified to the extent of 0.35 group per chain). The data show that the rate of depolymerization is decreased about 4-fold in the modified enzyme when GTP is added to the enzyme-NADPH complex. A similar decrease is observed when NADPH is added to the enzyme-GTP complex (data not shown). Such data are consistent with a 4-fold decrease in the rate of a GTP induced conformational change of the enzyme-coenzyme complex. It should be noted that the extent of depolymerization in the modified enzyme is smaller because partial modification does lead to changes in the ability of the enzyme to polymerize.

When measuring the effect of GTP on the activity of partially modified enzyme, it was noted that not only does the inhibition constant for GTP change as a function of modification, but so also does the extent of inhibition by GTP. Thus native enzyme is inhibited at least 95% by GTP, but enzyme modified to the extent of 0.75 group per chain is inhibited only 75%. However, as pointed out above, such modification does result in loss of activity of the enzyme. As a consequence, the residual activity in the presence of excess GTP is not markedly altered by modification with pyridoxal phosphate. In fact, as shown in Table I, when assayed in the presence of a saturating concentration of GTP, the enzymatic activity appears to increase by about 50% as modification proceeds up to 0.8 group per polypeptide chain. Under these assay conditions, therefore, the consequences of pyridoxal phosphate modification are quite different than observed earlier (Fig. 1).

ADP—Since pyridoxal phosphate modification resulted in large changes in the inhibition constant for GTP, as described
TABLE I
Activity of native and pyridoxal phosphate-modified glutamate dehydrogenase in presence of saturating level of GTP

GTP concentration in the assay was 500 μM. The activity was measured with 100 μM NADPH and the modification was carried out as described in Fig. 1 except that pyridoxal phosphate concentration was 600 μM.

<table>
<thead>
<tr>
<th>Relative activity</th>
<th>Group modified per polypeptide chain</th>
<th>Activity of modified enzyme in presence of excess GTP</th>
<th>Relative to native enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative to modified enzyme in absence of GTP</td>
<td>Relative to native enzyme in absence of GTP</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>3.5%</td>
<td>3.5%</td>
</tr>
<tr>
<td>75</td>
<td>0.30</td>
<td>6.2%</td>
<td>4.5%</td>
</tr>
<tr>
<td>35</td>
<td>0.60</td>
<td>15.0%</td>
<td>5.7%</td>
</tr>
<tr>
<td>30</td>
<td>0.75</td>
<td>22.0%</td>
<td>6.6%</td>
</tr>
<tr>
<td>25</td>
<td>0.80</td>
<td>26.0%</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

Fig. 6. First order plot of rate of inactivation of glutamate dehydrogenase by pyridoxal phosphate in the absence or presence of NADH plus GTP. The reaction was carried out as described in the legend to Fig. 1 except that the pyridoxal phosphate concentration was 300 μM and the glutamate dehydrogenase 1.65 mg per ml (30 μM based on molecular weight of 56,000). The reaction was stopped by dilution into cold Tris buffer, pH 7.15. Rates of inactivation when reaction mixture contained no added ligands (O--O); 30 μM NADH and 250 μM GTP (□--□); 30 μM NADH and 250 μM GTP (△--△). 60 μM NADH and 250 μM GTP (O--O).

Effect of Ligands on Modification of Glutamate Dehydrogenase

Since various ligands have been previously shown to protect against loss of activity when the enzyme was reacted with acetic anhydride (4), TNBS (6), or other reagents, it was of interest to test the effect of these ligands on the rate of activity loss in the presence of pyridoxal phosphate. Fig. 6 shows that NADH plus high concentrations of GTP decreases the rate of inactivation by at least 6-fold. In order to obtain this effect only 1 eq of NADH per polypeptide chain is necessary. It should be noted that GTP alone does not appreciably affect the rate of inactivation. This is also the case for α-ketoglutarate, ADP, NAD, and NADP, although high concentrations of NADH or NADPH do decrease the rate of inactivation somewhat.

TABLE II
Rate of pyridoxal phosphate inactivation of native and TNBS-modified glutamate dehydrogenase

The TNBS enzyme was modified to the extent of 0.5 group per polypeptide chain as described in the text. Pyridoxal phosphate modification was carried out as described in the legend to Fig. 1. The activity was measured with NADPH as coenzyme.

<table>
<thead>
<tr>
<th>Time of reaction with pyridoxal phosphate</th>
<th>Percentage of remaining activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Native enzyme</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>84</td>
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<tr>
<td>40</td>
<td>12</td>
</tr>
</tbody>
</table>

DISCUSSION

Smith et al. have shown (7) that pyridoxal phosphate reacts predominately with the lysyl residue at position 97 of the proposed tentative sequence (7, 9). That the reaction appears to be specific has also been confirmed by our own data although under extreme conditions, more than one group does appear to react. However, the specific modification of lysine-97 results in at least three dramatic changes in the kinetic characteristics of the enzyme. First, as shown previously (8) and confirmed here, it results in loss of enzyme activity. However, it is possible that

*B. R. Goldin and C. Frieden, unpublished observations.
loss of activity is not complete as a consequence of modification by pyridoxal phosphate. This is indicated by the data shown in Fig. 1 as well as those in Table I. These latter data, in particular, show that pyridoxal phosphate modification has a slight activating effect on the enzymatic activity when measured at saturating concentrations of the inhibitor GTP. Although any residual activity after modification of lysine-97 would probably be less than 10% that of the native enzyme, it would appear to be more correct to assign to this group the role of an important residue in the catalytic reaction rather than essential one. However, its role in the mechanism of the reaction is certainly not clear at this time. Also it should be noted that no combination of substrates and effectors will completely protect the enzyme against modification. Furthermore, NADPH appears to bind to the pyridoxal phosphate enzyme as well as to native enzyme. Thus, it is possible that pyridoxal phosphate does not sterically interfere with substrate- or coenzyme-binding sites of the enzyme.

The second major kinetic change is the loss of excess NADH inhibition. As discussed with respect to the data of Fig. 2, complete loss of such inhibition occurs when only a fraction (2 to 3) of the lysyl residues at position 97 within a given monomeric enzyme of six chains are modified. This result is similar to that observed when the enzyme is reacted with trinitrobenzenesulfonate where we have shown that a loss of excess NADH inhibition occurs when only three of the six chains are modified (6). In this latter case, however, the lysine residue so modified is at position 426 in the tentative sequence and it is not possible to modify more than three of the six chains at this position (5).

Both the pyridoxal phosphate and TNBS modification data imply some type of subunit-subunit interaction with respect to excess NADH inhibition. As a consequence of the fact that the complete modification would not lead to a total loss of activity, this conclusion is supported by the data of Fig. 1 and Table I as well as by the fact that some activity remains even after readdition of pyridoxal phosphate. This is indicated by the data shown in Fig. 1 as well as those in Table I. These latter data, in particular, show that pyridoxal phosphate and TNBS (97 and 428, respectively) may play different roles with respect to coenzyme-purine nucleotide-induced conformational changes. Certainly they play very different roles in the catalytic reaction since modification of lysine-428 does not lead to loss of enzymatic activity.

Finally, it should be noted that modification of either lysine-97 or -428 leads to a species of enzyme which is unable to polymerize to higher molecular weight forms (6, 8), although only half of the chains need be modified at lysine-428 for complete depolymerization.

It is clear that some kinetic and molecular properties change markedly on modification. Some of these must be related to subunit-subunit interactions, while others, such as the loss in enzymatic activity, are not. Although we can describe the changes which occur at this point, we are not able at present to postulate a mechanism which would account for all these changes occurring as a consequence of the modification of a single residue in the polypeptide chain.

Note Added in Proof—After this paper was submitted for publication, Piszkiwecz and Smith presented data on the kinetics of imine formation between lysine-97 and pyridoxal phosphate (13). Their extensive analysis of this reaction, based on the assumption that modification leads to a total loss of activity, would suggest that the pyridoxal phosphate modification is incomplete due to the equilibrium between various ionic forms of the enzyme and pyridoxal phosphate, thus accounting for some of the residual activity under the conditions used here. Our data, however, suggest that some of this residual activity is a consequence of the fact that the complete modification would not lead to a total loss of activity. This conclusion is supported by the data of Fig. 1 and Table I as well as by the fact that some activity remains even after readdition of pyridoxal phosphate to enzyme containing pyridoxal phosphate covalently attached by treatment with sodium borohydride.

REFERENCES
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