Effect of Trinitrophenylation of Specific Lysyl Residues on the Catalytic, Regulatory, and Molecular Properties of Bovine Liver Glutamate Dehydrogenase*

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ABSTRACT: The molecular, kinetic, and regulatory characteristics of bovine liver glutamate dehydrogenase have been examined as a function of modification of lysyl residues by trinitrobenzenesulfonate. It is shown that modification of three of the six chains of the monomer at lysine-428 of the tentative sequence (Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Jr., Langley, T. J., and Melamed, M. D., *Proc. Nat. Acad. Sci. U. S. 67*, 724 (1970)) results in the loss of excess NADH inhibition, an apparent change in the dissociation constant for GTP and ADP when using NADH as coenzyme, some loss in NAD activation, and a dissociation of the polymeric form of the enzyme to the monomer. Further trinitrophenylation, resulting in the modification of lysine-425 up to three groups per six chains, as well as small extents of modification of a number of other lysyl residues, gives rise to an apparently homogeneous polymerized form of the enzyme and it is concluded that the polymerization arises from the modification of lysine-425 or the sum of modification of lysine-428 and -425. Although some activity is lost under these conditions, similar results are obtained under conditions where ligand addition prevents loss of activity. In contrast to native enzyme, the sedimentation behavior of the modified polymerized enzyme, whether active or not, is unaffected by the presence of coenzyme and purine nucleotides. Furthermore, this enzyme appears to have characteristics more closely related to native monomer than polymer. The results obtained in these experiments are compared to those of others. It is noted that complete kinetic studies as well as identification of reacted residues should accompany chemical modification experiments if correct interpretation of the data is desired, particularly for complex enzyme systems.

As a means of determining the relationship between the structure and the complex kinetic properties of the bovine liver glutamate dehydrogenase, chemical modification experiments have been performed by a number of workers. Treatment of the enzyme with reagents such as acetic anhydride (Colman and Frieden, 1966a,b), pyridoxal phosphate (Anderson *et al.*, 1966), fluorodinitrobenzene (di Prisco, 1967), trinitrobenzenesulfonate (Freedman and Radda, 1969; Clark and Yielding, 1971), and bromopyruvate (Baker and Rabin, 1969) have indicated that modification of lysyl residues may lead to changes in enzymatic activity, in regulatory properties, and in the ability of the enzyme to polymerize at high protein levels.

So far, however, only lysine-97 (Smith *et al.*, 1970) has been identified as related to enzymatic activity as such since modification of this residue by pyridoxal phosphate (Piszkiewicz *et al.*, 1970) leads to essentially complete loss of activity.

In the preceding paper (Coffee *et al.*, 1971) it has been shown that trinitrobenzenesulfonate (TNBS)[1] leads to the modification of particular lysyl residues of this enzyme. As shown in that paper, lysyl-428 reacts most rapidly and to the extent of 0.5 group/polypeptide chain (*i.e.*, three of the six subunit chains in the active monomer). Somewhat less rapidly and apparently mutually exclusive with the lysine-428, lysine-425 reacts with TNBS also to the extent of 0.5 group on different polypeptide chains. Further trinitrophenylation results in the slower modification of other, as yet unidentified, lysyl residues.

The purpose of the present paper is to discuss the changes in the kinetic, regulatory, and physical properties of glutamate dehydrogenase as a consequence of modification of these specific residues. A few of these changes in properties after trinitrophenylation have been previously reported by Freedman and Radda (1969) and by Clark and Yielding (1971).

Materials and Methods

Materials. Bovine liver glutamate dehydrogenase was obtained as a crystalline suspension in ammonium sulfate from Boehringer-Mannheim Corp. or from Sigma Chemical Co. The crystals were centrifuged and dissolved in 0.04 M potassium phosphate buffer (pH 8.0). The solution was centrifuged 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.

Rat liver glutamate dehydrogenase was prepared as described previously (King and Frieden, 1970). All coenzymes and purine nucleotides were obtained from Sigma Chemical Co., as were DTNB (Ellman's reagent) and TNBS. These compounds were used without further purification. Stock solutions of TNBS were prepared immediately before use.

Kinetic Studies. Unless otherwise noted, all experiments concerning the kinetic properties of the enzyme were performed at 25° and pH 8.0 in 0.01 M Tris-acetate (0.01 M with respect to acetate) containing 10 μM EDTA. For NADH oxidation,

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[1] Abbreviations used are: TNBS, trinitrobenzenesulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

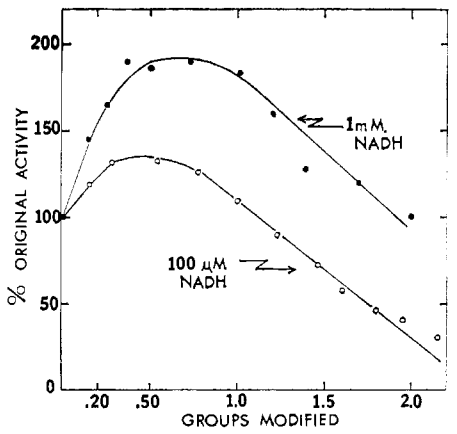

FIGURE 1: Glutamate dehydrogenase activity as a function of groups modified by TNBS using NADH in the assay system. The enzyme (1 mg/ml) was reacted with 200 μM TNBS at 25° at pH 8.0 in 0.04 M phosphate buffer. Aliquots were withdrawn and assayed for glutamate dehydrogenase activity using 100 μM (○) or 1 mM (●) NADH present in the assay.

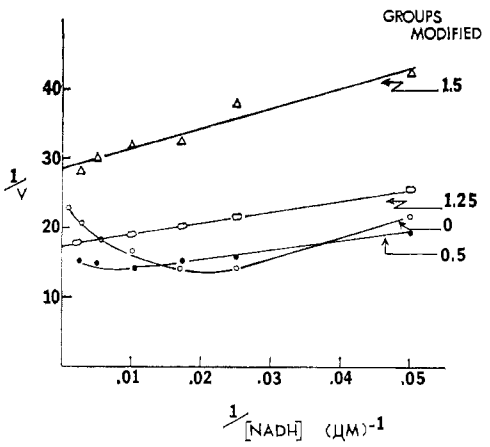

FIGURE 2: Plot of reciprocal concentration *vs.* reciprocal velocity at different extents of modification by TNBS. The conditions used for modification are described in the legend to Figure 1.

the α-ketoglutarate concentration was 5×10^{-3} M and ammonium chloride was 5×10^{-2} M. When pyridine nucleotide reduction was measured, the glutamate concentration used was 5×10^{-3} M. 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 (0–0.1-absorbance full scale). The presence of coenzymes or nucleotides during the modification reaction did not affect initial velocity measurements after dilution into the assay mixture.

Method of Trinitrophenylation. TNBS was incubated with glutamate dehydrogenase in 0.04 M phosphate buffer (pH 8) at 25°. The calculation of the number of groups modified (based on a subunit molecular weight of 56,100) is described in the preceding paper (Coffee *et al.*, 1971). When kinetic assays were performed by removing aliquots during the modification reaction, the trinitrophenylation reaction was effectively stopped by dilution into cold 0.1 M Tris-acetate buffer (pH 7.15) containing 1 mM phosphate, the enzyme being relatively stable in this buffer. When modified derivatives were to be used in other experiments, the reaction was stopped either by passing the enzyme over a Sephadex G-25 column (when the rate of modification was slow) or by rapid addition of 10-fold excess β-mercaptoethanol, followed by passage over a Sephadex G-25 column.

Sedimentation Experiments. Sedimentation velocity experiments were performed at 5–7° in 0.04 M phosphate buffer (pH 8.0) at a speed of 42,000 rpm using the Spinco Model E analytical ultracentrifuge. Schlieren optics were used when the protein concentration was greater than 1 mg/ml. Sedimentation coefficients were measured from the apex of the schlieren peak even for those cases where the peak was asymmetric. When $s_{20,w}$ values were measured at a protein concentration of 0.1 mg/ml, uv optics were employed.

The partition cell method described by Yphantis and Waugh (1956) was used to determine the $s_{20,w}$ values at low enzyme concentrations (<0.1 mg/ml) by using enzymatic activity to measure changes in concentration.

Stopped-flow experiments were performed using a Durrum stopped-flow spectrophotometer (2-cm light path).

Results

Since trinitrophenylation is not absolutely specific for one given residue (Coffee *et al.*, 1971), the effect of trinitrophenylation on the kinetic, regulatory, and physical properties of glutamate dehydrogenase will be discussed first in terms of the total number of groups modified per polypeptide chain. Correlation of specific changes in these properties with specific lysyl residues modified will be given in the Discussion.

It was found that enzymatic activity after modification depends not only on which coenzyme is used but, in some cases, the concentration of coenzyme employed in the assay. These differences are discussed below.

NADH as Coenzyme. Using NADH, the activity of glutamate dehydrogenase trinitrophenylated to different extents depends on the concentration of coenzyme in the assay. Figure 1 shows results at two assay levels of NADH. At 1 mM NADH, a marked activation by 0.5 group modified per chain is followed by a decrease in activity as the modification proceeds to two groups. At lower NADH levels (100 μM), activation is considerably less and a relatively greater loss of activity is observed at the point at which two groups are modified.[2]

Since it is known that high levels of NADH inhibit glutamate dehydrogenase activity (Frieden, 1959), it was considered possible that the activation noted in Figure 1 could be explained by the loss of this inhibition. In Figure 2, double-reciprocal plots of several trinitrophenylated derivatives are shown using NADH as the variable substrate. When an average of 0.5 group is modified per polypeptide chain of 56,100 molecular weight, there is an almost total loss of the excess NADH inhibition. At 1.25 groups modified, even the small amount of NADH inhibition remaining at 0.5 group had been abolished. It is difficult to determine whether the K_m for NADH is altered upon modification since the Michaelis constant for the native enzyme is not known with great accuracy. The K_m values for the derivatized enzymes are between

[2] The results observed here are different from those noted by Freedman and Radda (1970) who found that enzymatic activity using 100 μM NADH as coenzyme only decreased as a function of groups modified. The reason for this difference is not clear, but it should be noted that their data are internally inconsistent. Thus, the data of their Figure 3 indicate 80% loss of activity when 1.2 ± 0.2 groups are modified, but Table II indicates only 44% activity lost when 1.8 groups are trinitrophenylated. This latter value is consistent with the data presented in our Figure 1 using 100 μM NADH.

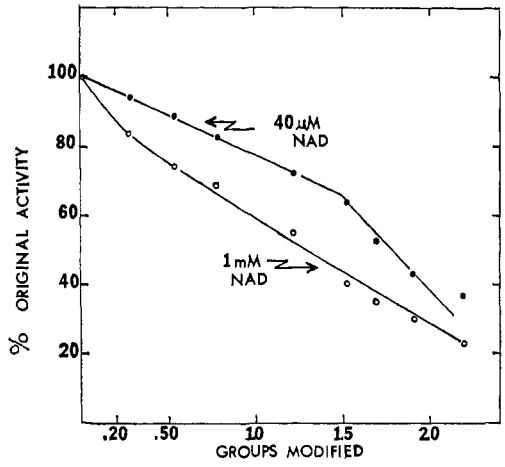

FIGURE 3: Glutamate dehydrogenase activity as a function of groups modified by TNBS using NAD in the assay system. Modification conditions the same as used in the legend to Figure 1.

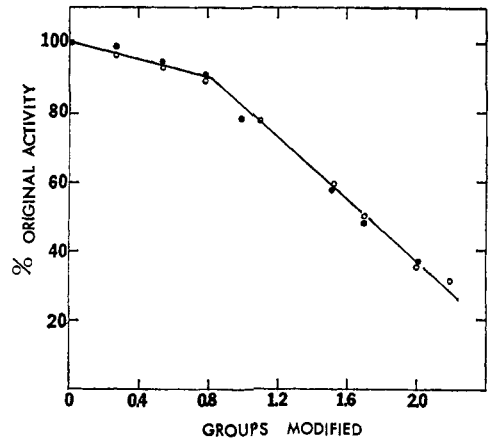

FIGURE 4: Glutamate dehydrogenase activity as a function of groups modified by TNBS using either 100 μM NADPH (○) or 500 μM NADP (●) in the assay system. Modification condition the same as described in the legend to Figure 1.

15 and 25 μM, somewhat less than the estimated value of 40 μM for the native enzyme.

In any case, the loss of the NADH inhibition adequately explains the initial activation noted for the enzymatic activity at a low extent of modification. As will be shown later, this loss of excess NADH inhibition is also responsible for an increase in the inhibition constant for GTP.

NAD as Coenzyme. In Figure 3, the effect of TNBS modification on glutamate dehydrogenase activity is shown when NAD was used as the coenzyme in the assay. In this case, the enzyme activity only decreases as the trinitrophenylation proceeds. As with NADH, the loss in activity as a function of groups modified was found to depend on the concentration of NAD in the assay, although in a very different way. At high NAD concentration there was a substantially larger drop in activity at small extents of trinitrophenylation than noted under conditions where the enzyme was assayed at low NAD concentration. The decrease in activity at high NAD concentrations suggested the possibility of a change in the extent of NAD activation which has previously been observed at high NAD levels (Frieden, 1959; Engel and Dalziel, 1969). That this is the case can be seen more clearly in Table I where the ratio of velocities of modified to native enzyme are shown at several different NAD levels, indicating a differential loss of activity as the NAD concentration is changed. When plots of reciprocal velocity against reciprocal NAD concentration are analyzed, a decrease in substrate activation was noted for the modified enzyme, as expected from the data on Table I. However, even the modified enzyme was still activated to some extent by high concentrations of NAD.

NADP and NADPH as Coenzyme. It has previously been shown (Frieden, 1959) that at pH 8 in 0.01 M Tris-acetate buffer, glutamate dehydrogenase exhibits normal Michaelis–Menten kinetics over a fairly wide substrate concentration range when using NADP or NADPH as coenzyme. In Figure 4 the activity as a function of groups modified is shown using 100 μM NADPH or 500 μM NADP as coenzyme. It can be seen for both coenzymes that the loss of activity is biphasic. Only a slight decrease in activity occurs up to 0.8 group modified per 56,100 molecular weight subunit, although there is almost a 65% loss by the point at which two groups are modified. These data differ markedly from those described above for NADH or NAD.

Effect of Trinitrophenylation on the Regulatory Properties of Glutamate Dehydrogenase. Figure 5 shows the effect of tri-

TABLE I: Activity Ratio of Modified to Native Enzyme at Different NAD Concentrations.[a]

Extent of Trinitrophenylation (Groups/56,100)	NAD Concn (μM)			
	40	200	1000	2000
	v'/v[b]			
0	1.00	1.00	1.00	1.00
0.28	0.98	0.75	0.78	0.78
0.9	0.84	0.73	0.70	0.60
2.0	0.40	0.32	0.24	0.19

[a] The conditions of trinitrophenylation were the same as described in the legend to Figure 1. Glutamate dehydrogenase activity was assayed with NAD at the indicated concentrations. [b] v', activity of the modified enzyme; v, activity of glutamate dehydrogenase, before modification.

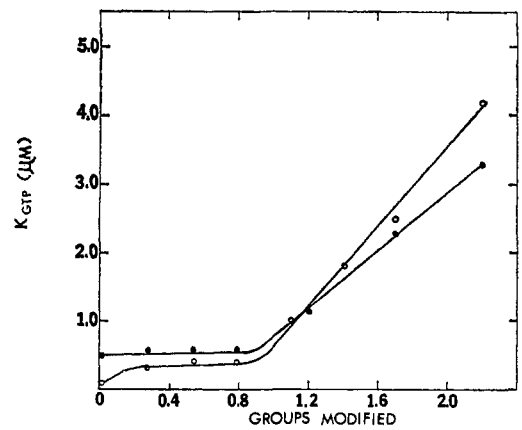

FIGURE 5: The dissociation constant for GTP as a function of the extent of trinitrophenylation using either 100 μM NADPH (●) or 100 μM NADH (○) in the assay system. The conditions of trinitrophenylation are described in the legend to Figure 1.

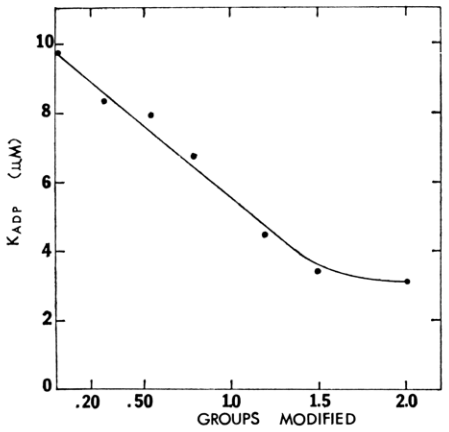

FIGURE 6: The dissociation constant for ADP as a function of the extent of trinitrophenylation using 100 μM NADPH as coenzyme in the assay. The conditions of trinitrophenylation are the same as described in the legend to Figure 1.

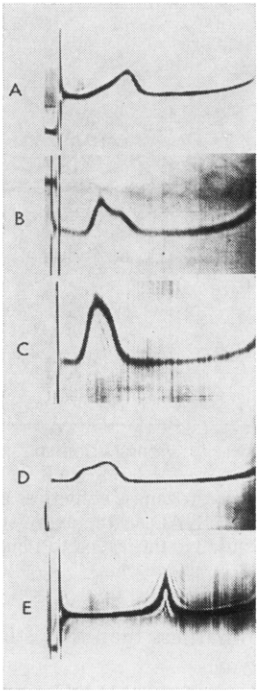

FIGURE 7: Sedimentation velocity patterns of bovine liver glutamate dehydrogenase reacted with TNBS to varying extents. The sedimentation experiments were performed at an enzyme concentration of 2 mg/ml in 0.04 M potassium phosphate buffer (pH 8.0). Pictures were taken 40 min after reaching speed (42,000 rpm). (a) Unmodified enzyme; (b) 0.45 group modified per 56,100 g; (c) 0.54 group modified per 56,100 g; (d) 1.2 groups modified per 56,100 g; (e) 2.2 groups modified per 56,100 g.

nitrophenylation on the inhibition constant for GTP using either NADH or NADPH as coenzyme. When measured at 100 μM NADH, K_{GTP} increased 40-fold when an average of 2.2 groups/subunit was modified. In addition (not shown) the extent of inhibition at high levels of GTP decreased from 95 to 80%.

Examination of the alteration in the K_{GTP} indicates a rise of four- to fivefold occurs when less than 0.4 group/56,100 subunit is modified, similar to results observed using acetic anhydride (Colman and Frieden, 1966a). In contrast, when NADPH was used as the coenzyme, essentially no effect on K_{GTP} was noted until the modification exceeded 0.8 group/subunit. The significance of this difference will be discussed later, but in view of the results above, it would appear likely that this change is related to the loss of NADH inhibition at low extents of modification. Above 0.8 group modified, K_{GTP} increases markedly when using either NADH or NADPH as coenzymes. Earlier experiments (Frieden, 1965, 1969) indicated that K_{GTP} for native enzyme was approximately fivefold greater when NADPH rather than NADH was the coenzyme and this observation was confirmed in these experiments.

In Figure 6 the effect of trinitrophenylation on K_{ADP} is shown using NADPH as coenzyme. In contrast to the results for GTP, K_{ADP} decreases from 10 μM to a value of 3.5 μM when 1.5 groups are trinitrophenylated. Although ADP binds more tightly to the modified enzyme, the extent of activation at high ADP concentrations decreases from three- to twofold. Consequently, the ability to determine K_{ADP} accurately is diminished and it difficult to carefully analyze the decrease in K_{ADP} as a function of groups modified.

Effect of Trinitrophenylation on the Physical Properties. Both Freedman and Radda (1969) and Clark and Yielding (1971) have noted that there were changes in the apparent sedimentation coefficient of glutamate dehydrogenase after trinitrophenylation. However, the relation between the number of groups modified and the changes in sedimentation coefficient was not clear. Our studies of these changes as a function of the number of groups modified are shown in Figure 7. At a protein concentration of approximately 2 mg/ml and in 0.04 M phosphate buffer, a single broad peak ($s_{20,w}$ = 23 S) is consistently observed for unmodified glutamate dehydrogenase (Figure 7a). At 0.45 group modified (per polypeptide chain) two peaks are observed, the major one with a $s_{20,w}$ of 13 S and a smaller peak of 18 S (Figure 7b). This smaller peak disappears on addition of NADH and GTP, ligands which normally dissociate native enzyme to 13S material.

Glutamate dehydrogenase trinitrophenylated to the extent of 0.55 group/polypeptide chain exhibits a single peak with a sedimentation coefficient of 13 S (Figure 7c). When the enzyme was modified to the extent of 1.2 group/56,100, two peaks again are apparent, the major peak having an $s_{20,w}$ value of 23 S while the minor peak has a value of 13 S (Figure 7d).

Glutamate dehydrogenase modified to the extent of 2.2 groups/56,100 shows a symmetrical sedimentation pattern with an $s_{20,w}$ value of 34 S (Figure 7e). Under these conditions of modification, the enzyme has lost considerable activity when assayed with any of the coenzymes (Figures 1, 3, and 4). No further change in the sedimentation pattern or coefficient is observed upon modification to as much as 4.5 group/subunit. It should be noted that preliminary experiments indicate that under these conditions polymerization is not quite as complete in Tris-acetate buffers as in the phosphate buffer used for these experiments.

In Table II, the sedimentation coefficients of enzyme modified to 0, 1.2, and 2.2 are given for several protein concentrations. In contrast to the native enzyme, enzyme modified sufficiently to form the polymer shows only a small tendency to dissociate under conditions where the native enzyme is completely dissociated. Only at very low concentrations is there an indication of depolymerization.

The presence of coenzyme and purine nucleotides has no effect on the molecular weight characteristics of trinitrophenylated derivatives. At either 1.2 or 2.2 groups modified per chain, neither ADP and NADH (which normally cause association) nor GTP and NADH (which normally cause depolym-

TABLE II: Comparison of the Effect of Protein Concentration on the Sedimentation Coefficient of Native and Modified Glutamate Dehydrogenase.

Extent of Trinitrophenylation (Groups/56,100)	Protein Concn (mg/ml)	$s_{20,w} \times 10^{13}$ (sec^{-1})
Unmodified	2.0	23
	0.1	13
	0.01	13
1.2	1.0	24
		13 (minor peak)
	0.1	24
		13 (minor peak)
2.2	2.0	34
	0.1	34
	0.01	27

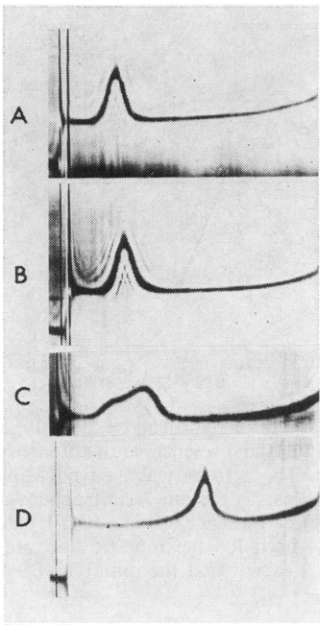

FIGURE 8: Sedimentation velocity patterns of rat liver glutamate dehydrogenase reacted with TNBS to varying extents. The sedimentation experiments were performed at an enzyme concentration of 2 mg/ml in 0.04 M potassium phosphate buffer (pH 8.0). Pictures were taken 40 min after reaching speed (42,000 rpm). (a) Unmodified enzyme; (b) 0.45 group modified per 56,100 g; (c) 1.2 groups modified per 56,100 g; (d) 2.2 groups modified per 56,100 g.

erization) change the sedimentation patterns seen in Figure 7d,e. In addition, the sedimentation coefficient of enzyme depolymerized by trinitrophenylation, *i.e.*, at 0.5 group modified, was unchanged in the presence of ADP and NADH. Because the presence of NADH and GTP results in the disappearance of a small 18S peak in material modified to 0.45 group (Figure 7b), it seems likely that this minor peak represents either native or very lightly modified enzyme.

Effect of Trinitrophenylation on the Sedimentation Coefficient of Other Dehydrogenases. King and Frieden (1970) have shown that rat liver glutamate dehydrogenase does not undergo a concentration dependent polymerization and that nucleotides such as ADP and GTP in the presence or absence of NADH do not affect the molecular weight. On the other hand, it appears that the rat and bovine liver enzymes are kinetically similar in many respects (King and Frieden, 1970) and preliminary results suggest a high degree of homology in the amino acid sequence of the two enzymes.[3] While it is not known yet which lysine residues of the rat liver enzyme react with TNBS, it would seem likely that similar residues would be involved and it was therefore of interest to see whether modification of the rat liver enzyme would affect its molecular weight. In Figure 8, it can be seen that at 1.2 groups trinitrophenylated, there is an increase in the sedimentation coefficient, but that the pattern of this derivative is the same as for the bovine liver enzyme modified to the same extent (Figure 1e). At 2.2 groups modified per 56,100 molecular weight, the enzyme had polymerized to material which had a sedimentation coefficient of 34 S, and again appeared similar to the bovine liver enzyme modified to 2.2 groups.

In order to test whether trinitrophenylation *per se* induced aggregation of enzymes which normally do not polymerize, four enzymes (yeast and liver alcohol dehydrogenase, muscle glyceraldehyde 3-phosphate dehydrogenase, and lactic dehydrogenase) were modified to the extent of three TNP groups per polypeptide chain under the same conditions as used for the glutamate dehydrogenases. Ultracentrifugation experiments show that none of these enzymes have any tendency to polymerize as evidenced by lack of change in the sedimentation coefficient at a concentration of 3 mg/ml before and after modification. Although this is a limited sample of enzymes, the data suggest that trinitrophenylation does not appear to induce aggregation and that the effects with respect to glutamate dehydrogenase are specific.

Modification in the Presence of Ligands. During the course of conducting these trinitrophenylation experiments it was found that, with variable effectiveness, a variety of compounds could prevent loss of enzymatic activity. In Table III, the per cent of residual activity (using NADH as coenzyme) is shown after trinitrophenylation in the presence of substrates and/or effectors. In all these experiments, the enzyme was modified to the extent of 3.0 groups/56,100 molecular weight, an extent sufficient to almost completely inactivate the enzyme in the absence of added ligands. It can be seen from Table III that high concentrations of NADH or NAD prevented activity loss. Protection was also afforded when GTP is present with NADH, but lower NADH levels can be used in these cases. Figure 9 shows the difference in activity loss during trinitrophenylation in the presence or absence of NADH and GTP. The results, as presented in Table III, indicate that the coenzymes NADP and NADPH were not very effective in preventing loss of activity and that α-ketoglutarate, GTP, or ADP alone had almost no effect. Further examination of Table III indicates that ADP may eliminate some, but not all, of the protection afforded by NADH. Thus, these studies show that only when the enzyme has undergone conformational changes associated with inhibition of enzymatic activity is complete protection obtained. This conclusion is supported by the fact that neither NADP or NADPH, which bind only to the active site, prevent loss of enzymatic activity. On the other hand, NADH or NAD can prevent inactivation but only at concentrations far above the K_m for these coenzymes and at levels in which activation (using NAD) or inhibition (using NADH) are noted. Thus, the amino groups being trinitrophenylated are not directly in the coenzyme active site. The

[3] C. J. Coffee, R. A. Bradshaw, and C. Frieden, work in progress.

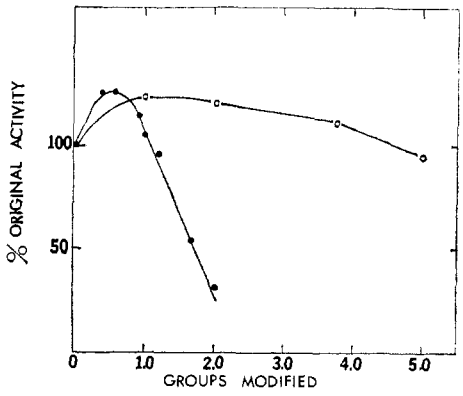

FIGURE 9: Glutamate dehydrogenase activity as a function of groups modified in the presence and absence of ligands in the reaction mixture. The activity was assayed using 100 μM NADH as coenzyme in the assay system. Trinitrophenylation reaction run in the absence (●) or presence (○) of 150 μM NADH and 200 μM GTP. Conditions used for the modification are described in the legend to Figure 1 except that the initial TNBS concentration was 3 mM.

fact that α-ketoglutarate does not prevent loss of enzymatic activity indicates either that trinitrophenylation does not occur within the binding site for this substrate or that α-ketoglutarate is unable to bind in the absence of the other substrates.

Kinetic and Molecular Properties of Enzyme Trinitrophenylated in the Presence of NADH and GTP. Since NADH and GTP prevent loss of activity, it was of interest to compare the properties of this enzyme to the native enzyme and with enzyme modified to the same extent but in the absence of these ligands. Figure 9 would indicate that NADH inhibition is lost under conditions where the enzyme is modified either in the presence or the absence of NADH and GTP. More extensive kinetic studies show that at 0.5 group modified, a small degree of inhibition by excess NADH remains in both cases. When the enzyme is modified in the presence of NADH and GTP, this slight inhibition by excess NADH remains even at extents of trinitrophenylation up to two groups per subunit.

Trinitrophenylation in the presence of NADH and GTP results in a four- to fivefold increase in the inhibition constant for GTP (using NADH as coenzyme) after only 0.4 group is modified. However, in contrast to the results shown in Figure 5, no further change in the inhibition constant occurs even after two groups are modified in the presence of NADH and GTP. Similarly, K_{ADP} changes only slightly (10–8 μM) after trinitrophenylation to two groups in the presence of NADH and GTP.

The sedimentation properties of glutamate dehydrogenase modified in the presence of NADH and GTP appear to be identical, within experimental error, with those observed after modification in the absence of these ligands. Thus, it is possible to prepare fully active enzyme with very different molecular weight characteristics compared to native enzyme. It has been previously shown that, because of the reversible polymerization reaction, some of the kinetic properties of native glutamate dehydrogenase depend on protein concentration (Frieden and Colman, 1967; Spivey, 1969). Thus, it seemed of interest to compare the kinetic differences between native enzyme and the trinitrophenylated, polymerized, active enzyme.

Stopped-flow kinetic experiments (in 0.1 M Tris-acetate buffer, pH 7.3, 10°) as a function of enzyme concentration up to 1 mg/ml how that the change in inhibition constant for GTP is considerably less for the modified (two groups), protected enzyme than for native glutamate dehydrogenase (Frieden and Colman, 1967). Since this change is believed to arise from the reversible polymerization, it would appear that modified enzyme has lost some of the regulatory characteristics which arise from the association–dissociation reaction of the native enzyme. Because excess NADH inhibition is lost in both the native enzyme at high protein concentrations (Spivey, 1969) and in the trinitrophenylated enzyme, it was not possible to use this criterion to test for the differences between the polymerized native and trinitrophenylated enzyme.

TABLE III: Effect of Substrates and Purine Nucleotides on the Loss of Activity Due to Trinitrophenylation.[a]

Additions During Trinitrophenylation		Residual Act.[b] (%)
None		23
ADP	1 mM	22
GTP	200 μM	22
NADH	50 μM	35
	100 μM	67
	+ 100 μM GTP	117
	+ 1 mM ADP	35
	400 μM	107
NAD	100 μM	31
	500 μM	60
	4 mM	91
NADPH	100 μM	40
	400 μM	45
	1 mM	49
NADPH	1 mM	34
	4 mM	50
α-Ketoglutarate		
	5 mM	34
	+ 100 μM NADPH	48
	+ 1 mM NADPH	64

[a] Glutamate dehydrogenase was trinitrophenylated to the extent of 3.0 groups/subunit. The conditions were the same as described in the legend to Figure 1 except for the additions indicated and that 1 mM TNBS was used. [b] Activity measured using NADH as coenzyme. Dilutions into the assay mixture were sufficient so that compounds present during trinitrophenylation did not affect activity measurements.

Discussion

Because TNBS reacts most rapidly with lysine residues which affect the regulatory properties of the enzyme, the effect of low extents of modification on enzymatic activity depends on which coenzyme is used in the assay. Thus, when an average of 0.5 group is modified per chain, the rate of NADH oxidation increases, the rate of NAD reduction decreases slightly, and the enzyme appears under some conditions to be desensitized to GTP inhibition. There is little doubt that the kinetic effects are directly related to one another. Thus, the rate of NADH oxidation increases because excess NADH inhibition is lost (Figure 2). The rate of NAD reduction decreases because some NAD activation has been

lost (Table I). The fact that the inhibition constant for GTP is unaltered at low extents of trinitrophenylation when using NADPH as coenzyme but increased when using NADH as coenzyme is accounted for by assuming that high levels of NADH, in addition to inhibiting NADH oxidation, potentiate GTP inhibition to some extent. It has already been shown that NADH or NADPH binding to the active site of the enzyme causes tighter GTP binding (Colman and Frieden, 1966a,b) and the present results would imply that in native enzyme, NADH binding to a second, nonactive, site (which normally results in the inhibition by excess NADH (Frieden, 1959)) increases GTP binding by another four- to fivefold. It is of interest that modified enzyme (at 0.5 group/chain) is kinetically similar when using either NADH or NADPH as coenzyme, whereas the kinetic properties of the native enzyme are quite different under these conditions, illustrating the important consequences of NADH binding to the second, nonactive site. Clark and Yielding (1971) have recently observed a slight change in GTP inhibition after TNBS modification to (presumably) about one group per polypeptide chain. They interpreted these data as a desensitization toward GTP, rather than loss of excess NADH inhibition, as shown here. Our data show the importance of complete characterization of the kinetic properties of the enzyme after chemical modification.

The loss of the ability of the enzyme to polymerize at a low extent of trinitrophenylation (Figure 7) may certainly reflect changes in subunit interactions. Although possible, it seems unlikely that one lysine residue in three of the six chains of the monomer is critically important *per se* to the polymerization process. Rather, it is tempting to speculate that the molecular, as well as the kinetic, changes observed are the consequence of a conformational change (or the loss of the ability to undergo a conformational change) due to trinitrophenylation. Such an argument would strongly indicate the importance of subunit–subunit interactions within the monomeric form of the enzyme and suggest that the kinetic changes which occur are the result of alterations in quaternary, as well as the tertiary, structure.

Reference to the preceding paper (Coffee *et al.*, 1971) shows that when an average of 0.5 group/chain are modified by trinitrophenylation, most (72%) of the modification has occurred at lysine-428 in the sequence proposed by Smith *et al.* (1970). Furthermore, this represents over 90% of the maximum extent of modification which can occur at this residue even at high extents of trinitrophenylation (Coffee *et al.*, 1971). These observations suggest that the kinetic and molecular changes which occur at low extents of trinitrophenylation are a consequence of the modification of lysine-428. The suggestion that conformational changes which influence the subunit–subunit interactions are altered by trinitrophenylation is strongly supported by the results of the preceding paper (Coffee *et al.*, 1971) in which it is shown that residue 428 must become modified before residue 425, that both are modified only to the extent of 0.5 group/chain (*i.e.*, 3 groups/6 chains), and that no single chain becomes modified at both positions.

Very few changes in the kinetic properties appear to be associated with modification of lysine-425, since the kinetic data, especially for enzyme modified in the presence of NADH and GTP, show only relatively small changes between 0.5 and 2.0 groups modified which would be equivalent to the modification of residue 425 from 14 to 97% of the total possible extent of modification of that residue. On the other hand, the extent of modification of the 425 residue (or the sum of modification of residues 428 and 425) correlates quite well with the formation of the high molecular weight (34 S) polymer. Thus, polymer formation begins at slightly over 0.5 group modified and is completed by 1.8 to 2.0 groups modified, similar to the modification of lysine 425 (Coffee *et al.*, 1971). At this point, lysine-428 and -425 are modified to a total of one group per chain and the other 0.8–1.0 group modified per chain are actually spread over several other groups, with no one group accounting for more than 20–30% of the total (Coffee *et al.*, 1971). More extensive trinitrophenylation, resulting in the modification of other lysine groups, does not alter the molecular weight further, and it seems unlikely that groups other than 425 (or 425 plus 428) are responsible for the polymerization process.

The polymer formed by trinitrophenylation (presumably due to modification of residue 425 or 425 plus 428) is different from native polymer in a number of ways. It is not dissociated by NADH and GTP; it depolymerizes only at very low protein concentrations; and its kinetic properties appear to be more similar to the native monomeric, rather than to the native polymeric, form. It is not possible at this point to explain the polymerization process which occurs after trinitrophenylation. It occurs in the rat liver enzyme (which is normally a monomer) as well as the bovine liver enzyme and in the presence or absence of ligands. Freedman and Radda (1969) suggest that the changes in molecular weight on trinitrophenylation result from a slowing down of the rate of equilibration between different molecular weight forms, but there is no support for that argument since the molecular weight distributions obtained at any given extent of modification appear unaltered under a variety of conditions. It seems more likely that modification of lysine-425 induces a conformational change of the protein, albeit a different one from that induced by trinitrophenylation at lysine-428. In this regard, it should be noted that toluene increases the degree of polymerization of bovine liver glutamate dehydrogenase (Eisenberg and Reisler, 1970) and that this polymer is much more stable than native polymer. It is possible that the two polymeric forms may be similar.

Trinitrophenylation of the native enzyme beyond one group per chain results in other kinetic changes. There is a loss of activity (regardless of which coenzyme is used) and a marked rise in the inhibition constant for GTP using either NADH or NADPH as coenzyme. This latter change had been observed earlier after modification of amino groups by acetic anhydride (Colman and Frieden, 1966a,b) and appeared to be related to the loss of enzymatic activity. This relationship still appears to be valid and is further strengthened by results of pyridoxal phosphate modification. Anderson *et al.* (1966) demonstrated that modification of a specific lysyl residue with pyridoxal phosphate resulted in loss of activity. Recently Piszkiewicz *et al.* (1970) have shown this residue to be lysine-97. Neither Anderson *et al.* nor Piszkiewicz *et al.* investigated the effect of pyridoxal phosphate on the inhibition constant for GTP.

It would be expected, however, in accordance with the TNBS and acetic anhydride data, that K_{GTP} should rise markedly upon pyridoxal phosphate modification and loss of activity. Indeed, this is the case and the change (as measured by the ratio of activity loss to increase in K_{GTP}) is similar to that observed at the higher extents of trinitrophenylation.[4] There is, therefore, a clear relationship between loss of activity and an increase in K_{GTP}.

On the other hand, the activation by ADP is relatively un-

[4] B. R. Goldin and C. Frieden, unpublished results.

changed on loss of activity. Several investigators have concluded from chemical modification studies that the ADP and GTP binding sites are different since the response to these nucleotides differs as a consequence of the modification. Our results suggest that the observed differences in response may be a kinetic, rather than a binding, argument and do not necessarily indicate the sites for ADP and GTP to be different. Of course, the marked changes in K_{GTP} which occur on activity loss are unrelated to the smaller change in K_{GTP} (of about fourfold) which occurs using NADH as coenzyme and is a consequence of the loss of excess NADH inhibition upon trinitrophenylation at lysine-428.

It is not clear why there is activity loss at higher extents of trinitrophenylation. Reaction of lysine-97 with pyridoxal phosphate results in such loss, but a peptide trinitrophenylated at this position has not been isolated. While trinitrophenylation at position 97 may occur (as one of the "other" peptides described by Coffee *et al.*, 1971), it would probably be insufficient to account for the loss of activity. Thus loss of activity in this case may arise from general modification of lysine residues. In any event, it is clear that lysine residues 97, 428, and 425 have very different reactivities toward different modifying reagents (Coffee *et al.*, 1971).

Activity loss and the subsequent change in K_{GTP} may be conveniently prevented by the presence of NADH and GTP. The same results are observed whether the modifying reagent is TNBS, acetic anhydride (Colman and Frieden, 1966), or pyridoxal phosphate.[4] In the latter case, we have observed that the native enzyme in the presence of NADH and GTP reacts very slowly, if at all, with pyridoxal phosphate.[4] This must account for the protection against activity loss, since lysine-97 cannot react under these conditions.

There have been a number of studies on the properties of chemically modified glutamate dehydrogenases. Other than those in the preceding paper (Coffee *et al.*, 1971) and those of Piszkiewicz *et al.* (1970), specific lysine groups modified have not been identified in these studies. On the other hand, the modification of tyrosine-412 by tetranitromethane has been reported by Piszkiewicz *et al.* (1971). It is of interest that this residue is quite close to residues 425 and 428 which react with TNBS. Further, the studies by Piszkiewicz suggest that the kinetic properties of tyrosine-412- and lysine-428-modified enzymes might be similar. For example, some of the GTP inhibition, when tested at low levels of GTP, is lost and enzymatic activity is not markedly altered in either case. Unfortunately, these were the only kinetic properties reported by Piszkiewicz *et al.* (1971) and by Price and Radda (1969) and the tyrosine-modified enzyme needs further kinetic characterization.

Finally, it should be noted that the NADH binding ability of enzyme modified with either TNBS or pyridoxal phosphate does not appear to be markedly different from native enzyme.[4] It would be incorrect to conclude, therefore, that modification occurs within the binding site of the coenzyme utilized for enzymatic activity or within the second binding site for NADH. Similarly, GTP is capable of binding as well to acetic anhydride modified enzyme as to native enzyme (Colman and Frieden, 1966) and the binding site for this nucleotide (or for ADP) is probably not directly altered by chemical modification. One must therefore proceed with some caution in the attempts to correlate changes in activity with alteration of binding sites in subunit containing enzymes.

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