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Identification of the Sites of Modification of Bovine Liver Glutamate Dehydrogenase Reacted with Trinitrobenzenesulfonate*

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ABSTRACT: Bovine liver glutamate dehydrogenase has been reacted with trinitrobenzenesulfonate and the primary sites of modification identified as lysine-428 and lysine-425 in the tentative amino acid sequence proposed by Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Jr., Langley, T. J., and Mclained, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724). Lysine-428 reacts most rapidly but only to the extent of 0.5 group/polypeptide chain (*i.e.*, three of the six subunits in the active monomer are modified). The reaction at lysine-425 proceeds more slowly but also reaches an end point of only 0.5 group/polypeptide chain. Examination of the rate and extent of incorporation of trinitrobenzenesulfonate into these residues indicates that lysine-425 may be essentially unavailable for modification until the available lysine residues as position 428 have been trinitrophenylated. Sequence analysit of the tryptic peptides containing these residues shows that the modifications at positions 428 and 425 occur on different polypeptide chains. There appears to be no polypeptide chain in which both these residues are modified. No difference in the extent of modification of lysyl residues 428 or 425 was observed when nucleotide ligands which protect against loss of catalytic activity during trinitrophenylation were added to the reaction mixture. Procedures for the purification of relatively large trinitrophenylated peptides on a preparative scale in good yields, which have been largely developed in these experiments, are described.

Bovine liver glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) is a complex enzyme with respect to both its regulatory and physical properties. Substrate inhibition and activation by excess NADH and NAD^+, respectively, as well as the modulation of enzyme activity by the guanosine and adenosine nucleotides have been well established (Frieden, 1959, 1965). The smallest active molecular unit (the monomer) appears to consist of six identical polypeptide chains each having a molecular weight of 56,100 (Smith *et al.*, 1970). It is well known that the active monomer undergoes a concentration dependent polymerization which has been extensively investigated (Eisenberg and Tomkins, 1968; Colman and Frieden,

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1966; Sund *et al.*, 1969; Cassman and Schachman, 1971). Furthermore, the equilibrium between the monomer and polymer can be strongly affected by the purine nucleotides. In the presence of NADH, GTP binds preferentially to the monomer while ADP binds preferentially to the polymer (Frieden and Colman, 1967), and it was proposed that the ability of the enzyme to associate may be an important factor in the control of enzymatic activity by excess substrate or purine nucleotides.

In an effort to relate the effects of excess NADH and purine nucleotides to the structure of the enzyme, chemical modification studies have been carried out using trinitrobenzenesulfonate (TNBS).[1] The choice of TNBS was prompted by both its reported specificity for lysyl residues (Kotaki *et al.*, 1964) and the apparent ease with which the modifications could be quantitated (Goldfarb, 1966).

Freedman and Radda (1969) have previously used TNBS to chemically modify glutamate dehydrogenase and concluded that there is a rapidly reacting lysyl residue which, when modified, results in the loss of catalytic activity, altered polymerization properties, and desensitization to GTP inhibition. More recently, Clark and Yielding (1971) have reported that the reaction with TNBS to the extent of one group per polypeptide chain results in a modified enzyme which is desensitized to GTP inhibition and has altered polymerization properties but still retains 80% of its catalytic activity. However, no attempt was made to identify the reactive lysyl residues in either of these studies.

In this investigation, it will be shown that treatment of bovine liver glutamate dehydrogenase with TNBS results in the modification of lysyl residues 428 and 425 in the proposed tentative sequence of Smith *et al.* (1970). In the subsequent paper (Goldin and Freiden, 1971), it will be shown that modification of lysine-428 results in depolymerization of the enzyme and altered regulatory properties but little or no effect on catalytic activity, whereas modification of lysine-425 has no apparent effect on either the regulatory or catalytic properties but may be related to the altered polymerization properties of the modified enzyme.

Materials and Methods

Materials. Bovine liver glutamate dehydrogenase was purchased as a crystalline preparation from Sigma. Trypsin, treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, carboxypeptidases A and B, and chymotrypsin were obtained from Worthington. Thermolysin was obtained from Daiwa Kasei K. K., Osaka, Japan.

TNBS, NADH, GTP, and α-*N*-acetyllysine were supplied by Sigma. Ultra Pure guanidine hydrochloride was purchased from Mann. Iodoacetic acid was recrystallized from petroleum ether (bp 30–60°). Pyridine was redistilled from a solution containing solid ninhydrin (1 g/l.). Methyl isothiocyanate was obtained from Eastman and recrystallized by vacuum sublimination. ϵ-TNP-lysine and α-TNP-alanine were synthesized according to the method of Okuyama and Satake (1960). Polyamide sheets were purchased from Gallard-Schlesinger. All solvents used in the Edman degradations were purified according to the procedures of Edman and Begg (1967).

Determination of Sulfhydryl Content. The sulfhydryl content of native and modified enzyme was determined spectrophotometrically by the procedure of Ellman (1959). Sulfite produced during trinitrophenylation was found to interfere with the sulfhydryl determination. Correction for this effect was made by dividing the reaction mixture into two equal parts, adding DTNB to both solutions and sodium dodecyl sulfate or guanidine hydrochloride to the sample cell. The denaturant-free solution was used as a reference when reading the absorbance at 410 nm. Bitensky *et al.* (1965) have shown that DTNB does not react with native glutamate dehydrogenase.

Measurement of Extent of Trinitrophenylation. It was essential that the extent of trinitrophenylation in both the kinetic studies and the stoichiometry studies be measured accurately. These measurements were based on the spectral characteristics of the trinitrophenylated derivatives, although some problems were encountered since trinitrophenylation results in the formation of sulfite which, in turn, changes the absorbancy characteristics of the trinitrophenyl group. However, as shown by Goldfarb (1966) and confirmed in our studies, there is an isosbestic point at 367 nm which is independent of sulfite concentration. The molar extinction coefficient of ϵ-TNP-α-acetyllysine in neutral solutions is 1.05×10^4 at this wavelength. In formic acid it has been shown (Goldfarb, 1966) that this derivative has a molar extinction coefficient of 1.45×10^4 at 345 nm. The ratio of the absorbance of trinitrophenylated glutamate dehydrogenase at 367 nm at neutral pH values to that at 345 nm in formic acid is only slightly different from that of ϵ-TNP-α-acetyllysine, indicating that the protein had little effect on the spectral characteristics of the trinitrophenyl group. Assuming that the extinction coefficients of trinitrophenylated enzyme and the model compound are identical in formic acid, a molar extinction coefficient of 1.08×10^4 for trinitrophenylated glutamate dehydrogenase at 367 nm was calculated. The extent of trinitrophenylation as a function of time can therefore be conveniently monitored at 367 nm.

It is also possible to follow the trinitrophenylation reaction at 420 nm. Measurements at this wavelength were necessary when the TNBS modification was performed in the presence of high levels of NADH which absorbs strongly at 367 nm. The difficulty encountered at 420 nm arises from the fact that sulfite, produced by the trinitrophenylation reaction, not only alters the absorbance of the trinitrophenyl group but also may bind to the enzyme. That this is the case is shown by the fact that the 367:420 ratio changes (approximately 25%) linearly as the course of trinitrophenylation proceeds. Therefore, when the reaction was monitored at 420 nm, the change in the 367:420 ratio was measured and the appropriate corrections were made.

When the reaction was carried out in the presence of NADH, an additional correction at 420 nm was necessitated by the fact that glutamate dehydrogenase catalyzes the desulfonation of TNBS to trinitrobenzene and sulfite. The resulting TNB–sulfite complex also absorbs at 420 nm (Bates *et al.*, 1970). This correction can be made by following the desulfonation reaction at 530 nm and correcting the absorbance at 420 nm, having previously determined the 530:420 ratio for the trinitrobenzene–sulfite complex.

These corrections were necessary only when following the time dependence of the modification reaction. In the experiments which required only a measurement of the extent of modification, the modified enzyme was subjected to filtration through columns of Sephadex G-25 followed by exhaustive dialysis in order to remove the sulfite and NADH.

The fact that the extent of modification determined on enzyme preparations which were free of sulfite and NADH

[1] Abbreviations used are: TNBS, trinitrobenzenesulfonate; TNP-, trinitrophenyl-; MITC, methyl isothiocyanate; MTH, methylthiohydantoin; DNS-, 1-dimethylaminonaphthalene-5-sulfonyl-; ASPM, *N*-(*N'*-acetyl-4-sulfamoylphenyl)maleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

agreed with the extent of modification determined by the procedures outlined above underscores the validity of corrections used in the kinetic studies. Values of the number of groups modified by TNBS obtained without these corrections (Clark and Yielding, 1971) may be slightly incorrect.

Preparation of TNP-glutamate Dehydrogenase. Crystalline glutamate dehydrogenase was centrifuged; the crystals were dissolved in a minimal volume of 0.04 M potassium phosphate buffer (pH 8.0). The residual ammonium sulfate was removed by filtration through Sephadex G-25. Unless otherwise indicated, the desalted enzyme was diluted to a concentration of 1 mg/ml in 0.04 M potassium phosphate at pH 8.0 and 25°. Protein concentration was determined by the microbiuret procedure of Itzhaki and Gill (1964). When the modification was carried out in the presence of NADH and GTP, the concentrations of these reagents in the reaction mixture were 600 and 500 μM, respectively. The trinitrophenylation reaction was initiated by the addition of TNBS (10 mM) to give a final concentration of 1 mM. The reaction was terminated at the desired extent of modification by the addition of 10-fold excess β-mercaptoethanol. The resulting solution was passed over a column of Sephadex G-25 (2.5 × 50 cm) which had been previously equilibrated with 0.04 M potassium phosphate buffer (pH 8.0).

Preparation of TNP-peptides. Cysteinyl residues were carboxymethylated in 0.05 M potassium phosphate (pH 8.0) by treatment with iodoacetate for 5 min in the presence of 6 M guanidine hydrochloride. The alkylation was terminated with 10-fold excess β-mercaptoethanol, and the protein was dialyzed exhaustively against deionized water and recovered after lyophilization. The lyophilized protein was suspended in 5 ml of deionized water and the pH was raised to 11.0 with 1 M NaOH. After approximately 5 min at the elevated pH, complete solubilization was achieved and 1 N HCl was added dropwise until the desired pH was reached. All digestions were carried out in a pH-Stat (Radiometer TTT1) at 37°. Tryptic digestions were carried out at pH 8.9 with 2% (w/w) trypsin for 6 hr. Digestion with thermolysin was performed at pH 8.0 for 16 hr with 5% (w/w) thermolysin. Chymotryptic fragments were prepared from purified tryptic peptides by digestion, with 1% (w/w) chymotrypsin at pH 8.9 for 3 hr. In each case, the reaction was terminated by acidification to pH 2.0 with 6 N HCl. Any insoluble material was removed by centrifugation.

Fractionation of TNP-peptides. The acid-soluble peptides from both the tryptic and thermolytic digests were fractionated on columns (2.0 × 85 cm) of Sephadex G-50. The column was equilibrated with 0.1 M acetic acid prior to the addition of the sample. Elution was carried out with 0.1 M acetic acid at a flow rate of 24 ml/hr. For the large-scale preparation of tryptic peptides, a 5 × 150 cm column of Sephadex G-50 was used and the column was eluted at a flow rate of 150 ml/hr. The total peptide in the effluent was monitored by its absorbance at 220 nm and the TNP-peptide was located by its absorbance at 345 nm.

Subfractionations of tryptic peptides were carried out on SE-Sephadex C-25, Dowex 50-X8 (Spinco amino acid analyzer resin AA-15), or Dowex 1-X2. The SE-Sephadex C-25 columns (2 × 25 cm) were equilibrated with 0.05 M pyridine–acetate (pH 2.4) and developed at 55° with a linear gradient as previously described by Bradshaw *et al.* (1969a) of 0.05 M pyridine–acetate (pH 2.4) to 0.5 M pyridine–acetate (pH 3.75). The total volume of the gradient was 500 ml. When a second fractionation on SE-Sephadex was required to obtain pure tryptic peptides, the column (2.0 × 25 cm) was equilibrated with 0.05 M pyridine–acetate (pH 4.0). After the sample was applied, the column was washed with 80 ml of the same buffer and then developed at 55° with a linear gradient from 0.05 M pyridine–acetate (pH 4.0) to 0.50 M pyridine–acetate (pH 7.0). The total volume of the gradient was 500 ml. All SE-Sephadex columns were eluted at a flow rate of 20 ml/hr.

Dowex 50-X8 columns (0.9 × 17 cm) were equilibrated with 0.05 M pyridine–acetate (pH 2.4) before addition of the sample. Elution was carried out at 55° and a flow rate of 30 ml/hr. A continuous gradient composed of 150 ml of 0.05 M pyridine–acetate (pH 2.4), 150 ml of 0.50 M pyridine–acetate (pH 3.75), and 150 ml of 2.0 M pyridine–acetate (pH 5.0) was used.

Dowex 1-X2 columns (0.9 × 50 cm) were equilibrated with 3% pyridine and developed at 35° with a 400-ml linear gradient from 3% pyridine (pH 9.0) to 0.5 M pyridine–acetate (pH 5.5). A flow rate of 30 ml/hr was used.

Subfractionations of thermolytic peptides were carried out on 0.9 × 17 cm columns of Dowex 50-X8. The columns were equilibrated with 0.05 M pyridine–acetate (pH 2.4) prior to sample application. Elution was carried out at 55° and a flow rate of 30 ml/hr. A continuous gradient consisting of 150 ml of 0.05 M pyridine–acetate (pH 2.4), 150 ml of 0.20 M pyridine–acetate (pH 3.1), 150 ml of 0.50 M pyridine–acetate (pH 3.75), and 150 ml of 2.0 M pyridine–acetate (pH 5.0) was used.

In all of the subfractionations, the eluent was monitored for TNP-peptide by reading the absorbance at 345 nm. The profile of the total eluted peptide was monitored at 570 nm with a Technicon AutoAnalyzer equipped for alkaline hydrolysis and ninhydrin analysis (Hill and Delaney, 1963).

Amino Acid Compositions. Samples were hydrolyzed in evacuated glass tubes at 110° in 6 N HCl for 24 hr. The HCl was removed by rotary evaporation and the hydrolysates were analyzed on a Spinco 120 automatic amino acid analyzer. The elution time of the trinitrophenylated amino acid was determined on a 0.9 × 8 cm column eluted with sodium citrate buffer (pH 5.28). TNP derivatives were monitored by diverting the flow from the bottom of the column to an external fraction collector. Fractions were collected at 1-min intervals and read at 345 nm in a Zeiss spectrophotometer. The measured time lag between elution and print-out in the normal operating mode of the analyzer was 8 min, which was added to obtain an elution time for the trinitrophenylated amino acid.

End-Group Analyses. Edman degradations were carried out according to the procedures of Waterfield and Haber (1970). MTH-amino acids were identified by gas chromatography on a Varian Model 2100 gas chromatograph. If a positive identification of the MTH-amino acid could not be made, an aliquot of the residual peptide was hydrolyzed and a subtractive amino acid analysis was performed. In some cases, the N terminus of the peptide was determined as the DNS-amino acid prior to Edman degradation. The dansylation reactions were carried out using a modified version[2] of the Hartley procedure (1970).

Digestions with carboxypeptidases A and B were carried out under the conditions described by Bradshaw *et al.* (1969b). Aliquots were removed after 30 min and 4 hr, acidified to pH 2.2, and analyzed for released amino acids.

Results

Nature of Groups Modified with TNBS. Since TNBS can react with both thiol and amino groups (Okuyama and Satake,

[2] Personal communication from Dr. Keith Brew.

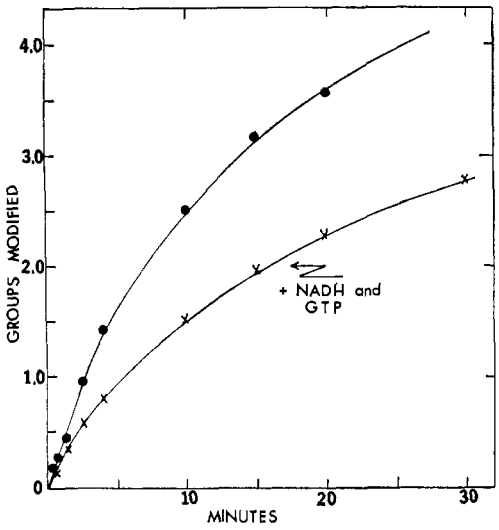

FIGURE 1: Kinetics of trinitrophenylation of bovine liver glutamate dehydrogenase. The reaction was carried out both in the absence and presence of NADH (600 μM) plus GTP (500 μM). In both cases, the enzyme concentration was 1 mg/ml in 0.04 M potassium phosphate (pH 8.0) and 25°. The extent of modification was calculated from the extinction coefficients at 367 nm (in the absence of NADH and GTP) and 420 nm (in the presence of NADH and GTP). The details are described in Methods.

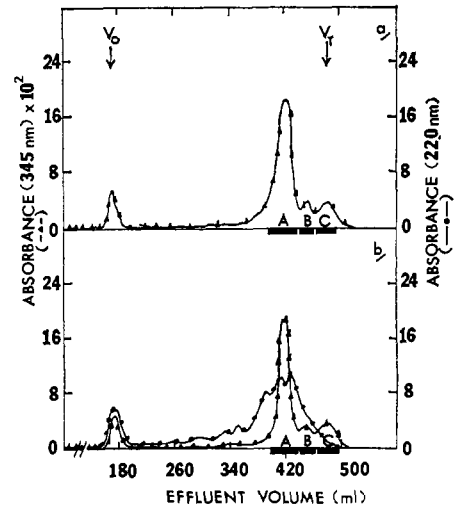

FIGURE 2: Elution profiles of the soluble tryptic TNP-peptides from bovine liver glutamate dehydrogenase on a 2.0 × 85 column of Sephadex G-50. The enzyme was modified to the extent of 0.57 group/polypeptide chain. Modification was performed (a) in the presence of 600 μM NADH and 500 μM GTP, and (b) in the absence of NADH and GTP. Fractions were monitored for TNP-peptides and total peptide by the absorption at 345 and 220 nm, respectively. Modification was carried out as described in the legend to Figure 1.

1960), it was important to establish the specificity of the reaction with glutamate dehydrogenase. Determination of the sulfhydryl groups using Ellman's reagent indicates about 6.1 groups/56,000 g of enzyme regardless of the extent of modification by TNBS. These results agree with the number of cysteinyl residues known to be present in the subunit (Smith *et al.*, 1970) and confirm a similar observation by Freedman and Radda (1969) that TNBS does not react with the cysteinyl residues of this enzyme.

To determine the residue(s) modified, the elution times of the modified amino acid obtained after acid hydrolysis of TNP-peptides were compared to those of several trinitrophenyl derivatives. The TNP-amino acid of glutamate dehydrogenase had an elution time of 47 min, identical with that of an acid hydrolysate of authentic ϵ-TNP-lysine. About 12% of the effluent absorbing at 345 nm had an elution time equal to that of trinitrophenol (16 min), in good agreement with the results of Kotaki and Satake (1964) who reported a 17% reversion of ϵ-TNP-lysine to trinitrophenol and free lysine after 6-hr hydrolysis in 6 N HCl at 110°. However, the acid hydrolysate of ϵ-TNP-lysine has a different elution time (47 min) from that of unhydrolyzed ϵ-TNP-lysine (14 min), indicating that although acid hydrolysis results in only a slight degree of reversion to trinitrophenol and lysine, there is a conversion of ϵ-TNP-lysine into another product. This degradation product was not further characterized. The absence of significant amounts of trinitrophenol in the acid hydrolysates of modified enzyme would appear to eliminate the N-terminal alanine as a site of modification since TNP-alanine has been reported (Kotaki and Satake, 1964), and confirmed in our studies, to revert quantitatively to trinitrophenol and alanine upon acid hydrolysis. Thus, the trinitrophenylation of glutamate dehydrogenase appears to be specifically directed only to ϵ-NH_2-lysyl residues.

Kinetics of Trinitrophenylation. Figure 1 shows the time course of modification of glutamate dehydrogenase by 1 mM TNBS in the presence and absence of ligands which prevent loss of enzymatic activity (Goldin and Frieden, 1971). These experiments were performed at pH 8 in 0.04 M potassium phosphate buffer at 25°. In neither case can the data be expressed as a simple first-order rate process. However, analysis of the data through use of first-order plots (Ray and Koshland, 1960) indicates that initially the curves are practically identical. Thus, in both cases, between 0.5 and 0.8 group appear to be modified per polypeptide chain with an apparent first-order rate constant of 0.4–0.5 min^{-1}. In the absence of ligands, another 1.2 groups appear to be modified with an apparent first-order rate constant of 0.18 min^{-1}. This can be seen from the data in Figure 1 in that at 20–30 min there is a difference of 1.0–1.5 in the number of groups modified. At longer time periods, both reactions proceed slowly and with approximately the same apparent rate constant (3×10^{-3} min^{-1}).

The fact that the first 0.5–0.8 group reacts essentially identically in both cases would suggest that the residues modified may be the same. The data shown below confirm this conclusion.

Identification of Sites of Modification. NUMBER OF REACTIVE LYSYL RESIDUES. In view of the fact that bovine liver glutamate dehydrogenase has 33 lysyl residues (Smith *et al.*, 1970), the most reliable means of determining the number of residues modified by TNBS would be to examine the distribution of the trinitrophenyl label in peptides derived from the enzyme modified to varying extents. For these experiments, 100 mg of enzyme was reacted with TNBS both in the presence and absence of ligands (*i.e.*, NADH and GTP) which protect the enzyme against loss of activity. The elution profiles from Sephadex G-50 columns of the tryptic peptides from enzyme modified to 0.57 and 1.4 groups per polypeptide chain are shown in Figures 2 and 3, respectively. In Figures 2a and 3a, the modification was carried out in the presence of NADH and GTP, and in Figures 2b and 3b, these ligands were omitted. Recovery of the labeled peptides from Sephadex G-50 columns was greater than 90%.

It is clear from Figure 2 that at a limited extent of modification (0.57 group) most of the label elutes in a single peak

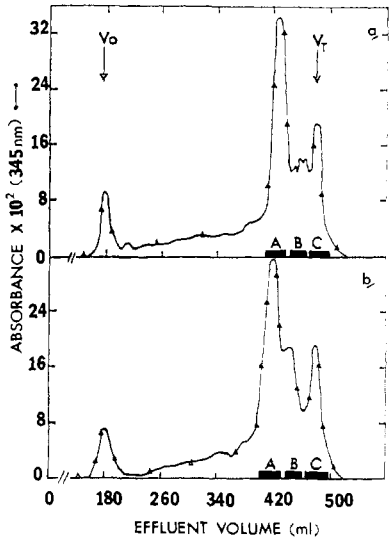

FIGURE 3: Elution profiles of the soluble tryptic TNP-peptides from bovine liver glutamate dehydrogenase on Sephadex G-50. The enzyme was modified to the extent of 1.4 group/polypeptide chain. Modification was performed (a) in the presence and (b) in the absence of NADH and GTP. Details as described in Figure 1.

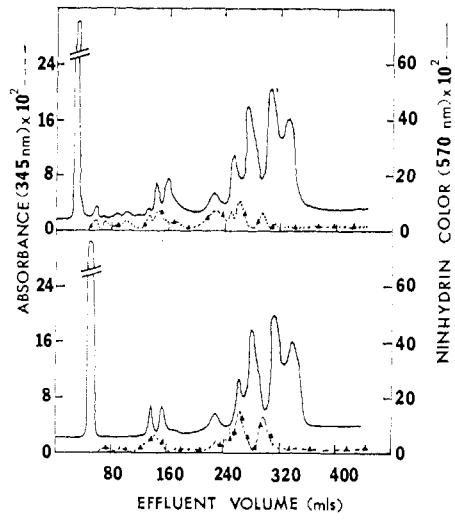

FIGURE 5: Elution profile of the separation of pool B (Figure 2) on 2.0 × 25 cm columns of SE-Sephadex C-25. Details as in Figure 4.

(designated A). By comparison of Figure 2a,b, it would appear likely that NADH and GTP do not alter the specificity of modification. The most prominent difference in the peptide profiles for 1.4 groups modified per polypeptide chain (Figure 3a,b) and 0.57 group (Figure 2a,b) is the change in the relative amounts of labeled peptide in the regions designated as A and C. For example, in going from 0.57 to 1.4 groups, the fraction of the total label in region A is decreased from 63 to 35% while that in region C is increased from 14 to 20%. Although there is an appreciable increase of label in the intermediate region B, the shape of the profile in Figure 3a suggests that this may be a heterogeneous mixture of modified peptides. Except for the B region, there is a close similarity in the elution profiles of Figure 3a,b, suggesting that even at the higher extent of modification, NADH and GTP do not markedly affect either the residues which are reactive or the extent to which they are modified.

Subfractionations of the pools A, B, and C derived from enzyme modified to the extent of 1.4 groups (Figure 3a,b) are shown in Figures 4–6, respectively. These figures show pools A and C to be quite homogeneous while pool B contains a number of trinitrophenylated peptides, none of which is modified to a significantly greater extent than any other. Although yields of 50–60% were obtained in the subfractionations, these yields do not represent the loss of another highly labeled peptide but are attributed to general chromatographic losses (as will be shown later). Therefore, on the basis of these data, it can be concluded that at the extent of 1.4 groups modified per polypeptide chain, there are two lysyl residues which are highly reactive with TNBS and that these same

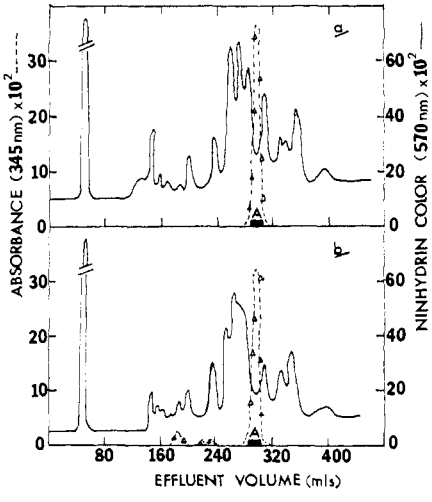

FIGURE 4: Elution profile of the separation of pool A (Figure 2) on 2.0 × 25 cm columns of SE-Sephadex C-25. The columns were developed at 55° with a gradient from 0.05 M pyridine–acetate (pH 2.4) to 0.5 M pyridine–acetate (pH 3.75) as described in the text. Modification was performed (a) in the presence and (b) in the absence of NADH and GTP. Fractions were monitored for TNP-peptide by the absorption at 345 nm and for total peptide by ninhydrin analysis at 570 nm after alkaline hydrolysis.

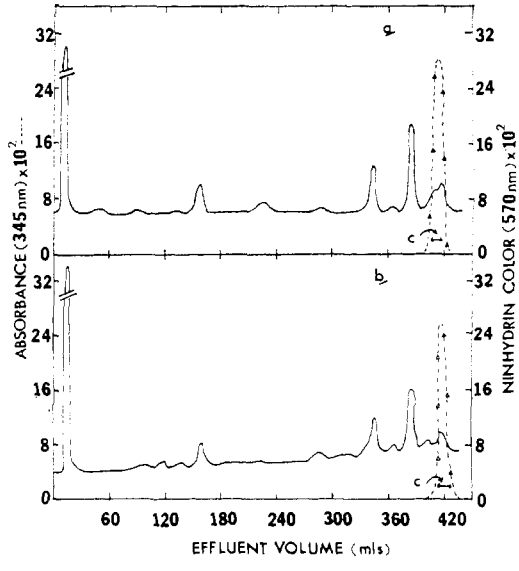

FIGURE 6: Elution profile of the separation of pool C (Figure 2) on 0.9 × 17 cm columns of Dowex 50-X8. The columns were developed with a pyridine–acetate gradient at 55° as described in the text. Other details as in Figure 4.

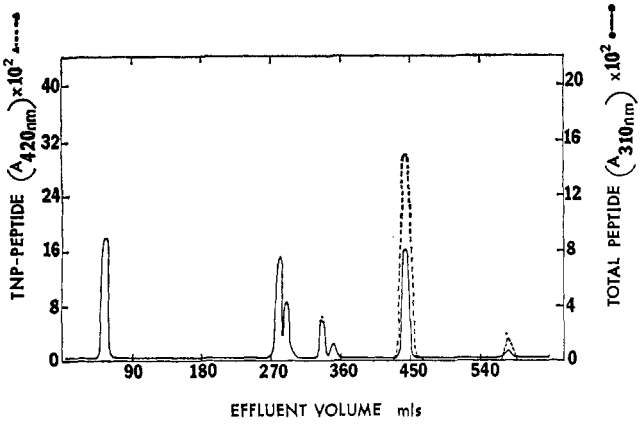

FIGURE 7: Elution profile of the fractionation pool A (Figure 4) on a 2.0 × 25 cm column of SE-Sephadex. The column was developed at 55° with a linear gradient from 0.05 M pyridine–acetate (pH 4.0) to 0.5 M pyridine–acetate (pH 7.0). Fractions were monitored for total peptide by the microbiuret assay (Itzahaki and Gill, 1964) and for TNP-peptide by the absorption at 420 nm.

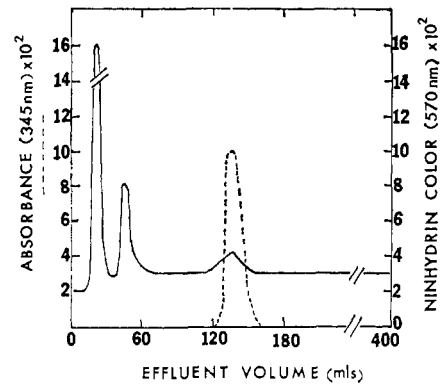

FIGURE 8: Elution profile of the fractionation of pool C (Figure 6) on a 0.9 × 50 cm column of Dowex 1-X2. The column was developed at 35° with a continuous gradient of pyridine–acetate as described in the text. The fractions were monitored for TNP-peptide by their absorption at 345 nm and for total peptide by ninhydrin analysis after alkaline hydrolysis at 570 nm.

residues are modified regardless of whether NADH and GTP are included in the reaction mixture. Furthermore, the differences in the profiles of Figures 2 and 3 suggest that at the lower extent of modification, one of these residues is considerably more reactive than the other.

Purification of the Trinitrophenylated Peptides. In order to determine the sequence of the peptide containing the more reactive residue, large-scale purifications were required. For this purpose, 500 mg of enzyme (5 mg/ml) was reacted with 500 μM TNBS to the extent of approximately 0.7 group/polypeptide chain. All subsequent steps in preparing the modified enzyme were carried out under the conditions described above.

The soluble tryptic peptides (over 90% of the total material) were applied to a 5 × 150 cm column of Sephadex G-50 and eluted with 0.1 N acetic acid at a flow rate of 150 ml/hr. The elution profiles of both the trinitrophenylated peptide and the total peptide were essentially identical with those shown in Figure 2b. Pools A and C (comparable to those in Figure 2b) were made and further purified as described below.

POOL A. After concentration, the mixture of peptides was subfractionated on SE-Sephadex C-25 under conditions identical with those described in Figure 4. Fractions containing the trinitrophenylated peptide were pooled. After standing overnight, the pool contained a white flocculent precipitate which was removed by filtration through a Millipore filter. The pool was found to contain at least five peptides as judged by high-voltage electrophoresis in pyridine–acetate buffer at pH 3.75 and descending chromatography in the second dimension using 1-butanol–pyridine–acetic acid–water (Bennett, 1967). However, only one peptide exhibited the characteristic yellow color of the trinitrophenyl group. Further fractionation was achieved on another column of SE-Sephadex which had been equilibrated with 0.05 M pyridine–acetate (pH 4.0) prior to applying the sample. The column was washed with 80 ml of the same buffer, then developed at 55° with a linear gradient from 0.05 M pyridine–acetate (pH 4.0) to 0.5 M pyridine–acetate (pH 7.0). The results of this fractionation are shown in Figure 7. The trinitrophenylated peptide was judged to be pure by the criteria of both high-voltage electrophoresis and amino acid composition.

POOL C. In view of the position of elution of this pool on Sephadex G-50 which suggested that the peptides present were in the range of two to five residues, it was considered probable that losses due to strong interaction of the TNP group with Dowex-type resins would not be nearly as great as with the larger peptides. Thus, in order to take advantage of the higher resolution of Dowex 50-X8, the first subfractionation of pool C was carried out on this resin as described in Figure 6. High-voltage electrophoresis (pH 3.75) of pool C (Figure 6) indicated a single trinitrophenylated peptide and one nonlabeled peptide. This mixture was separated on Dowex 1-X2. The elution profiles are shown in Figure 8.

A summary of the steps involved in the purification of these trinitrophenylated peptides and the recoveries at each step is given in Table I.

The modified peptides recovered from pools A and C are designated as TNP-peptide A and TNP-peptide C, respectively.

TABLE I: Purification of TNP-peptides from Bovine Liver Glutamate Dehydrogenase.

	TNP-peptide A		TNP-peptide C	
Step	TNP-peptide (μmoles)	% Recov[a]	TNP-peptide (μmoles)	% Recov[a]
Sephadex G-50	3.00	100	1.20[b]	100
SE-Sephadex (first fractionation)	1.40	47		
SE-Sephadex (final fractionation)	0.78	28		
Dowex 50-X8			0.74	62
Dowex 1-X2			0.18	15

[a] Since more than 90% of the total labeled peptide applied to the Sephadex G-50 column was recovered, the pools A and C were assumed to represent all of these two peptides. [b] TNP-peptide C was not purified from the large-scale preparation used for peptide A. Rather, the C pools from several small-scale columns were combined and further fractionated together.

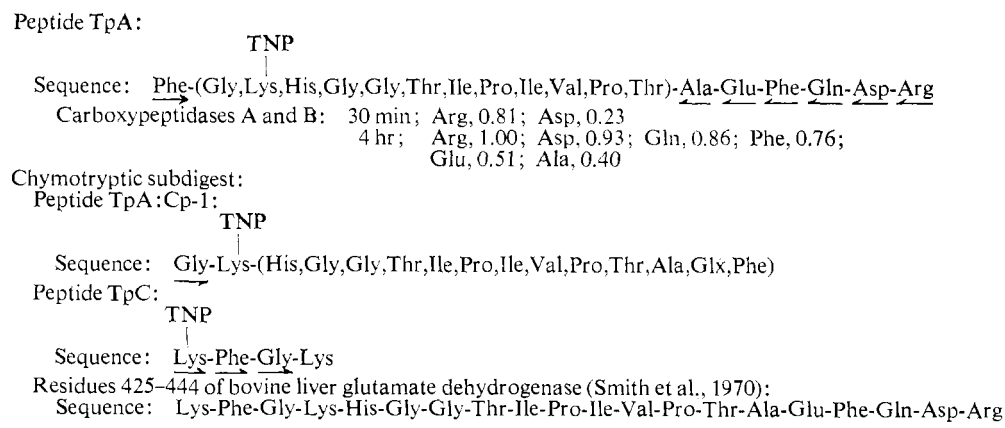

FIGURE 9: Characterization of TNP-peptides A and C. The single-headed arrows refer to assignments by dansylaiortion (—→) pr to Edman degradation, direct analysis (—→), or subtractive amino acid analysis (→) following Edman degradation, and carboxypeptidase A and B hydrolysis (←).

TABLE II: Amino Acid Compositions of TNP-peptides from Bovine Liver Glutamate Dehydrogenase.[a]

Amino Acid	TpA	TpA/Cp-1	TpA/Cp-2	Residues 426–444 of Glutamate Dehydrogenase[c]	TpC	Residues 425–428 of Glutamate Dehydrogenase[c]
Lysine				1	1.04 (1)	2
TNP-lysine	1.06[b] (1)	1.00[b] (1)			1.00[b] (1)	
Histidine	1.02 (1)	0.98 (1)		1		
Arginine	1.00 (1)		1.00 (1)	1		
Aspartic acid	1.06 (1)		0.96 (1)	1		
Threonine	2.03 (2)	1.97 (2)		2		
Glutamic acid	2.09 (2)	1.10 (1)	0.83 (1)	2		
Proline	1.94 (2)	1.92 (2)		2		
Glycine	3.08 (3)	3.06 (3)		3	1.09 (1)	1
Alanine	0.95 (1)	0.94 (1)		1		
Valine	0.96 (1)	0.90 (1)		1		
Isoleucine	2.03 (2)	2.04 (2)		2		
Phenylalanine	1.94 (2)	1.09 (1)		2	1.04 (1)	1
Per cent impurities	<5	<5	<5		<5	
Residue no.	426–444	427–441	442–444		425–428	

[a] Values are given in residues per mole. The assumed integral values are given in parentheses. [b] TNP-lysine content was quantitated from the molar extinction as described in the text. [c] Taken from Smith *et al.* (1970).

Sequence Analysis of TNP-peptide A. IDENTIFICATION OF LYSINE-428. The determination of the sequence of TNP-peptide A (TpA) is summarized in Table II and Figure 9. Amino acid analysis on the residual peptide after one cycle of Edman degradation showed the loss of a phenylalanyl residue and indicated this residue as the N terminus of tryptic peptide A. However, MTH-phenylalanine was found only in trace amounts on gas chromatography and further cycles through the Edman degradation procedure failed to give rise to detectable levels of any MTH-amino acid. Furthermore, the composition of the residual peptide after subsequent degradation was unchanged. These observations suggested that although the initial coupling reaction between the N-terminal residue and methyl isothiocyanate had occurred, the cyclization and concomitant cleavage reactions were blocked. Although the nature of the blockage was not pursued. the ease with which the thiocarbamyl group can be oxidized to prevent further degradation has been previously emphasized (Ilse and Edman, 1963). Because this peptide had been contaminated during Millipore filtration with trace amounts of copper which is known to catalyze the oxidation of sulfur groups, it seemed likely that oxidation had occurred. Further characterization of this peptide was therefore undertaken using chymotrypsin which would be expected to remove the blocked phenylalanine and give rise to two additional peptides by cleavage at the second phenylalanyl residue.

The composition of the two chymotryptic peptides (TpA/Cp-1 and TpA/Cp-2) obtained in this way is given in Table II. The presence of arginine in TpA/Cp-2 placed this peptide at the C terminus. Two Edman degradations on TpA/Cp-1 resulted in the release of MTG-glycine in step 1 and the disappearance of TNP-lysine from the residual peptide after step

2. Thus, in conjunction with the identity of phenylalanine in the N-terminal position and the location of TpA/Cp-2 at the C-terminus (*vide supra*), these data allow the assignment of Phe-Gly-Lys(TNP)- to the amino-terminal portion of the peptide.

The relative yields of amino acids released after digestion of TpA with carboxypeptidases A and B (Figure 9) would indicate the sequence of -Ala-Glu-Phe-Gln-Asp-Arg at the C-terminal end. The composition and partial sequence of TpA are in exact agreement with two adjacent tryptic peptides (Table II) suggested by Smith *et al.* (1970). These are residues 426–444 and have the following sequence: Phe-Gly-Lys-His-Gly-Gly-Thr-Ile-Pro-Ile-Val-Pro-Thr-Ala-Glu-Phe-Gln-Asp-Arg.

The data obtained in this study (Figure 9 and Table II) cannot be accommodated by any other two adjacent tryptic fragments in the molecule, and thus identifies the lysyl residue most reactive with TNBS as lysine-428.

Sequence Analysis of TNP-peptide C. IDENTIFICATION OF LYSINE-425. The amino acid composition and sequence data for the TNP-peptide C (TpC) are summarized in Table II and Figure 9. The first cycle of Edman degradation resulted in a quantitative release of TNP-lysine and positioned this amino acid as the N-terminus of TpC. Dansylation of the residual peptide followed by acid hydrolysis released DNS-phenylalanine. After a second round of Edman degradation and dansylation, DNS-glycine was identified in the acid hydrolysate. These data, combined with the composition of TpC (Table II), permit the assignment of Lys(TNP)-Phe-Gly-Lys as the sequence of the tetrapeptide. The only region in the entire sequence (Smith *et al.*, 1970) that corresponds to this peptide is the one comprising residues 425–428 and has the sequence Lys-Phe-Gly-Lys. Thus, these data identify lysine-425 as the other lysyl residue with an enhanced reactivity toward TNBS.

It should be noted that these two lysyl residues are separated from one another in the linear sequence by only two amino acid residues. *The fact that residue 428 is found in both of the trinitrophenylated peptides TpA and TpC shows that these two peptides must be derived from different polypeptide chains*, and the failure to find a peptide in which both lysine-425 and -428 are modified suggests that the reactions are mutually exclusive within a given subunit. Data supporting this conclusion will be presented below.

Stoichiometry of TNBS Incorporation. It is obvious from Figures 2 and 3 that a major fraction of the TNBS reacted is incorporated into lysyl residues 425 and 428. However, before any correlations can be made between the modification of these residues and changes in the physical and functional parameters (Goldin and Frieden, 1971), it is necessary to quantitate the extent to which each of these residues has been modified at any given extent of total trinitrophenylation. The difficulties encountered in such a quantitation are threefold: (1) when the tryptic digest is acidified there is some acid-insoluble material ("core") which is normally removed by centrifugation, (2) some trinitrophenylated peptide always appears in the void volume of Sephadex G-50 columns (also designated as "core"), and (3) the subfractionation procedures yielding pure TNP-peptides result in recoveries of only 50–60% at each step. Therefore, before a quantitation of TNBS incorporation into residues 428 and 425 is possible, the nature of the material described as "core" must be established and the losses suffered on subfractionations must be defined, *i.e.*, whether they represent general overall losses or the loss of a single, significantly labeled peptide.

That the labeled peptides appearing in both the acid-insoluble core (which can be solubilized by a two- to threefold dilution) and the material in the Sephadex G-50 void volume are actually an aggregate of the peptides appearing in Figure 3 was shown by refractionation of this material on Sephadex G-50. The TNP profiles were essentially identical with those shown in Figure 3.

However, the difficulties described above were best overcome by thermolysin digestion. The average sized peptide expected from the specificity of thermolysin (Bradshaw, 1969) is considerably smaller than that for trypsin, and consequently the amount of insoluble material after acidification of a thermolytic digest would be expected to be small.

Glutamate dehydrogenase (in 100-mg batches) was reacted with TNBS to the extent of 0.5 and 1.5 groups per polypeptide chain. The modified enzyme was digested with thermolysin (5% w/w) and the reaction terminated after 16 hr by acidification to pH 2.0. There was no acid-insoluble material in either case. Aliquots of the digests were subjected to high voltage electrophoresis at pH 3.75 and 6.5 and the TNP-peptides were identified by their visible yellow color. At 0.5 group modified, one strong and one very weak spot were seen. At the higher level of modification, two discrete spots were visible at both pH 3.75 and 6.5. In addition, at the higher extent of modification there was a trail of weak yellow color which was spread over several ninhydrin-positive spots. These results suggested, as did the above experiments, that the TNP-labeled material found in the core of the tryptic digests were large fragments resulting either from incomplete tryptic digestion or from aggregation of the smaller peptides.

These conclusions were verified by fractionation of the thermolytic digests. The digests were chromatographed on Sephadex G-50 columns under conditions identical with those described in Figure 2, and subfractionations were made on columns of Dowex 50-X8. At 0.5 group modified, essentially all of the trinitrophenyl label was localized in a single peptide. At the higher extent of modification (1.5 groups), approximately 50% of the total label was contained in only two peptides, one of which was more heavily labeled than the other (~30 and ~20%). The remaining 50% of the trinitrophenyl label was distributed over several peptides. These data, in conjunction with the data presented in Figure 5, clearly show that all of the trinitrophenyl label in the tryptic digest (both acid soluble and acid insoluble) which cannot be accounted for in lysyl residues 425 and 428 is distributed over a large number of peptides. Furthermore, the appearance of only one or two highly labeled peptides either in high-voltage electrophoresis or in the fractionations on Dowex columns (for 0.5 and 1.5 groups modified, respectively) indicates that the losses suffered on subfractionation are general rather than specific losses.

Thus, for the purpose of quantitating the amount of TNBS incorporated into residues 425 and 428, it was assumed that the profiles of Figures 2 and 3 (omitting the peptides eluting at V_0) were representative of the entire digest. Therefore, the sum of the TNP label over the column was defined as the total label in the tryptic digest.

Quantitation of Modification at Lysine-428 and Lysine-425. The most striking change in the TNP-peptide profiles as the extent of modification is extended beyond 0.5 group is the disproportionate increase of modification at residue 425 relative to 428.

This is illustrated quite clearly in Figures 10 and 11 where the relative distributions between residues 428 and 425 are examined at three different extents of modification. In these experiments, pools from Sephadex G-50 columns containing

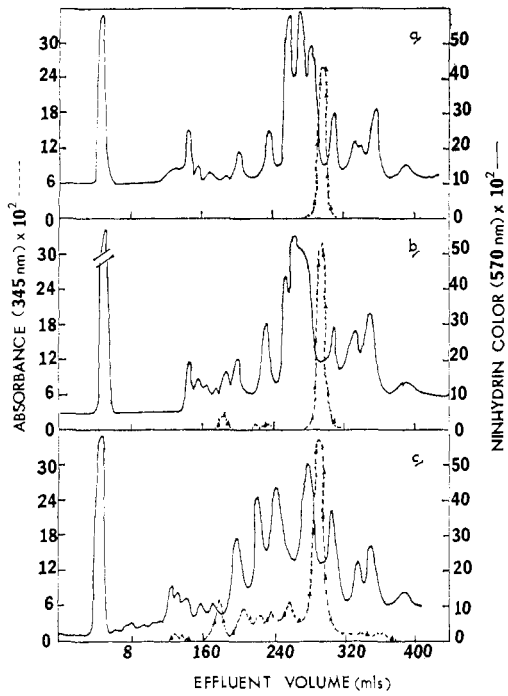

FIGURE 10: Elution profiles of ε-TNP-lysine-428 peptide from SE-Sephadex C-25 at varying extents of modification. The enzyme was modified to (a) 0.57 group, (b) 1.4 groups, and (c) 2.6 groups per polypeptide chain. Other details as in Figure 4.

the tryptic peptides modified at positions 428 and 425 were subfractionated on columns of SE-Sephadex C-25 and Dowex 50-X8 as previously described. In Figure 10, it can be seen that in going from 0.57 group (Figure 10a) to 1.4 groups (Figure 10b), the amount of label incorporated into lysine-428 is increased by only 20%. Upon extended modification from a total of 1.4 groups to 2.6 groups (Figure 10c), there is no further increase. Therefore, 80% of all the reaction which occurs at this residue appears to have occurred by the time 0.57 group/polypeptide chain has been modified.

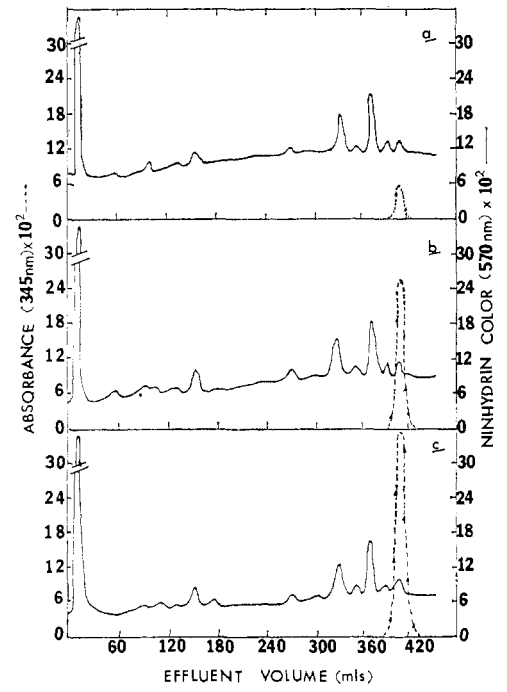

FIGURE 11: Elution profiles of ε-TNP-lysine-425 peptide from Dowex 50-X8 at varying extents of modification. The enzyme was modified to (a) 0.57 group, (b) 1.4 groups, and (c) 2.6 groups per polypeptide chain. Other details as in Figure 6.

TABLE III: Stoichiometry of TNBS Incorporation into Lysine-428 and Lysine-425 of Bovine Liver Glutamate Dehydrogenase.

Extent of Modification (Groups of TNBS Incorpd/ Polypeptide Chain)	Lysine-428		Lysine-425	
	% of Total Label	Residues/Six Subunits[a]	% of Total Label	Residues/Six Subunits[a]
0.40	83	1.99	10	0.24
0.45	77	2.08	13	0.35
0.53	67	2.13	14	0.44
0.57	63	2.16	17	0.66
0.80	58	2.78	18	0.86
1.12	46	3.09	18	1.21
1.41	35	3.01	19	1.63
2.03	25	3.04	24	2.92
2.60	21	3.19	20	3.12

[a] Results given on the basis that the lowest molecular weight form of active enzyme contains six subunits.

On the other hand, the modification of lysine-425 proceeds quite differently. The elution profile in Figure 11a shows that at the level of 0.57 group the amount of label in residue 425 is quite small relative to the amount in residue 428 (Figure 10a). Furthermore, as the total extent of modification is increased from 0.57 to 2.6 groups (Figure 11c) there is a fivefold increase in the amount of label appearing in lysine-425. These data suggest that residue 425 may not be available for modification until lysine-428 has been modified. It should be emphasized that the numbers 0.57, 1.4, and 2.6 refer to the extent of trinitrophenylation of the entire subunit and not to the individual residues. Therefore, the fact that the modification at lysine-428 appears to be 80% complete at the extent of 0.57 group suggests that a maximum of two to three of the subunits per hexamer may be modified at lysine-428.

In order to more accurately assess the extent of modification of each of these lysyl residues, the tryptic peptide profiles from a large number of G-50 columns were examined. The percentage of the total label in the peaks containing residues 425 and 428 was calculated by assuming Gaussian distributions. From the percentage of the total label in each peak and the overall extent of modification, the number of residues modified per hexamer can be calculated. These results are summarized in Table III. It is clear that between two and three residues per six chains are modified very rapidly at lysine-428. On the other hand, the modification of residue 425 proceeds very slowly, if at all, in the initial stages of the reaction. These results are in excellent agreement with the kinetic data (Figure 1) which indicated that approximately 0.5–0.8 group/polypeptide chain are rapidly modified with an apparent constant about five times greater than any other residues. Furthermore, the data in Table III indicate that only half of the subunits are modified

at either residue 428 or 425. The sequence analysis presented earlier clearly demonstrates that the two tryptic peptides containing TNP-lysine-428 and TNP-lysine-425 could not have been derived from the same polypeptide chain. Therefore, three of the six subunits in the active hexamer must be modified at lysine-428 and the other three subunits are modified at lysine-425.

Discussion

The results given in Figures 10 and 11 plus those calculated from a number of separate modification experiments are summarized with respect to the extent of modification of residues 428, 425, and "others" in Figure 12. It is clear that the most reactive residue is lysine-428 and that this residue is modified only to the extent of 0.5 group/polypeptide chain (*i.e.*, three of the six subunits in the active enzyme are modified). The observed lag in the extent of trinitrophenylation of lysine-425 implies that this residue may be available for modification only after lysine-428 has been modified. This implication is strengthened by plots of the log of the extent of modification of each of the residues (428 and 425) *vs.* time. The first-order plots show that modification of residue 428 is strictly first order while there is a clear lag in reactivity of lysine-425. Furthermore, the rate of reaction of lysine-425 does not approach the maximal rate until an average of 0.45 residue of lysine-428 has been modified. If a random distribution of label over the available lysine-428 residues is assumed, it is possible to calculate the rate of increase in lysine-425 reactivity and it appears to be directly related to the number of enzyme molecules having three of the lysine-428 residues modified.

It is important to note that the modification of residues 428 and 425 does not occur on the same polypeptide chain. This is clearly shown by the fact that the tryptic peptide containing TNP-lysine-425 also contains unmodified lysine-428. In addition, no single peptide has been found which is modified at both the 428 and 425 positions. The results in Figure 12, which were quantitated on the basis of TNBS incorporation into two different tryptic peptides, would appear to rule out the possibility of modification occurring at both positions on the same polypeptide chain.

There are two different aspects of structure–function relationships related to these observations. First, only half of the polypeptide chains in the active enzyme can be trinitrophenylated at either position 428 or 425, and second, residue 425 is essentially unreactive until residue 428 has been modified. However, the six subunits are presumably identical (Smith *et al.*, 1970). There are several possible explanations for these results. For example, there may be a conformational change which accompanies the modification of three of the six chains at lysine-428 which prevents further reaction at that residue but at the same time makes lysine-425 available for modification. On the other hand, the relative positions of the six subunits in the active monomer may sterically prevent modification at all six residues at either position. A third possibility, which should not be ignored, is that the six subunits may not be identical in this region of the polypeptide chain.

Of these possible alternatives, it seems that the results obtained here can best be explained in terms of a conformational change in the active monomer, since not only does the lysine-425 depend on previous modification at lysine-428, but there are marked changes in the kinetic and molecular properties of the enzyme after three of the six subunits have been modified at lysine-428 (Goldin and Freiden, 1971). Thus,

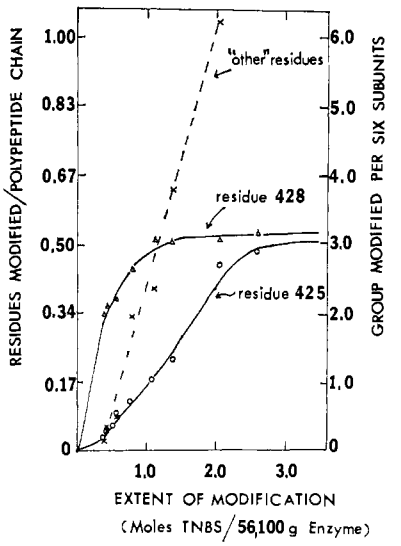

FIGURE 12: Stoichiometry of modification of lysine-428 and -425 as a function of total modification by TNBS. The incorporation of TNBS into the individual residues was calculated as described in the text from a number of different modification experiments.

after modification to the extent of 0.5 group/polypeptide chain, the enzyme depolymerizes and loses some of its regulatory properties such as excess NADH inhibition. Such observations may reflect strong negative cooperativity between subunits which makes subsequent trinitrophenylation at lysine-428 difficult, but allows modification at lysine-425 to proceed easily.

On the other hand, it is possible that the geometry of the subunits in the active enzyme is such that only three of the lysine-428 or lysine-425 residues are exposed at any one time. On the basis of the data available, such a possibility certainly cannot be excluded, and further studies are needed to clarify the situation. In any case, it should be noted that these results may be related to observations with other enzymes in which it would appear that structurally identical subunits are not functionally identical (O'Leary and Westheimer, 1967; Trentham and Gutfreund, 1968; Bernhard *et al.*, 1970).

The data of Figures 5 and 12 show that in addition to residues 428 and 425, there are a number of other lysyl residues modified to some extent by TNBS. However, none of these appears to represent a significant amount of the total label incorporated even when the extent of modification is as high as 2.6 groups/polypeptide chain. It is of interest that the modification of residues 428 and 425 appears to be unaffected by the presence of NADH and GTP, although there are definite changes in the rate of modification of the other less reactive residues. For example, Figure 1 shows an apparent difference of one group in the absence *vs.* the presence of NADH and GTP after 15-min reaction and this must represent a change in reactivity of a number of residues. That there must be some difference in the residues modified in the presence and absence of these ligands is indicated by the fact that the enzyme is protected against loss of activity by NADH and GTP, as discussed in the accompanying paper (Goldin and Freiden, 1971). However, at this point, the less reactive residues have not been characterized.

It is clear from these studies that different lysine residues have different reactivities toward various lysine-modifying reagents. For example, Piszkiewicz *et al.* (1970) have shown that pyridoxal phosphate reacts essentially stoichiometrically

with lysine-97. This same residue has been reported to react with ASPM (Holbrook and Jeckel, 1969). In both of these studies, it was concluded that lysine-97 was essential for catalytic activity. On the other hand, TNBS does not appear to react to a significant extent with lysine-97, but rather is directed toward lysine-428 and -425. Conversely, pyridoxal phosphate or ASPM must not react significantly with residues 428 or 425. It is not clear why pyridoxal phosphate and ASPM should differ from TNBS in their rate of reaction with particular lysyl residues but the most likely explanation would be that there is some specificity arising from the conformation of the protein in the vicinity of these different residues.

It is of interest that residues 428 and 425, aside from being near the C-terminal end of the polypeptide chain (of 506 residues), are quite close, at least in the linear structure, to tyrosine-412. This residue has been reported to react rapidly with tetranitromethane (Piszkiewicz *et al.*, 1971). The similarity of the kinetic properties of the enzyme modified at tyrosine-412 and lysine-428 is discussed in the following paper.

Finally, in view of the difficulties which were encountered in finding chromatographic systems which resulted in reasonable yields of large TNP-peptides, it is noteworthy that SE-Sephadex, eluted at elevated temperatures, appears to be particularly well suited for such purposes. It was found that the ion-exchange resins which are commonly used in peptide purification (Schroeder, 1967) resulted in yields too low to be of practical use. Presumably because of the strong interaction of the trinitrophenyl group with the resin, recoveries of less than 5% were usually found. In some cases, the peptide was irreversibly absorbed and could not be recovered even under drastic stripping conditions. Similar low yields of peptides modified with large hydrophobic groups are obtained when isolation is carried out on paper. The procedure described here, which results in clean fractionations with high yields, does not appear to have been used previously for the fractionation of trinitrophenylated peptides. Since many of the reagents which are used in chemical modification studies introduce aromatic and/or nitro groups (Spande and Witkop, 1970), we suggest that SE-Sephadex under the conditions used here may be a useful tool for the purification of such peptides. A similar suggestion has been made by Walsh *et al.* (1970) for the fractionation of disulfide peptides.

Acknowledgments

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