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# Tyrosyl and Lysyl Residues Involved in the Reactivity of Catalytic and Regulatory Sites of Crystalline Beef Liver Glutamate Dehydrogenase*

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ABSTRACT: Beef liver glutamate dehydrogenase has been chemically modified with 1-fluoro-2,4-dinitrobenzene, a reagent of functional groups of proteins. Dinitrophenylation has been shown to cause both inactivation and desensitization of the enzyme to ADP activation and GTP inhibition: these effects could be prevented by the presence of NAD (which protected the active site), ADP, and GTP (each of which had been previously shown to afford selective protection to its specific regulatory site). Reacting glutamate dehydrogenase under these conditions yielded a dinitrophenylated enzyme, which retained essentially the same kinetic behavior as that of native glutamate dehydrogenase. The results of a second dinitrophenylation with [$^{14}$C]1-fluoro-2,4-dinitrobenzene, performed under conditions of partial protection of the enzyme, in which no more than one site at a time has been exposed to the action of the reagent, have shown that site reactivity is lost concomitantly with dinitrophenylation of nine amino acid residues per site per mole of active oligomer. Following total hydrolysis, electrophoretic and chromatographic identification of the radioactive dinitrophenylated amino acids has indicated lysyl and tyrosyl, in the ratio of approximately two to one, as the only residues whose chemical modification causes loss of activity and/or allosteric response. One can therefore suggest that the reactivity of the active and the two regulatory sites of the active oligomer of beef liver glutamate dehydrogenase is dependent on three separate but identical sets of amino acid residues, each composed of six lysyl and three tyrosyl residues.

In a previous communication (di Prisco, 1967), it was reported that reacting crystalline beef liver glutamate dehydrogenase (L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) with 1-fluoro-2,4-dinitrobenzene (FDNB)[1] caused a time-dependent loss of catalytic activity and of the propensity for allosteric activation by ADP and inhibition by GTP. The presence of the reaction coenzyme NAD during dinitrophenylation prevented the inactivation, and the presence, in addition, of one of the two allosteric modifiers prevented the loss of its specific allosteric effect. Although previous kinetic studies (Frieden, 1963) had supported the view that both allo-

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[1] Abbreviation used is: FDNB, 1-fluoro-2,4-dinitrobenzene.

TABLE I: Comparison of Properties of Native and Protected Dinitrophenylated Glutamate Dehydrogenase.[a]

| | Native Enzyme | DNP Enzyme (Active) |
|---|---|---|
| Michaelis constant (NAD; μM) | 66 | 63 |
| Activation constant (NAD; mM) | 2.0 | 2.1 |
| ADP response (% activation) | 210 | 185 |
| GTP response (% inhibition) | 82 | 78 |

[a] Assay conditions were 0.05 M Tris-HCl buffer, pH 9.0, 8.3 mM potassium glutamate, 50 μM NAD (when used at fixed concentration); when used, ADP and GTP were 0.5 mM and 20 μM, respectively; 10 μg of enzyme was used in a final volume of 3 ml.

steric modifiers interacted at the same site of the enzyme, the selective protection observed with either ADP or GTP suggested on the contrary that this interaction takes place at two separate or perhaps partially overlapping regulatory sites, in agreement with a similar suggestion (Colman and Frieden, 1966a), based on the modifications induced on the enzyme by acetylation. Further evidence of the existence of more than one type of regulatory site has been obtained also by exposing glutamate dehydrogenase to high concentrations of inorganic phosphate at pH 8–9, which caused a loss of GTP response, whereas the activating effect of ADP remained unaltered (di Prisco and Strecker, 1969). Finally, studies on polarization of eosin fluorescence in the presence of the enzyme (Dodd and Radda, 1967; Brocklehurst *et al.*, 1970) also confirmed the concept of two different allosteric sites.

Use of [$^{14}$C]FDNB has allowed the identification of the amino acids involved in the reactivity of the active site and of the two allosteric sites. The data reported in this paper show that, following exposure to the radioactive reagent, the catalytic activity was lost as a result of dinitrophenylation of 9 amino acid residues per mole of active oligomer, whose molecular weight has been reported by Eisenberg and Tomkins (1968) to be 313,000. Desensitization to either ADP or GTP was also found associated with the uptake of 9 DNP equiv/mole of active oligomer. Furthermore, the three sets of amino acid residues involved in the reactivity of the active and the two allosteric sites appeared to be separate but identical; in fact, differential labeling indicated that the loss of each of the three functions was associated with dinitrophenylation of six residues of lysine and three residues of tyrosine per oligomer unit.

## Experimental Procedure

The activity of crystalline beef liver glutamate dehydrogenase, supplied by Sigma Chemical Co., as a sodium phosphate–glycerol solution, was measured in the direction of glutamate oxidative deamination, as described previously (Strecker, 1955).

NAD, ADP, and GTP were supplied by Sigma Chemical Co.; [$^{14}$C]FDNB was purchased from The Radiochemical Centre, Amersham; DNP-amino acid standards were kindly supplied by Dr. C. Baglioni. Other reagents were of the highest purity commercially available. All preparations were made in glass-distilled water.

Ethanol solutions of FDNB were freshly prepared before each experiment; the reagent was added to the incubation solutions to a final concentration of 1 mM; dinitrophenylation was carried out in the dark at 32° in 0.05 M phosphate buffer, pH 8.0 (di Prisco, 1967). The reaction was stopped either by addition of saturated ammonium sulfate solution to a final concentration of 80% saturation or, when it was not necessary to recover the enzyme in undenatured form, of trichloroacetic acid to a final concentration of 10%.

Radioactivity was measured by means of a Selo Model CH 3 low-background gas-flow counter. After electrophoresis or chromatography of radioactive samples, the paper strips containing the radioactivity were cut into 1-cm portions and counted in a Packard Tri-Carb Model 314 EX liquid scintillation counter, using a toluene solution containing 4 g/l. of 2,5-diphenyloxazole and 0.1 g/l. of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

High-voltage electrophoresis was carried out on Whatman No. 3MM paper in 6.9% formic acid, at 5000 V for 2 hr. When necessary, spots were revealed by wetting the dried strip with a solution of 0.25% ninhydrin in acetone; when quantitive evaluation of an amino acid was required, the cadmium–ninhydrin reagent (Atfield and Morris, 1961) was employed, followed by elution of the color spot.

Decending paper chromatography was employed; Whatman No. 1 paper was used for 16 hr with a solvent system of 1-butanol–acetic acid–water (4:1:5, v/v, upper phase) (Kilgour *et al.*, 1957).

Dinitrophenylated glutamate dehydrogenase was hydrolyzed in sealed evacuated vials by heating at 110° for 16 hr with constant-boiling 6 N HCl. The protein sample was first precipitated with 10% trichloroacetic acid, and the centrifuged sediment was washed twice with trichloroacetic acid, then with a mixture of ethanol–ether (1:1, v/v). Hydrolyzed samples were then evaporated to dryness in a Büchler rotary evapomix connected with a sodium hydroxide trap, and residues were dissolved in a minimal volume of water and extracted twice with peroxide-free ether. Aliquots of the aqueous phase were used for electrophoretic and chromatographic identification of the radioactive DNP-amino acids.

The specific activity of [$^{14}$C]FDNB was determined as described by Rosen and Rosen (1966); in all experiments the added reagent had a specific activity of 480 cpm/nmole.

## Results

The uptake of DNP residues during inactivation and desensitization of glutamate dehydrogenase was measured according to the general method for site labeling, described by Koshland *et al.* (1959). An aliquot of enzyme was pretreated with nonradioactive FDNB, according to the described procedure, in the presence of 1 mM NAD, 1 mM ADP, and 0.1 mM GTP together, thus allowing dinitrophenylation of those residues which were not related to the reactivity of either catalytic or regulatory sites (di Prisco, 1967). After 2 hr, ammonium sulfate was added to the enzyme solution to 80% saturation; the yellow precipate was collected, washed several times with saturated ammonium sulfate solution, and finally dissolved in 0.05 M potassium phosphate buffer, pH 8.0. This dinitrophenylated enzyme showed catalytic and allosteric properties virtually identical with those of native glutamate dehydrogenase (Table I). A second dinitrophenyla-

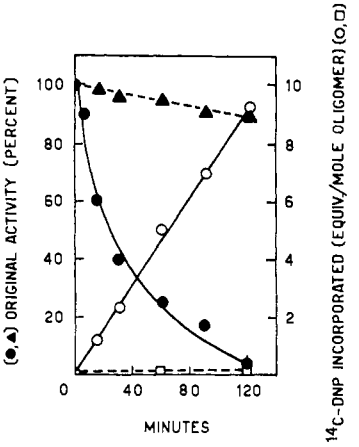


FIGURE 1: [$^{14}$C]DNP uptake (○) during inactivation of glutamate dehydrogenase (●). Incubation with 1 mM [$^{14}$C]FDNB was carried out in the presence of 1 mM ADP, 0.1 mM GTP. Aliquots (10 μg) were removed at different times and assayed (assay conditions as in Table I). Other aliquots (100 μg) were removed at the same times and precipitated with 10% trichloroacetic acid; the precipitates were collected on a Millipore filter, washed with 5% trichloroacetic acid, and counted. The dotted lines refer to measurements of activity (▲) and incorporation of radioactivity (□) in the presence, in addition to ADP and GTP, of 1 mM NAD.

tion was then carried out, this time using [$^{14}$C]FDNB, in which only one of the three specific sites at a time was exposed to the radioactive reagent. This condition was achieved by selective protection of the dinitrophenylated active enzyme; the enzyme solution was divided into three aliquots: 1 mM ADP and 0.1 mM GTP were added to the first one, 1 mM NAD and 0.1 mM GTP to the second, 1 mM NAD and 1 mM ADP to the third; each mixture was then incubated at 32° in the dark with 1 mM [$^{14}$C]FDNB. In parallel with activity measurements, samples were removed from each aliquot at different times, precipitated with trichloroacetic acid, and counted. The results of these experiments are outlined in Table II and Figures 1–3. As shown in Figure 1, after incubating for 2 hr the enzymatic activity was almost completely lost; this loss was associated with the uptake of 9 equiv of [$^{14}$C]DNP per oligomer mole.

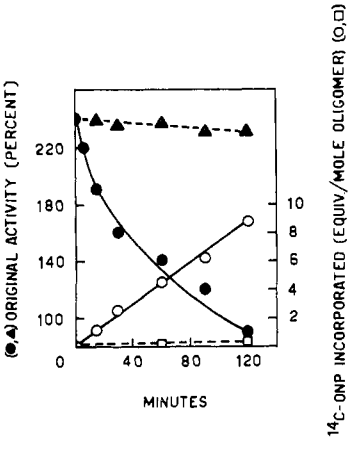


FIGURE 2: [$^{14}$C]DNP uptake (○) during desensitization of glutamate dehydrogenase to ADP activation (●). Incubation with 1 mM [$^{14}$C]FDNB was carried out in the presence of 1 mM NAD, 0.1 mM GTP. See Figure 1 for further details. The dotted lines refer to measurements of activity (▲) and incorporation of radioactivity (□) in the presence, in addition to NAD and GTP, of 1 mM ADP.

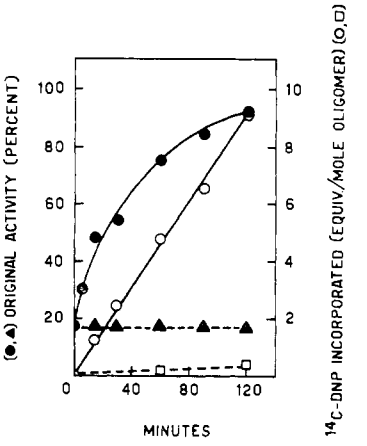


FIGURE 3: [$^{14}$C]DNP uptake (○) during desensitization of glutamate dehydrogenase to GTP inhibition (●). Incubation with 1 mM [$^{14}$C]FDNB was carried out in the presence of 1 mM NAD, 1 mM ADP. See Figure 1 for further details. The dotted lines refer to measurements of activity (▲) and incorporation of radioactivity (□) in the presence, in addition to NAD and ADP, of 0.1 mM GTP.

Figures 2 and 3 show that desensitization of regulatory sites specific for ADP and GTP, respectively, was also virtually complete after the uptake of 9 DNP residues per mole of oligomer. In all cases, the rate of [$^{14}$C]DNP incorporation was linear, as other investigators have described in the study of inactivation of fructose 1,6-diphosphatase with FDNB (Rosen and Rosen, 1966). The control curves in Figures 1–3 (dotted lines) show that, when the enzyme was dinitrophenylated in the simultaneous presence of all three protecting agents, a negligible amount of radioactivity was found associated with the enzyme (indicating no further reaction with FDNB) and either inactivation or desensitization to ADP or GTP was almost totally prevented. Table II also shows that, in the absence of all protecting agents, namely, when all three sites were exposed simultaneously to [$^{14}$C]FDNB, 24–30 DNP residues were incorporated per mole after a 2-hr incubation; in this experiment, inactivation and desensitization occurred together with partial precipitation of the totally unprotected enzyme. (Evidence pointing against the possibility that the same residues were reacting each time was obtained by carrying out

TABLE II: Selective Desensitization of ADP and GTP Sites of Beef Liver Glutamate Dehydrogenase, and [$^{14}$C]DNP Uptake during Desensitization to ADP (Line 3), to GTP (Line 4), and Inactivation (Line 5).[a]

| | Protecting Agents during Dinitrophenylation | % Residual ADP Effect | % Residual GTP Effect | [$^{14}$C]DNP Uptake (equiv/ Mole of Oligomer) |
|---|---|---|---|---|
| 1 | No addition | 0 | 0 | 24–30 |
| 2 | NAD | 3 | 5 | |
| 3 | NAD, GTP | 12 | 98 | 8.8 |
| 4 | NAD, ADP | 97 | 6 | 9.2 |
| 5 | ADP, GTP | | | 9.2 |
| 6 | NAD, ADP, GTP | 96 | 98 | 0.3 |

[a] Assay conditions, as in Table I.

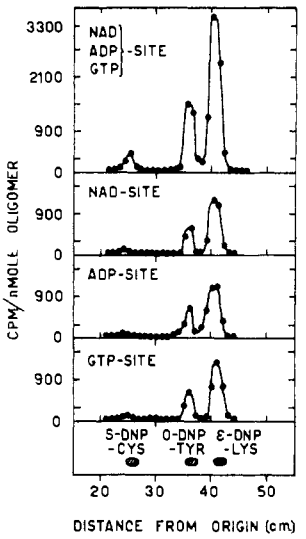


FIGURE 4: Identification of [$^{14}$C]DNP amino acids by high-voltage electrophoresis in the presence of samples of authentic specimens, following total hydrolysis of glutamate dehydrogenase, dinitrophenylated in the absence and presence of protecting agents in different combinations. Details are given in the Experimental Procedure.

dinitrophenylation in the presence of one set of two protecting agents and subsequently removing these agents, followed by dinitrophenylation of the same sample in the presence of a second set of ligands: the number of DNP residues after the first dinitrophenylation increased twofold after the second treatment.)

At the end of the incubation with [$^{14}$C]FDNB, a portion of each reaction mixture was precipitated with trichloroacetic

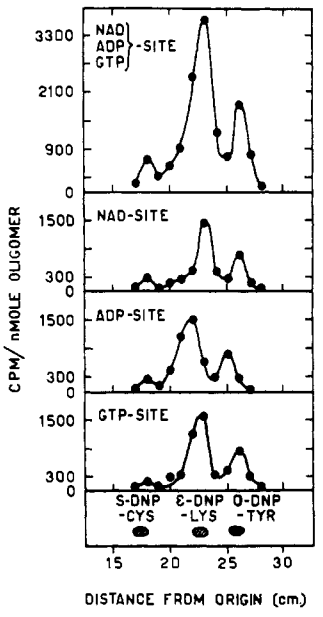


FIGURE 5: Identification of [$^{14}$C]DNP amino acids by paper chromatography in butanol–acetic acid–water (4:1:5, v/v, upper phase) in the presence of samples of authentic specimens, following total hydrolysis of glutamate dehydrogenase, dinitrophenylated in the absence and presence of protecting agents in different combinations. Details are given in the Experimental Procedure.

TABLE III: Amino Acid Residues Dinitrophenylated during Inactivation and Desensitization.[a]

| Nucleotides Present during Treatment with [$^{14}$C]-FDNB | [$^{14}$C]DNP Uptake (equiv/Mole of Oligomer) | | [$^{14}$C]DNP Amino Acids (equiv/Mole of Oligomer) | | | |
|---|---|---|---|---|---|---|
| | | | ε-DNP-Lys | | *O*-DNP-Tyr | |
| | Expt 1 | Expt 2 | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| ADP, GTP | 8.5 | 9.0 | 5.9 | 6.0 | 3.0 | 3.0 |
| NAD, GTP | 9.0 | 8.7 | 6.0 | 5.7 | 3.0 | 3.0 |
| NAD, ADP | 8.8 | 8.5 | 6.0 | 5.7 | 2.8 | 2.8 |

[a] Before [$^{14}$C]FDNB treatment, the enzyme was incubated with nonradioactive FDNB, in the simultaneous presence of NAD, ADP, and GTP.

acid and hydrolyzed in 6 N HCl. Over 90% of the radioactivity was not ether extractable. The radioactive DNP-amino acids were identified by high-voltage electrophoresis (Figure 4) as *O*-DNP-tyrosine and ε-DNP-lysine, in the ratio of approximately one to two for each type of site. (A negligible amount of *S*-DNP-cysteine was also detected.) These findings were confirmed by paper chromatography of the hydrolyzed samples (Figure 5). Data are expressed as cpm/nmole of glutamate dehydrogenase (oligomer); since the amino acid composition of the enzyme is known (Appella and Tomkins, 1966), internal controls with the hydrolysates were obtained each time after electrophoresis, by measurement of eluted glycine (see Experimental Procedure).

Table III summarizes these findings. The reactivity of the catalytic and each of the two allosteric sites of glutamate dehydrogenase was found to depend on three identical sets of amino acid residues, each composed of six lysyl and three tyrosyl residues per mole of active oligomer.

## Discussion

FDNB, a compound known to react with functional groups present in proteins (Fraenkel-Conrat *et al.*, 1955) has been used by several investigators as a tool in the study of the mechanism of action of enzymes (Cremona *et al.*, 1965; Hirs, 1962; Massey and Hartley, 1956; Pontremoli *et al.*, 1965; Ronca *et al.*, 1966; Rosen and Rosen, 1966). Treatment of glutamate dehydrogenase with this reagent brought about a time-dependent inactivation and desensitization to certain activators and inhibitors; it has been suggested that the enzyme molecule contains, in addition to the active site, two separate or perhaps partially overlapping allosteric sites (di Prisco, 1967). This paper deals with the identification and quantitative determination of the amino acid residues responsible for the reactivity of the catalytic and the two allosteric sites. It was found that, following exposure to [$^{14}$C]FDNB, an equal uptake of DNP occurred with each type of site, namely, 9 equiv per mole of enzymatically active oligomer of mol wt 313,000. The dinitrophenylated amino acids were identified as ε-DNP-lysine and *O*-DNP-tyrosine; on a quantitative basis, three identical but separate sets of six residues of lysine and three residues of tyrosine appeared to be responsible for the reactivity of the catalytic and the two allosteric sites. The twofold increase in the number of residues dinitro-

phenylated accompanying the exposure of the same sample of enzyme to FDNB in the successive presence of two different sets of protecting agents, seems to indicate that the sets of reactive amino acid residues are indeed separate. It seems pertinent to note that Rosen and Rosen (1966) have reported that two identical but separate sets of four residues (two lysyl and two tyrosyl) are required for catalytic activity and allosteric properties of fructose 1,6-diphosphatase.

According to recent findings, the active oligomer of glutamate dehydrogenase is composed of six noncovalently linked subunits, probably identical, with mol wt 53,000 (Marler and Tanford, 1964; Appella and Tomkins, 1966; Eisenberg and Tomkins, 1968); thus, each hypothetical functional subunit on average should have three identical and separate sets of one lysyl and one-half tyrosyl residue associated with catalytic activity and regulatory properties. Therefore, although the subunits may be identical in primary structure, they probably differ in some way in terms of function, since only one tyrosyl residue per pair of subunits appears to be necessary for the reactivity of each type of site. The fact that the subunits do not seem to be equivalent may arise from their spatial assembly in the oligomer. One cannot, however, rule out the possibility that the subunits do indeed differ in primary structure from one another, since it is conceivable that the criteria used to establish that they are identical (end-group analysis, "fingerprinting") would fail to reveal fine differences, such as replacement or different location of a few amino acid residues.

Acetylation of glutamate dehydrogenase has been reported by Colman and Frieden (1966b) to cause complete inactivation when 5.3 amino groups per subunit had been acetylated. The enzyme was maintained in active form by performing acetylation in the presence of GTP and $NADH_2$; this active enzyme contained only 3.5 acetylated amino groups per subunit and therefore the authors concluded, in agreement with our findings, that one to two amino groups per peptide chain are involved in the catalytic function of the enzyme. Since the absorption at 280 $m\mu$ remained unaltered, Colman and Frieden also concluded that tyrosyl residues were not acetylated.

Involvement of $\epsilon$-amino groups of lysine residues in the catalytic activity of the enzyme is substantiated by the findings of Anderson *et al.* (1966), who reported that glutamate dehydrogenase is inactivated by incubation with pyridoxal 5′-phosphate. This reagent was found combined in a Schiff base with $\epsilon$-amino groups of lysine; after reduction, acid hydrolysis of the pyridoxyl enzyme yielded $\epsilon$-pyridoxyllysine as the major derivative present in the hydrolysate; furthermore, Holbrook and Jeckel (1969) determined the sequence of a peptide containing an essential lysyl residue, following inactivation with N-substituted maleimide. Recently, Brocklehurst *et al.* (1970) confirmed the presence on the enzyme molecule of catalytically essential amino groups, on the grounds of inactivation occurring during reaction with a specific amino group reagent. Since the response to GTP inhibition was lost by reacting the enzyme with tetranitromethane or *N*-acetylimidazole, the authors also suggested that tyrosine forms part of the GTP site, in agreement with our results.

Whether any or some of the lysyl and tyrosyl residues found associated with the catalytic and allosteric properties of glutamate dehydrogenase are directly involved in the binding of NAD, ADP, and GTP, or whether they are merely essential to maintain the proper conformation of the sites, cannot yet be established unequivocally from the present data. In view of the similarities in chemical structure of the respective ligands, the fact that these sets of residues are identical is not surprising; an analogous situation has been reported (Rosen and Rosen, 1966) in desensitization and inactivation of fructose 1,6-diphosphatase. Future studies will be aimed at clarifying whether this peculiarity reflects the existence of three regions of rather similar sequence in the polypeptide chain, or whether the major role is played by secondary and tertiary structure.

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