Biochem. J. (1971) **123**, 421–426
Printed in Great Britain

The Inhibition of Glutamate Dehydrogenase by L-Serine *O*-Sulphate and Related Compounds and by Photo-oxidation in the Presence of Rose Bengal

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(*Received* 19 *February* 1971)

1. Glutamate dehydrogenase was inhibited by L-serine *O*-sulphate, β-chloro-L-alanine, *O*-phospho-L-serine and β-chloro-L-alanine methyl ester. With the exception of β-chloro-L-alanine methyl ester which was an irreversible inhibitor, it was possible to reverse the inhibitory effects by dialysis. 2. Both NAD^+ and glutamate afford some protection against the inhibition due to the methyl ester. No change in the normal stimulatory effect exhibited by ADP was observed in the presence of β-chloro-L-alanine methyl ester but the effect due to GTP was modified. 3. Irradiation of glutamate dehydrogenase in the presence of Rose Bengal produced rapid inactivation. Amino acid analysis of the inactivated enzyme showed that eight histidine residues had been destroyed in the process.

Studies on the chemical modification of glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] by a number of investigators have yielded results that implicate an impressive array of functional amino acid residues. In most instances it has not been possible unequivocally to relate these residues to a specific functional site in the active oligomer.

As a result of examining difference spectra of systems containing glutamic acid, glutamate dehydrogenase and NADH, Fisher & Cross (1965) suggested that tryptophan was involved in binding the γ-CO_2H grouping of the amino acid substrate, a finding which received some substantiation by the work of Brocklehurst *et al.* (1970), utilizing 4-iodoacetamidosalicylic acid as a site-specific probe for glutamate dehydrogenase. These latter workers further implicated, on the basis of inactivation experiments with tetranitromethane, a specific tyrosine residue, which was thought to form part of the GTP binding site. The involvement of both tyrosine and lysine as functional amino acids has also emerged from the investigations of di Prisco, Banay-Schwartz & Strecker (1970) though it has been stressed that a differentiation between a purely functional or structural role for these amino acids was not possible. Holbrook & Jeckel (1969) and Freedman & Radda (1969) have also detected reactive lysyl residues in glutamate dehydrogenase, but again found difficulty in deciding on their exact locations. More recently the numbers of amino acid residues capable of being specifically modified has been increased by the finding of Malcolm & Radda (1970) that *S*-carboxymethylcysteine may be isolated from hydrolysates of glutamate dehydrogenase which has been alkylated by 4-iodoacetamidosalicylic acid. The presence of a relatively large number of important amino acid residues is not surprising, particularly when due account is taken of the fact that the active enzyme has a molecular weight of approx. 310000 and is believed also to possess at least six distinct sub-sites (Cross & Fisher, 1970).

Investigations into the metabolism and enzymic degradation of L-serine *O*-sulphate have shown that this substituted amino acid possesses many features in common with glutamic acid, particularly in the stereochemical arrangement of functional groupings (N. Tudball, J. P. G. Richards & H. Davies, unpublished work). Moreover, L-serine *O*-sulphate has been reported to be capable of acting as a 'quasi' substrate in at least two systems which utilize glutamate as a substrate, namely aspartate aminotransferase (John & Fasella, 1969) and alanine aminotransferase (see Tudball, Thomas & Fowler, 1969). With aspartate aminotransferase, L-serine *O*-sulphate, in addition to performing the role of a substrate, also acted as an irreversible inhibitor, which resulted in the complete inactivation of the enzyme, probably as a result of alkylation at or near the active site.

The ability of L-serine *O*-sulphate and related compounds to effect changes in the activities of other enzymes which employ glutamic acid as a substrate

have therefore been investigated as part of our continuing programme of investigation into the chemistry and biochemistry of L-serine *O*-sulphate. The present report deals with preliminary results obtained when the reactivity of glutamate dehydrogenase was examined in the presence of L-serine *O*-sulphate and related compounds.

MATERIALS AND METHODS

Chemicals. The sodium salt of L-glutamic acid, the nucleotides ADP and GTP and *O*-phospho-L-serine were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. The coenzymes NAD^+ and $NADP^+$ and ox liver glutamate dehydrogenase, as a solution in 50% glycerol (10 mg/ml), were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Rose Bengal was obtained from Eastman Organic Chemicals, Rochester 3, New York, N.Y., U.S.A.

The potassium salt of L-serine *O*-sulphate was prepared by the method of Tudball (1962). β-Chloro-L-alanine and its methyl ester were prepared according to the procedure of Fischer & Raske (1907).

Assay of enzyme activities. Assays involving NAD^+, $NADP^+$, $NADP^+$ (ADP) and $NADP^+$ (GTP) were performed essentially as described by Baker & Rabin (1969). Progress curves were obtained by using a Unicam SP. 800 recording spectrophotometer fitted with a scale-expansion accessory. The rate plots obtained were linear over 2–3 min and initial velocities were determined from their slopes.

EXPERIMENTAL AND RESULTS

The effect of incubating L-serine *O*-sulphate, β-chloro-L-alanine, β-chloro-L-alanine methyl ester and *O*-phospho-L-serine with glutamate dehydrogenase was examined initially by using the NAD^+ assay system, employing glutamate in final concentrations ranging from 0.625 to 20 mM. All reactions were initiated by the addition of 12.5 μg of enzyme (obtained by diluting the stock solution with 0.1 M-tris–HCl buffer, pH 7.6) to the assay mixture, which contained, in addition to the substrates, a known concentration of test compound. All compounds markedly inhibited enzyme activity. The degree of inhibition remained unaltered in systems which contained L-serine *O*-sulphate, β-chloro-L-alanine or *O*-phospho-L-serine when the NAD^+ concentration was doubled. The results obtained, plotted as the double-reciprocal plot of Lineweaver & Burk (1934), are shown in Fig. 1.

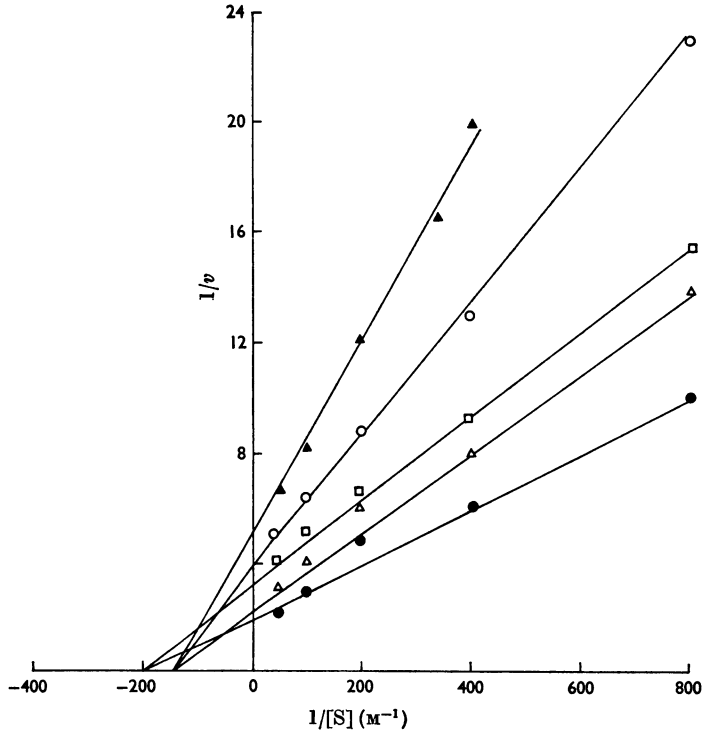

Fig. 1. Inhibition of glutamate dehydrogenase by using inhibitors at a final concentration of 10 mM. ●, No inhibitor present; ○, L-serine *O*-sulphate present; △, β-chloro-L-alanine present; □, *O*-phospho-L-serine present; ▲, β-chloro-L-alanine methyl ester present. Experimental details are given in the text.

Only with *O*-phospho-L-serine was it possible to classify the type of inhibitory effect, namely non-competitive, the other compounds being neither competitive nor non-competitive. However, the reasons for these observed inhibitory effects were not further investigated.

Preincubation of enzyme with β-chloro-L-alanine, L-serine *O*-sulphate or *O*-phospho-L-serine at 25°C for 5min did not alter the degree of inhibition observed and full activity could be restored by dialysis. In the case of β-chloro-L-alanine methyl ester preincubation with inhibitor markedly affected the enzyme activity, which could not be subsequently restored after dialysis.

Inactivation of glutamate dehydrogenase by β-chloro-L-alanine methyl ester

The inactivation of glutamate dehydrogenase by β-chloro-L-alanine methyl ester was followed by incubating enzyme (0.5mg) and inhibitor (at final concentrations 5, 10, 20mM) in 0.1M-tris–HCl buffer, pH7.6 (2ml) at 25°C. Portions (50μl or 100μl) were removed at various time-intervals and pipetted into the standard NAD^+ assay mixture at 25°C. The time-course of the inactivation under different experimental conditions is illustrated in Fig. 2. The curves obtained are biphasic indicating that the inactivation process is due to two reactions, a fast primary process followed by a slower secondary process. It can further be shown that the two reactions are first-order with respect to inhibitor.

Modification of inhibition effect by substrates and allosteric effectors

The effect of NAD^+ and glutamate on the inactivation of glutamate dehydrogenase was followed using the $NADP^+$ assay system. When the enzyme was incubated in the presence of β-chloro-L-alanine methyl ester and either NAD^+ (330μM final concentration) or glutamate (0.01M final concentration) then considerable protection against inactivation was afforded in either case (Fig. 3), the NAD^+ affording greater protection than glutamate under the conditions employed. In the absence of NAD^+ and glutamate, the progress curve, apart from a slight deviation at the start, indicates an essentially first-order reaction, which is almost identical to the second phase obtained by using the NAD^+ assay system. This suggests that the primary event in the latter system is the alkylation of the NAD^+ binding site which perhaps explains the

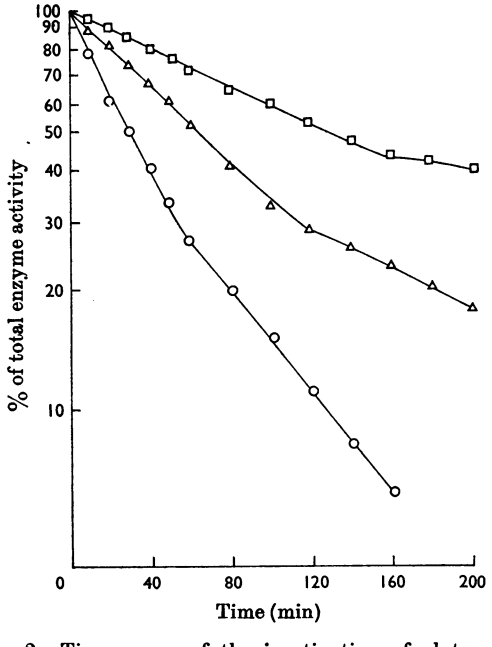

Fig. 2. Time-course of the inactivation of glutamate dehydrogenase by β-chloro-L-alanine methyl ester. Activity at zero time was taken as 100%. Concentration of β-chloro-L-alanine methyl ester: ○, 20mM; △, 10mM; □, 5mM. Experimental details are given in the text.

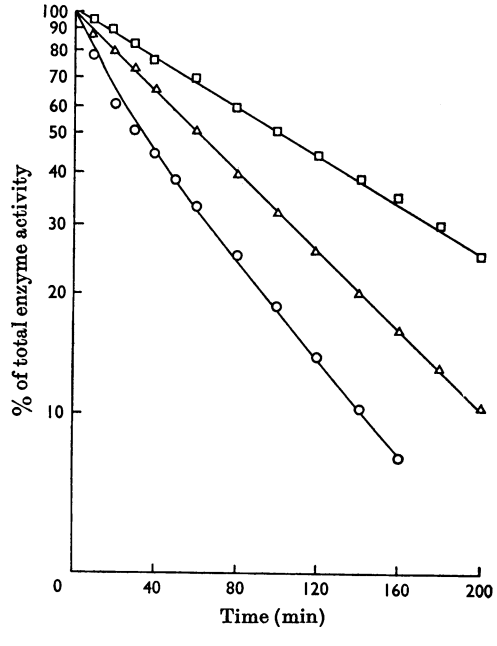

Fig. 3. Time-course of the inactivation of glutamate dehydrogenase by β-chloro-L-alanine methyl ester (20mM final concn.) in the presence of NAD^+ and glutamate, by using the $NADP^+$ assay. ○, Inhibitor alone; △, in the presence of 10mM-glutamate; □, in the presence of 330μM-NAD^+. Experimental details are given in the text.

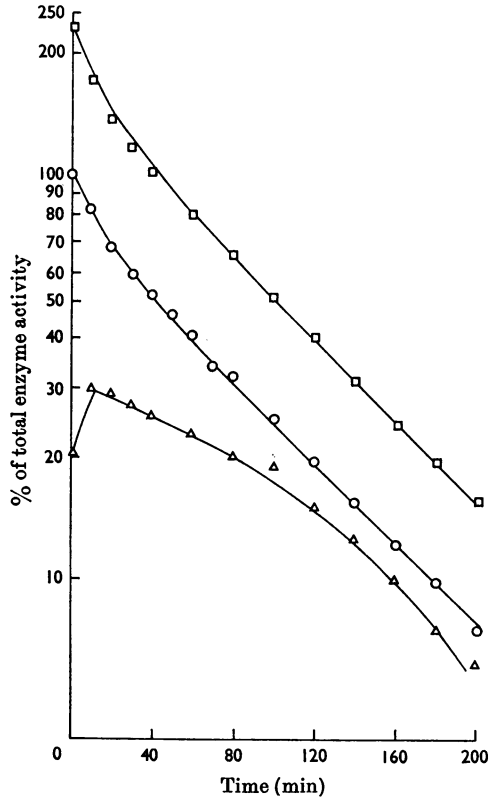

Fig. 4. Time-course of the inactivation of glutamate dehydrogenase by β-chloro-L-alanine methyl ester (20 mM final concn.) in the presence of ADP and GTP, by using the $NADP^+$ assay. ○, Inhibitor alone; △, in the presence of 20 μM-GTP; □, in the presence of 500 μM-ADP. Experimental details are given in the text.

protection observed with NAD^+ (see Baker & Rabin, 1969).

It is well known that the nucleotides GTP and ADP act as modifiers of the oxidation of glutamate by glutamate dehydrogenase, the former bringing about a decrease, the latter an increase, in the initial velocity. These effects may be readily examined by using $NADP^+$ as the cofactor in the assay medium. Fig. 4 illustrates the results obtained when enzyme was incubated with inhibitor in the presence of either GTP (20 μM final concentration) or with ADP (500 μM final concentration). The activation effect normally associated with ADP still persisted in the presence of inhibitor, the progress curve paralleling that observed when the effector was omitted from the incubation medium.

In the presence of GTP, the activity observed at zero time increased to reach a maximum value after 10 min after which time the observed activity decreased in such a way as to parallel the decrease when effector was omitted. Clearly the effect of ADP is retained but that of GTP is lost in the presence of β-chloro-L-alanine methyl ester, and it would be worth while to investigate further the changes undergone in the protein molecule after the chemical interaction with this compound.

Photoinactivation of glutamate dehydrogenase

During preliminary experiments on the analysis of glutamate dehydrogenase that had been chemically modified by interaction with β-chloro-L-alanine methyl ester we were led to believe that a histidine residue may be important as a functional amino acid. There are now many well-documented examples of other dehydrogenase enzymes that require histidine residues for action (see Robinson, Stollar, White & Kaplan, 1963). Moreover it is well known that they are sensitive to illumination in the presence of a photoactive dye. It has been reported that photoinactivation in the presence of the photoactive dye, Rose Bengal, specifically brings about the photo-oxidation of histidyl residues (Westhead, 1965). The effect of irradiating glutamate dehydrogenase in the presence of Rose Bengal was therefore investigated.

The standard procedure employed for the study of the photoinactivation was as follows. Rose Bengal (20 μg) and glutamate dehydrogenase (1 mg) were dissolved in 0.1 M-tris–HCl buffer, pH 7.6 (2 ml), contained in a quartz cell of 1 cm path length. The system was then irradiated at 16°C with the light from a standard 1000 W lamp placed 10 cm away from the cell. Portions (100 μl) of the irradiated mixture were taken at various time-intervals and added to an assay mixture consisting of the above tris–HCl buffer (0.9 ml) containing glutamate and NAD^+ at final concentrations of 0.011 M and 330 μM respectively. Two control systems were employed in which either the Rose Bengal was omitted or alternatively the whole system was kept in the dark. The results obtained from these experiments are presented in Fig. 5. When the photoinactivation experiments were performed in the presence of either NAD^+ (330 μM final concentration) or glutamate (0.011 M final concentration), then the photoinactivation effect, although still observable, was considerably decreased with NAD^+ affording greater protection than glutamate.

Even though Rose Bengal has been used when it has been desirable to specifically photoinactivate a histidine residue, the simple demonstration of photoinactivation in its presence does not unequivocally demonstrate the oxidation of this amino acid. However, if the photoinactivation is performed over a range of different pH values, then the presence of a histidine residue can readily be

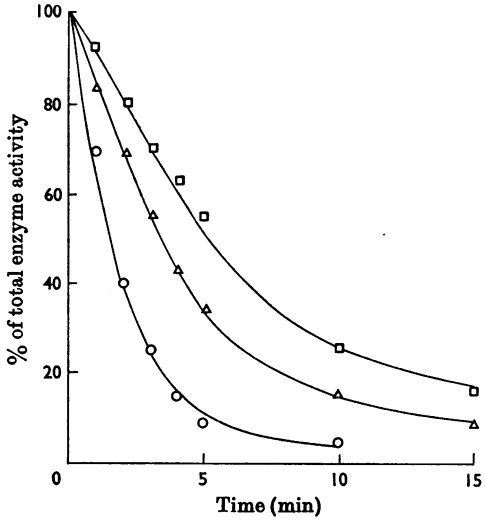

Fig. 5. Time-course of the photo-oxidation of glutamate dehydrogenase in the presence of Rose Bengal. The amount of enzyme activity is related to that at zero time which was taken as 100%. ○, Enzyme+Rose Bengal; △, enzyme+Rose Bengal+10mM-glutamate; □, enzyme+Rose Bengal+330μM-NAD$^+$. Experimental details are given in the text.

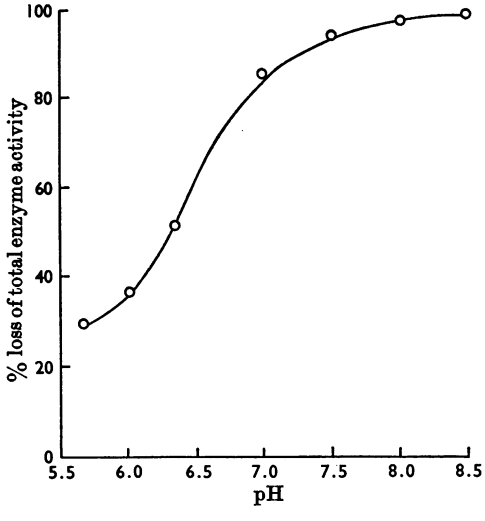

Fig. 6. Effect of pH on the photo-oxidation of glutamate dehydrogenase in the presence of Rose Bengal. Incubations were performed in 0.05M-sodium acetate–acetic acid buffer for pH values 5.5–6.2 and in 0.1M-tris–HCl buffer for pH values 7–8.5. Experimental details are given in the text.

indicated from a graphical presentation of the results (Westhead, 1965). The results obtained when the photoinactivation of glutamate dehydrogenase was performed over the pH range 5.5–8.5 are presented in Fig. 6. The curve obtained is typical of that to be expected if histidine was the amino acid residue governing the photo-oxidative inactivation.

Amino acid analysis of photoinactivated enzyme

The photoinactivation of glutamate dehydrogenase was performed essentially as described above, except that 2mg of the enzyme was employed. A small quantity of charcoal was added to remove the bulk of Rose Bengal. After removing the charcoal by centrifugation, the supernatant, which still contained traces of dye, was dialysed against five changes of water (total vol. 10 litres) and non-diffusible material freeze-dried. The resulting preparation was hydrolysed in 2ml of 6M-(constant-boiling) hydrochloric acid *in vacuo* for 24h at 110°C. Excess of hydrochloric acid was removed by repeated freeze-drying. For the subsequent determination of the amino acid composition, the dried sample was dissolved in 1.5ml of pH2.875 buffer (Technicon Auto Analyser Instruction Manual) and samples (0.5ml) were analysed by using the Technicon Amino Acid Analyser. A control experiment was performed in an identical manner, except that the sample was not subjected to illumination. The tryptophan content was estimated before and after photo-oxidation by the method of Goodwin & Morton (1946). Amino acid analysis revealed that eight histidine residues/subunit of mol.wt. 52000 had been lost in the photoinactivation procedure. There was no evidence for modification of any other amino acids.

DISCUSSION

β-Chloro-L-alanine methyl ester may now be added to the growing list of inhibitory agents that can be employed to study the functional groupings of glutamate dehydrogenase. The inactivation of glutamate dehydrogenase observed with this reagent is qualitatively similar in some respects to that observed by Baker & Rabin (1969) using bromopyruvate and by Freedman & Radda (1969) employing 2,4,6-trinitrobenzenesulphonic acid as inhibitory agents. In all three cases biphasic progress curves were obtained, the enzyme's normal response to the allosteric effector GTP was modified, whereas its response to ADP remained essentially unaffected. The sites of action of these agents may well be identical, though more direct comparison must await the amino acid analyses of glutamate dehydrogenase which has been modified by bromopyruvate and β-chloro-L-alanine methyl ester respectively.

On the basis of experiments performed in the

present series of investigations, it is not possible to determine whether or not histidine residues are directly concerned with the catalytic mechanism of the enzyme. It is eminently possible that photo-oxidation of histidine residues has simply resulted in an alteration of the basic enzyme structure, consequently leading to inactivation. There is in fact ample evidence in the literature to indicate the importance of histidine in maintaining the three-dimensional integrity of proteins. No attempt was made to correlate the photo-oxidation effect with a corresponding change in the conformation of the enzyme, though clearly such studies are desirable. The photo-oxidation of a tryptophan residue might also have been expected to occur concomitantly with histidine oxidation, particularly since it has been implicated in substrate binding (Fisher & Cross, 1965) but no such modification was observed.

It now seems reasonable to conclude that two-thirds of the histidine residues in the glutamate dehydrogenase oligomer are on the surface of the enzyme. What is not clear, however, is whether or not histidine can be further implicated in the catalytic mechanism. The photoinactivation is certainly a very rapid process so it is tempting to suggest that the oxidation of glutamate requires the participation of an imidazolium nitrogen. The inactivation of glutamate dehydrogenase is retarded by the presence of either NAD^+ or glutamate, suggesting that a histidine residue resides sufficiently close in space to both the glutamate and NAD^+ binding sites so as to act as a bridge for hydrogen transfer. Further, exchange with solvent hydrogen would not be expected in such a mechanism. The histidine residues undergoing oxidation in the presence of Rose Bengal must be present in the uncharged form which would not invalidate the idea that such a residue is involved in the catalytic mechanism. Further investigations are now in progress to resolve the problem of the specific role of histidine residues in glutamate dehydrogenase.

The present investigation, even though it specifically deals with glutamate dehydrogenase, nevertheless demonstrates the possible utility of L-serine *O*-sulphate and related compounds for the examination of the structures of enzymes which utilize glutamic acid as a substrate.

P.T. is grateful to the Science Research Council for a studentship. We thank Dr R. Bailey-Wood for performing the amino acid analyses.

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