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The Sulphydryl Groups of Ox Brain and Liver Glutamate Dehydrogenase Preparations and the Effects of Oxidation On Their Inhibitor Sensitivities*

Ivan Couée[1,2] and Keith F. Tipton[1,3]

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Glutamate dehydrogenase preparations from several sources have been shown to have suffered limited proteolysis during purification. This proteolysis has been previously shown to involve removal of the N-terminal tetrapeptide and to result in changes in the regulatory properties of the enzyme. In the present work the previously unidentified N-terminal residue of the unproteolysed enzyme from ox brain and liver is shown to be cysteine. The thiol group of this residue is masked in the native enzyme but it becomes accessible after reduction. Exposure of solutions of the unproteolysed enzyme to air oxidation causes large changes in its sensitivity to inhibition by the antipsychotic drug perphenazine, GTP and by high concentrations of NADH. No such changes occurred in the behaviour of preparations of the enzyme that had suffered proteolysis during purification under these conditions.

KEY WORDS: Glutamate dehydrogenase; sulphydryl groups; perphenazine, GTP; NADH; proteolysis; peptide sequence.

INTRODUCTION

The functions of glutamate dehydrogenase (EC 1.4.1.3) in brain are still far from clear and there is still controversy as to whether the net flux through the reaction is in the direction of ammonia formation or fixation (see 1,2). The enzyme activity is subject to complex allosteric regulation by a variety of effectors, including amino acids such as L-leucine (3,4), nucleoside di- and triphosphates (see 5) and phospholipids (6–8). It is also inhibited by some centrally-acting drugs, such as the phenothiazines and butyrophenones (9–12). Although this inhibition is unlikely to be important in the direct actions of these neuroleptic drugs, an involvement in some of their side-effects cannot be excluded.

Problems in understanding the behaviour of the enzyme have been compounded by the occurrence of limited proteolysis during its purification, which gives rise to an enzyme species with altered kinetic and regulatory properties (5,13-15). This proteolysis has been shown to have occurred in commercially-available preparations of the ox liver enzyme (13), but procedures involving affinity chromatography or affinity precipitation have been devised, that result in apparently homogeneous preparations of the enzymes from ox brain and liver in which the degradation has not occurred (13,16). The proteolysis results in the loss of a tetrapeptide from the amino-terminal end of the polypeptide chains, with the sequence H_2N-X-Asp-Ala-Ala- (13). The amino terminal residue was not identified in that study, although it was suggested that it might be cysteine or cysteic acid (13). This artefactual proteolysis should not be confused with

[1] Department of Biochemistry, Trinity College, Dublin 2, Ireland.
[2] Present address: INRA Centre de Recherche Bordeaux, Station de Physiologie Vegetale, BP 81, 33883 Villenave d'Ornon Cedex, France.
[3] Address reprint requests to: K. F. Tipton, Department of Biochemistry, Trinity College, Dublin 2, Ireland.
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the processing of the newly-synthesized enzyme precursor protein, which has been shown to be active (17), associated with its insertion into the mitochondrion. In the present paper we report the positive identification of this residue in ox brain and liver glutamate dehydrogenase as cysteine. Exposure of the enzyme to air is shown to result in decreased sensitivity to inhibition by perphenazine, GTP and high concentrations of NADH without any apparent change in the specific activity.

EXPERIMENTAL PROCEDURE

Ox brain and liver glutamate dehydrogenase were purified to apparent homogeneity and shown not to have suffered significant proteolytic cleavage by the procedures previously described (13). No significant differences in kinetic properties between the enzyme preparations from these two sources were found in any of the studies reported here or previously (5,13-15). Enzyme preparations were stored, at 4°C and at a concentration of at least 3 $mg.ml^{-1}$, in 20 mM phosphate buffer, pH 7.4, containing 30% (vol./vol.) glycerol and 1 mM sodium azide. During normal storage vials containing enzyme samples were frequently opened to remove samples for study and no precautions were taken to exclude air. Under such conditions there was no detectable loss of activity over a period of 6 months. Neither were there any detectable changes on storage in mobility on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), suggesting that N-terminal proteolysis did not occur on storage of the purified enzyme under these conditions (see 13). Preparations of the purified enzyme, which had suffered limited proteolysis, were obtained from Boehringer Corp. SDS-PAGE (13) showed these preparations to contain no detectable material with a mobility corresponding to that of the unproteolysed enzyme.

Activity was determined spectrophotometrically, at 30°C, by following the decrease in absorbance at 340 nm in a reaction mixture containing, unless otherwise stated, 50 mM phosphate buffer, pH 7.4, 100 mM NH_4Cl, 80 μM NADH, the enzyme and 5 mM 2-oxoglutarate in a total volume of 2.5 ml. Kinetic data were analysed by non-linear regression of initial-rate values obtained over a range of inhibitor concentrations. The inhibition by perphenazine was analysed in terms of the equation of Shemisa and Fahien (9), as previously reported (11), to allow the apparent inhibitor constant, K_3, to be determined. Fieller's theorem was used, as described previously (11), to calculate the standard errors of inhibitor constants.

Sulphydryl group determinations were performed by determining the formation of 2-nitro-5-thiobenzoate (TNB^{2-}), resulting from the reaction of 5.5'-dithiobis-(2-nitrobenzoate) (DTNB) with thiol groups, spectrophotometrically under alkaline conditions (18). Reactions were performed at 30 or 37°C in N_2-flushed 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and, where indicated, 2% sodium dodecyl sulphate (SDS). Enzyme samples (0.1-0.2 ml) at concentrations of 1-2 $mg.ml^{-1}$ were applied to a 13 × 1 cm (length × diameter) column of Sephadex G-25 (Fine), which had been equilibrated with the above buffer, and were eluted with that buffer. Nitrogen gas was continuously bubbled through the reservoir of eluting buffer to ensure minimal exposure to oxygen. In subsequent manipulations the fractions from gel-filtration were kept in sealed tubes to minimize diffusion of oxygen and evaporation. Aliquots of 0.8 ml of eluted protein were used for sulphydryl group determinations. The concentration of glutamate dehydrogenase protomers was determined from the absorbance at 280 nm, assuming a 1 $mg.ml^{-1}$ solution to give an absorbance of 0.97 in a 1 cm path-length cuvette (19). M_r values of 55,393 and 55,760 were used for the commercially-obtained (20) and laboratory-purified (13) enzyme preparations, respectively.

The formation of TNB^{2-} was determined by following the change in absorbance at 412 nm after addition of DTNB to the enzyme sample. A molar absorbance coefficient of 14,150 $M^{-1}.cm^{-1}$ was used for determinations in the absence of SDS (21) and a value of 13,600 $M^{-1}.cm^{-1}$ was used for data obtained in the presence of 2% (wt./vol.) SDS (22). Stock solutions of DTNB were prepared freshly each day in N_2-flushed 50 mM phosphate buffer, pH 7.4 containing 1 mM EDTA and were kept on ice. Decomposition of DTNB resulting in the formation of TNB^{2-} was found to be negligible over the experimental period. Enzyme protomer concentrations were varied in the range 0-3.3 μM and DTNB was added to givea final concentration in the range 10-60 μM. The complete reaction of the sulphydryl groups present was verified by the addition of a further aliquot of DTNB to the reaction mixture after the absorbance at 412 nm had reached a plateau. Data were analysed by plotting the concentration of TNB^{2-} against the enzyme protomer concentration. The slope of such a plot was equal to the number of sulphydryl groups reacting per protomer and the standard error was determined by linear regression. Blank samples contained neither dithiothreitol (DTT) nor prôtein. The control value for the increase in absorbance at 412 nm was the mean of at least 4 separate determinations.

Amino-terminal analysis was performed after reaction with dansyl chloride and hydrolysis (23). Enzyme samples, containing about 0.5 $mg.ml^{-1}$, were dialysed at room temperature overnight against 11 of 50 mM Tris-HCl buffer, pH 8.5, in order to facilitate unfolding of the enzyme (see 24). DTT was then added to 250 μg aliquots of the enzyme to give a final concentration of 10 mM, and the mixture was incubated at 30°C for 30 min. The protein was then precipitated by the addition of an equal volume of 20% trichloroacetic acid. After being allowed to stand at 4°C for 60 min, the precipitate was removed by centrifugation and washed with cold acetone and recentrifuged at least twice. After the complete evaporation of any remaining acetone, dansylation and subsequent hydrolysis was performed according to the procedure of Gray (23). Dansylamino acids were identified by two-dimensional thin-layer chromatography (tlc) on 7.5 × 7.5 cm polyamide sheets (23,25). The first dimension was developed with: 1. water-90% formic acid (100:1.5, vol./vol.) and the sècond dimension was developed by the sequential use of: 2. benzene-glacial acetic acid (90:10, vol./vol.) followed by: 3. ethylacetate-methanol-glacial acetic acid (20:1:1, by volume). The dansyl derivatives used as standards were prepared as described by Gray (23). Double-sided tlc plates were used so that samples and standards could be chromatographed under identical conditions.

RESULTS

Samples of glutamate dehydrogenase which have suffered limited proteolytic digestion during purification have been shown to be less sensitive than the native enzyme to inhibition by the antipsychotic drug perphenazine (12), the allosteric inhibitor GTP (14) or by high concentrations of NADH (14). However in studies on the kinetics of the laboratory-purified, unproteolysed, enzyme it was found that its sensitivity to inhibition by

high concentrations of NADH altered with time of usage. Figure 1 shows that, after storage at 4°C for about 3 months, the preparation had become less sensitive to this high-substrate inhibition than the commercially-obtained, proteolysed, sample. Comparison of these data with our previous results (14), which were obtained with fresh enzyme preparations, indicate that storage under these conditions resulted in a considerable decline in the sensitivity of the laboratory-purified preparation to inhibition by NADH. Similar changes occurred with the enzyme purified from liver, but there was no significant change in the sensitivity of the commercially-obtained liver enzyme under these conditions. There was no significant change in the specific activities of the enzyme samples, assayed in the presence of 80 μM NADH, during this period of storage. Electrophoresis in the presence of SDS (13) indicated that no detectable N-terminal proteolytic digestion had occurred. Storage also resulted in decreases in the sensitivities of the native enzyme, to inhibition by GTP and perphenazine, whereas those of the commercially-obtained preparation were not significantly affected (Figure 2).

These alterations in inhibitor sensitivity resulted from an oxidative process. This is shown in Figure 3 where the effects of incubation of preparations of the enzyme in an atmosphere of air or nitrogen are compared. The

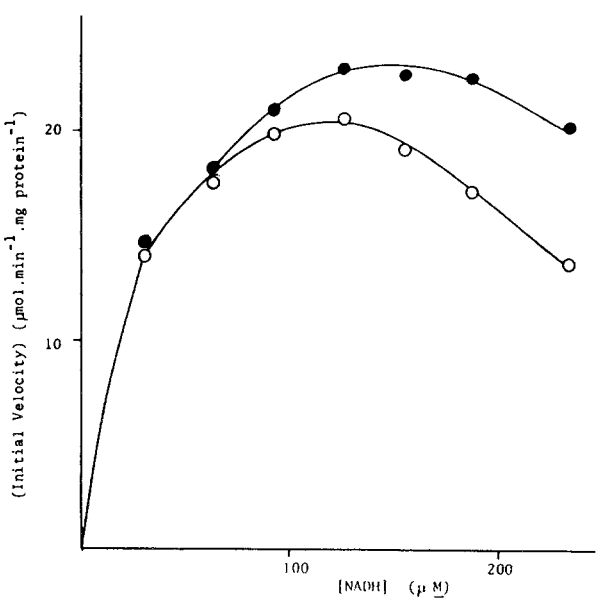

Fig. 1. The effects of NADH on the activities of glutamate dehydrogenase preparations. The activity of ox brain, non-proteolysed, glutamate dehydrogenase which had been stored for approximately 3 months (•) was compared with that of the commercially-obtained preparation of the enzyme from ox liver (○).

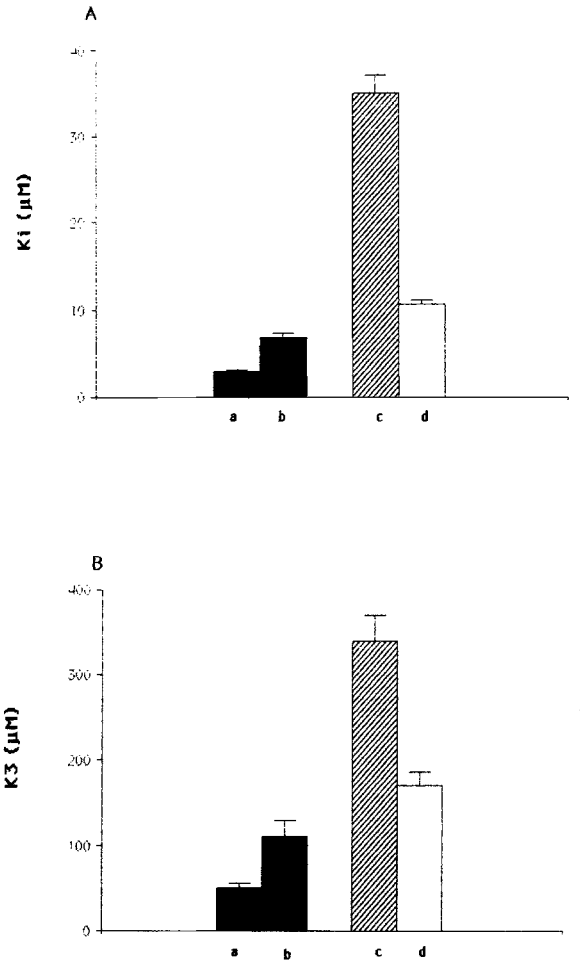

Fig. 2. The effects of storage on the sensitivities of glutamate dehydrogenase to inhibition by GTP (A) and perphenazine (B). Inhibitor constants and s.e. values were determined as described in the text. A freshly-prepared sample of the laboratory-purified ox brain enzyme (a), a similar preparation after storage for about 3 months (c) and commercially-obtained preparations of the ox liver enzyme (b) and (d) treated in the same way were compared. In neither case was the difference between (b) and (d) significant.

sensitivities of the glutamate dehydrogenase preparations to allosteric inhibition by high concentrations of NADH were determined by initial-rate measurements in the presence of either 70 μM or 160 μM NADH band are expressed as the ratios of these values. Any decrease in the sensitivity to inhibition by NADH would result in an increase in the ratio of velocity at 70 μM NADH divided by that determined at 160 μM (see Figure 1). Although preparations of the enzyme were stored in the presence of 1 mM azide, any contribution of this compound to the change in inhibitor sensitivity may be ruled out since incubation of a laboratory-purified sample of the enzyme with that concentration of sodium azide under an at-

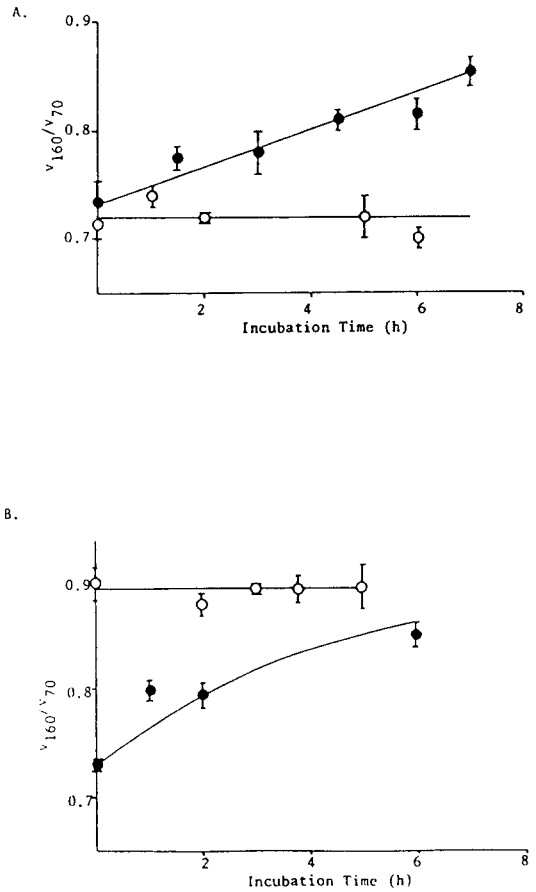

Fig. 3. The effects of exposure to air on the sensitivities of glutamate dehydrogenase preparations to inhibition by NADH. Samples of enzyme at a concentration of 1 mg.ml^{-1} in 20 mM phosphate buffer, pH 7.4, containing 30% glycerol were incubated at 30°C without shaking or stirring. At the times indicated 1 µl aliquots were withdrawn and diluted into N_2-flushed 50 mM phosphate buffer, pH 7.4, for assay. Activities were determined in the presence of either 70 or 160 µM NADH. The data are expressed as the ratios of these values. Each point is the mean, ± SEM of determinations with triplicate or quadruplicate samples. A: laboratory-prepared ox brain glutamate dehydrogenase incubated exposed to air (•) or protected from air in a stoppered vial flushed with nitrogen (○). B: Laboratory-purified ox brain (•) or commercially-obtained ox liver enzyme (○) exposed to air during the incubation.

mosphere of N_2 did not result in any change in the sensitivity to inhibition by NADH, whereas a sample exposed to air in the presence of 1 mM azide gave the same result as one incubated in its absence (results not shown). This air-dependent change could not be reversed by incubation of the enzyme preparation with 10 mM DTT for 30 min at 37°C

As a consequence of these observations vials containing stored glutamate dehydrogenase preparations were routinely flushed with N_2 after each time they were opened. Under such conditions the enzyme was found to retain its native properties, whereas samples stored and used without this precaution were found to lose inhibitor sensitivity in the same way as shown in Figures 1 and 2. Since this behaviour was not observed with commercially-obtained, proteolysed, preparations of the enzyme and those residues previously identified in the peptide removed by proteolysis should not be sensitive to oxidation, the nature of the amino-terminal residue was determined.

Under non-denaturing conditions ox brain and liver glutamate dehydrogenase purified in the laboratory and the commercially-obtained, proteolysed, preparation of the ox liver enzyme showed no significant reactivity with DTNB (see Table I). Thus there were no accessible sulphydryl groups in any of these preparations. After reduction with 10 mM DTT and subsequent gel-filtration the laboratory-purified ox brain and liver enzyme preparations reacted rapidly with DTNB to an extent corresponding to each containing about one reactive sulphydryl group (see Figure 4). In contrast, the commercially-obtained preparation, when treated in the same way, gave no significant reaction with DTNB (Figure 4). The absence of any reactivity with this preparation indicated that the reactions observed with the laboratory-purified preparations was not due to incomplete removal of the DTT by gel-filtration.

The total sulphydryl group content of the enzyme preparations was determined after reduction by 10 mM DTT in the presence of 2% (wt. vol.) SDS. Subsequent gel-filtration through Sephadex G-25 was performed as described earlier, but 2% SDS was included in the equilibration and elution buffer and the column temperature was maintained at 37°C. Under these conditions the commercially-obtained preparation of the ox liver en-

Table I. Sulphydryl Groups of Glutamate Dehydrogenase Preparations

Enzyme Sample	Source[1]	Sulphydryl content per protomer (mol/mol; ± SE)
Ox brain	a	0.01 ± 0.02
Ox liver	a	−0.04 ± 0.01
Reduced ox brain	a	0.85 ± 0.02
Reduced ox liver	a	0.78 ± 0.02
Denatured and reduced ox brain	a	7.0 ± 0.3
Reduced ox liver	b	0.02 ± 0.01
Denatured and reduced ox liver	b	6.2 ± 0.3

[1] Source (a) refers to laboratory-purified preparations which had not suffered limited proteolysis during purification, source (b) samples were purchased from Boehringer. Experimental details are given in the text.

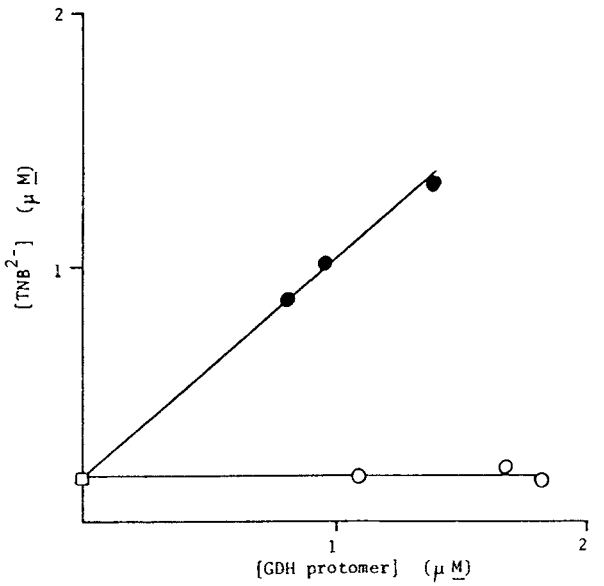

Fig. 4. Sulphydryl group determinations in reduced, non-denatured, preparations of glutamate dehydrogenase. Samples of the laboratory-purified ox brain (•) or commercially-obtained ox liver (○) enzyme were incubated with DTT and gel-filtered, as described in the text, before the sulphydryl group contents were determined by reaction with 10 μM DTNB.

zyme was found to contain approximately 6 sulphydryl groups per protomer (see Table I). This value is consistent with the published amino acid sequence of the commercially-obtained enzyme from this source, which showed it to contain 6 cysteinyl residues in each identical subunit (20). However, as shown in Table I, the unproteolysed, laboratory-purified ox brain enzyme was found to contain about 7 sulphydryl groups per protomer. Storage of the enzyme preparations in an atmosphere of air, under conditions that resulted in decreases in inhibitor sensitivity, before reduction and denaturation did not cause significant alterations in these values. Thus these data are consistent with the presence of an additional cysteinyl residue in each subunit of the enzyme preparation that had not suffered limited proteolysis during the purification procedure.

The possibility that this additional cysteinyl residue was at the N-terminus of the unproteolysed preparation was investigated by reaction with dansyl chloride followed by hydrolysis (23). As shown in Figure 5A dansylation of cysteine gave rise to two yellow-fluorescent spots (marked a and b) that could be seen under ultraviolet light after tlc. Spot (a) was found to migrate to only a small extent in solvents 1 and 3 but not at all in solvent 2, indicating that it corresponded to N,S-bis-DNS-cysteine (25,26). This was also consistent with the observation that no such spot could be detected when dansylation was performed at a dansyl chloride concentration of 1.4 $mg.ml^{-1}$, corresponding to a molar ratio of L-cysteine/dansyl chloride of 2, rather than the 4.25 $mg \cdot ml^{-1}$ used in the earlier preparation. Comparison with the mobilities of N(2)-DNS-lysine, N(6)-DNS-lysine and DNS-arginine, each of which has a free carboxyl- and a free amino-group, allowed the other spot, (b), to be identified as S-DNS-cysteine (23,27). When 0.5 mM dansylcysteine (20 μl) was mixed with 200 μg bovine serum albumin and the mixture was lyophilised and subsequently hydrolysed, by the same procedure used for the dansylated glutamate dehydrogenase samples, tlc analysis showed the same behaviour as before except for the presence of some minor additional spots. These results indicated that it should be possible to identify N-terminal cysteine in the glutamate dehydrogenase preparations if it were present. The chromatographic behaviour of dansyl-OH, dansylamine and dansylalanine was similar to that reported by others (23,25,27). When ox liver glutamate dehydrogenase from Boehringer was subjected to dansylation and hydrolysis the result illustrated in Fig. 5B was obtained. Three yellow fluorescent spots could be detected. That numbered (1) in the Fig. corresponds

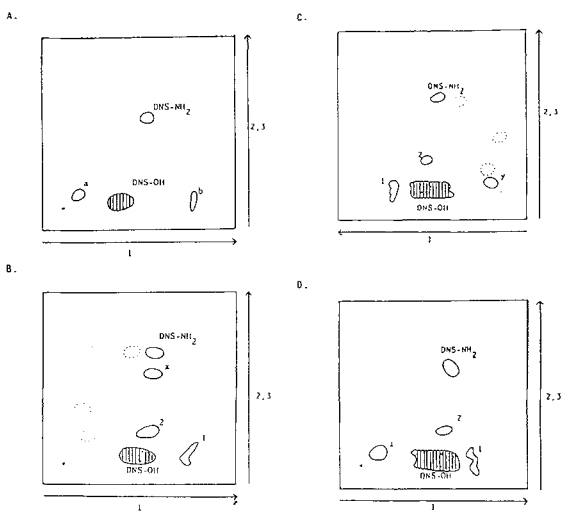

Fig. 5. Thin-layer chromatography of dansyl-derivatives. Chromatography was performed as described in the text. Solvent 1 was used for the first dimension and solvents 2 and 3 were used sequentially in the second. In all cases the origin (*), blue fluorescent (○), and major (○) and minor ( ) yellow fluorescent spots are shown. A: Dansylated L-cysteine, B: commercially-obtained ox liver glutamate dehydrogenase after reaction with dansyl chloride and hydrolysis. C and D: laboratory-purified ox brain and liver preparations, respectively, treated in the same way. Since double-sided tlc plates were used the results shown in C are the mirror image of those in D. The identities of the labeled spots are discussed in the text.

in behaviour to S-dansylcysteine, although in the systems used here it is not possible to exclude the additional presence of N(6)-dansyllysine (23). The spot numbered (2) would correspond to O-dansyltyrosine. However the most pronounced yellow fluorescent spot in the chromatogram, that indicated as x in Figure 5B, could be identified by comparison to the behavior of the authentic compound as dansylalanine. This identification is consistent with the published sequence of the commercially-obtained enzyme from this source (20). When samples of glutamate dehydrogenase purified in the laboratory from ox liver and brain were treated in the same way no dansylalanine spot could be detected. The two spots also seen with the proteolysed preparation, and identified above, could be seen together with an additional strongly fluorescent spot, not present in the chromatogram from the commercially-obtained enzyme, designated (y and z) in the chromatograms obtained from the brain and liver enzymes, which are illustrated in Figure 5C and D. The mobilities of this spot in the different developing solvents used (23,25,27) and its co-migration with the authentic compound permitted its identification as N,S-bis-dansylcysteine.

DISCUSSION

The presence of about one sulphydryl group per subunit that reacted with DTNB in native preparations of glutamate dehydrogenase, after reduction with DTT, but not in those that had suffered proteolysis during purification might have been a result of conformational differences affecting the accessibility of these groups. However determination of the total number of -SH groups after reduction of the enzyme in the presence of SDS showed the unproteolysed enzyme to contain one more of these groups per protomer than the proteolysed preparation. The identification of the amino-terminal residue as cysteine confirmed this difference. N,S-bis-DNS-cysteine was detected after reduction of the enzyme and reaction with dansyl chloride. Thus this residue cannot be in the form of cysteic acid in the native preparation, as sulphonic acid groups are not reduced by DTT.

Comparison of the N-terminal regions of other glutamate dehydrogenase preparations, shown in Table II, indicates a strong similarity between ox brain and liver glutamate dehydrogenase purified by the method of McCarthy et al. (13) and the enzymes from chicken, rat and human liver. Each of these enzymes shows an additional 3 or 4 amino acid residues at the N-terminal end, as compared with the commercially-obtained ox liver enzyme and the sequence of rat liver glutamate dehydrogenase reported earlier (see 30). The residues in these additional oligopeptides are either identical, or derived from single base changes in the amino acid codons. The N-terminal sequence first reported for the enzyme from rat liver (see 30) is similar to that of the proteolysed preparation from ox liver. However a preparation of the rat liver enzyme purified by affinity precipitation has been shown to have the same mobility on polyacrylamide gel electrophoresis as the native ox liver and brain enzymes (16). Furthermore the amino acid sequence derived from the sequence of cDNA clones shows the presence of an additional N-terminal tetrapeptide which is analogous to that found in the ox brain and liver enzymes (31). Thus it is possible that the preparation used for the earlier sequence studies had suffered limited proteolysis during purification. The limited proteolysis appears to have occurred at a similar position, suggesting that it resulted from similar causes, such as the extended periods of storage of crude enzyme-containing solutions that are involved in the purification of the enzyme by crystallization (see 14). The effects of this proteolysis on the enzyme from rat liver require evaluation in view of the significant differences in behavior of this and the ox liver enzyme that have been reported (33).

As might be expected, in view of the apparent homogeneity of the ox brain and liver enzymes purified in this laboratory (see 13) and the presence of azide in the medium, no proteolysis could be detected during storage, under the conditions described in the present work, as judged by the criteria of SDS-PAGE and cysteine content. The possibility that the proteolysis occurring during the preparation of the commercially-obtained enzyme might also involve some degradation of the C-terminal end of the polypeptide chain cannot be completely excluded, although the results of peptide-map-

Table II. Amino-Terminal Sequences of Glutamate Dehydrogenase Preparations

Source	Sequence	Reference
Ox liver	H_2N-Ala-Asp-Arg-Glu-	(20)[a]
Rat liver	H_2N-Thr-Asp-Lys-Glx-	(see 30)[b]
Rat liver	H_2N-Ser-Glu-Gly-Pro-Thr-Asp-Arg-Glu-	(31)[c]
Ox liver	H_2N-Cys-Asp-Ala-Ala-Ala-Asp-X^1-Glu-	(13)
Ox brain	H_2N-Cys-Asp-Ala-Ala-Ala-Asp-X^1-Glu-	(13)
Chicken liver	H_2N-Cys^2-Glu-Ala-^{3}-Ala-Asp-Lys-Glu-	(28)
Human liver	H_2N-Ser-Glu-Ala-Val-Ala-Asp-Arg-Glu-	(29 & 32[c])

Notes: [1] May be Arg; [2] cysteine or cysteic acid: [3] A special alignment has been made to show the similarity to the enzyme from other sources. [a] A commercially-available enzyme preparation; [b] the purification method was not described. In all other cases the purification procedure is given, or cited, in the reference given. [c] The amino acid sequence was derived from the nucleotide sequences of cDNA clones.

ping and SDS-PAGE studies (13) suggest this would be very limited, if it occurred at all. Clearly, if any C-terminal degradation had occurred during the preparation of the commercially-obtained enzyme, but not in those prepared in this laboratory, it could not involve regions containing cysteinyl residues. In view of the readiness with which N-terminal proteolysis can occur during the preparation of glutamate dehydrogenase, determination of the total sulphydryl-group content can provide a convenient way of checking for its occurrence in purified preparations of the ox liver and brain enzymes.

The identification of the amino-terminal residue of the ox brain and liver enzymes as cysteine makes it tempting to conclude that the changes in the regulatory properties which occur on exposure to air may result from the oxidation of this sulphydryl group. Such a conclusion would be consistent with the observation that commercially-obtained preparations of the enzyme were not affected by exposure to oxygen in this way. The observation that no sulphydryl groups in the native enzyme preparations were accessible to reaction with DTNB before reduction might indicate the terminal residue to be involved in a disulphide bond with one of the other sulphydryl groups in the enzyme. However, such behaviour might also result from the oxidation of the group to the sulphenic acid. The amino-terminal residue of the chicken liver enzyme has been reported to be cysteic acid, but the ability of DTT to expose the $-SH$ to reaction with DTNB indicates that this higher oxidation state cannot be involved. The existence of a single oxidized sulphydryl group has been reported in plasma amine oxidase (34). An alternative possibility is that the sulphydryl group might be masked by disulphide bond formation with low molecular weight thiols, as has been reported for some other enzymes (35,36). Further work will be necessary before the state of the sulphydryl groups and the nature of the residues that contribute to the oxidative changes in inhibitor sensitivity can be firmly identified.

These changes in the inhibitor sensitivity of non-proteolysed preparations of the enzyme as a result of exposure to air may make it difficult to obtain reproducible results and complicate attempts to infer its role in the tissues unless care is taken to prevent oxidation from occurring. The alterations in behaviour reported here might be expected to affect other aspects of the regulation of glutamate dehydrogenase activity, since the activatory behaviour of L-leucine is in part related to the inhibition of the enzyme by NADH (4). Since the changes affect inhibition by NADH, GTP and perphenazine without affecting the specific activity of the preparation or the apparent relative molecular mass of its subunits, they might go unobserved unless specific experiments were performed to test for oxidative modification.

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