The Role of Histidine Residues in Glutamate Dehydrogenase

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1. Glutamate dehydrogenase was subject to rapid inactivation when irradiated in the presence of Rose Bengal or incubated in the presence of ethoxyformic anhydride. 2. Inactivation in the presence of Rose Bengal led to the photo-oxidation of four histidine residues. Oxidation of three histidine residues had little effect on enzyme activity, but oxidation of the fourth residue led to the almost total loss of activity. 3. Acylation of glutamate dehydrogenase with ethoxyformic anhydride at pH 6.1 led to the modification of three histidine residues with a corresponding loss of half the original activity. Acylation at pH 7.5 led to the modification of two histidine residues and a total loss of enzyme activity. 4. One of the histidine residues undergoing reaction at pH 6.1 also undergoes reaction at pH 7.5. 5. The presence of either glutamate or NAD^+ in the reaction mixtures at pH 6.1 had no appreciable effect. At pH 7.5 glutamate caused a marked decrease in both the degree of alkylation and degree of inactivation. NAD^+ had no effect on the degree of inactivation at pH 7.5 but did modify the extent of acylation. 6. The normal response of the enzyme towards ADP was unaffected by acylation at pH 6.1 or 7.5. 7. The normal response of the enzyme towards GTP was altered by treatment at both pH 6.1 and 7.5.

The activity of glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] is subject to regulation by a wide variety of biologically important compounds. These include the coenzymes NAD^+ , NADP^+ , NADH and NADPH (Barton & Fisher, 1971; Cross & Fisher, 1970), the nucleotides GTP and ADP (Wolff, 1962; Frieden, 1959) and a large number of other compounds that act as competitive inhibitors (Caughey *et al.*, 1957). Clearly a number of distinct sites must be associated with the enzyme to accommodate the binding of effector molecules. A schematic model for the arrangement of these sites has been proposed (Cross & Fisher, 1970) based on the difference spectra obtained when the enzyme complexes with a variety of ligands. At present it is not possible to propose a similar model based on chemical evidence, although a number of amino acid residues have been shown to be important in the normal functioning of the enzyme. These include tryptophan (Fisher & Cross, 1965; Brocklehurst *et al.*, 1970), tyrosine (Brocklehurst *et al.*, 1970; di Prisco *et al.*, 1970), lysine (Freedman & Radda, 1969; Holbrook & Jeckel, 1969; di Prisco *et al.*, 1970) and cysteine (Malcolm & Radda, 1970). Preliminary investigations further indicated that histidine residues also perform an as yet ill-defined but essential role (Tudball & Thomas, 1971). Photo-

oxidation of glutamate dehydrogenase in the presence of Rose Bengal led to the oxidation of eight histidine residues with the concomitant loss of enzymic activity. This loss was modified in the presence of either glutamate or NAD^+ , suggesting that active-site residues were altered in the oxidation.

The present paper contains further results on the photo-oxidation of glutamate dehydrogenase coupled with investigations employing ethoxyformic anhydride, which offers considerable advantages over other reagents for the study of active-site histidine residues (see Mühlrad *et al.*, 1967; Thomé-Beau *et al.*, 1971).

Materials and Methods

Chemicals

The sodium salts of L-glutamic acid, ADP, GTP and 5,5'-dithiobis-(2-nitrobenzoate) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. The coenzyme NAD^+ and ox liver glutamate dehydrogenase, as a 10 mg/ml solution in aq. 50% (v/v) glycerol were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Rose Bengal and ethoxyformic anhydride were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Methods

Assay of enzyme activities. Enzyme assays were performed essentially as described by Baker & Rabin

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(1969). NAD^+ was the cofactor normally employed, though when it was necessary to examine the responses of the system to ADP and GTP, then NADP^+ was used. Progress curves were obtained by using either a Unicam SP.800 recording spectrophotometer fitted with a scale-expansion accessory or a Hilger Uvispek H700 fitted with a Hilger Gilford absorbance converter. The rate plots obtained were linear over the first 1 min of incubation and initial velocities were determined from their slopes.

Photo-inactivation of glutamate dehydrogenase in the presence of Rose Bengal. The method of Tudball & Thomas (1971) was used. The amount of inactivation obtained was always corrected for the inhibition obtained when glutamate dehydrogenase was incubated with Rose Bengal in the dark.

Alkylation of glutamate dehydrogenase by ethoxyformic anhydride. Ethoxyformic anhydride was used as a solution in propan-2-ol prepared immediately before use and kept at 4°C . The final concentration of propan-2-ol in the reaction mixture was always kept below 0.5%. Carboxylation of the enzyme was performed at pH 6.1 in the presence of 0.1 M-sodium acetate-acetic acid buffer and at pH 7.5 in 0.1 M-tris-HCl buffer. All reaction mixtures contained NaCl at a final concentration of 0.1 M. Reaction mixtures (1 ml) were placed in a quartz cuvette of 1 cm light-path maintained at 25°C .

The rate of formation of carboxylated histidine was followed with a Zeiss PMQ 11 spectrophotometer. Difference spectral readings were obtained at 242 nm, by taking $\epsilon = 3200$ at pH 6.1 (Ouali *et al.*, 1967) and 2900 at pH 7.5. The number of histidine residues undergoing modification were computed by using a value of 56 100 for the molecular weight of the monomeric unit of glutamate dehydrogenase.

Amino acid analysis of modified enzyme. The amino acid analyses of photoinactivated preparations of glutamate dehydrogenase were performed as described by Tudball & Thomas (1971).

Results

Photoinactivation of glutamate dehydrogenase in the presence of Rose Bengal

When glutamate dehydrogenase (1 mg) was subjected to photoinactivation in the presence of Rose Bengal (final concns. 3.8, 5.8 and $9.7\ \mu\text{M}$) the rate of inactivation was directly dependent on the concentration of dye. The rates exhibited first-order kinetics, suggesting that the loss of activity was caused by the alteration of a single amino acid residue.

Amino acid analyses of enzyme (2 mg) that had been subjected to photo-oxidation for different periods of time in the presence of $3.8\ \mu\text{M}$ -Rose Bengal indicated that three residues were rapidly oxidized (within the first 2 min period of irradiation) accom-

panied by a loss of approx. 20% of the initial enzyme activity. Oxidation of a further residue occurred much more slowly, though its modification led to a considerable decrease in enzyme activity. After 30 min four histidine residues had been oxidized and 90% of the initial enzyme activity had been lost.

One could conclude that at least three of the histidine residues that undergo photo-oxidation under the above conditions are not essential for enzyme activity. Alternatively, after the loss of the three rapidly oxidized residues, the protein may undergo a slower conformational change or depolymerization, either process leading to loss of enzyme activity. These suppositions can, however, be discounted, since after irradiation of the enzyme for 2 min as described above, no further loss of activity occurred when the reaction mixture was placed in the dark for periods of up to 60 min. Gel filtration of the same irradiated preparation on a column ($2.5\text{ cm} \times 100\text{ cm}$) of Sephadex G-200 showed that no alteration in molecular weight had occurred.

In the dark Rose Bengal behaved as a simple non-competitive inhibitor of glutamate dehydrogenase (dark inhibition), which can probably be attributed to the non-specific binding of the dye to the enzyme.

Reaction of glutamate dehydrogenase with ethoxyformic anhydride

The results from the photo-oxidation of glutamate dehydrogenase indicate that at least one histidine residue is involved in the catalytic mechanism of the enzyme. Ethoxyformic anhydride was used to obtain further information on the involvement of histidine in the functioning of this system.

Rate plots for the inactivation of glutamate dehydrogenase (final concn. $2.5\ \mu\text{M}$) at pH 6.1 in the presence of ethoxyformic anhydride (final concn. $20\ \mu\text{M}$) and the corresponding formation of ethoxyformyl histidine are presented in Figs. 1 and 2. After 2 min approximately half of the original activity remained, with a corresponding loss of one histidine residue. On prolonged reaction two other histidine residues underwent alkylation, but only a further 5% loss of original enzyme activity was observed. The initial loss of enzyme activity and the alkylation of the first histidine residue occurred at about the same rate. Pseudo-first-order rate constants of 0.55 min^{-1} and 0.49 min^{-1} respectively for these processes were obtained from the corresponding rate-plots.

When enzyme (final concn. $2.5\ \mu\text{M}$) and alkylating agent (final concn. $20\ \mu\text{M}$) were incubated at pH 7.5, over 80% of the original enzyme activity was lost during the initial 10 min of reaction, with the concomitant alkylation of 1.5 histidine residues (Fig. 3). Complete inactivation probably corresponds to the alkylation of two histidine residues (Fig. 2).

The alkylation at pH 7.5 differs quantitatively from

Fig. 1. Time-course of the inactivation and degree of acylation of glutamate dehydrogenase by ethoxyformic anhydride at pH 6.1

Activity at zero time was taken as 100%. Enzyme was used at a final concn. of $2.5\ \mu\text{M}$ and acylating agent at a final concn. of $20\ \mu\text{M}$. $\circ$, Loss of enzyme activity; $\bullet$, formation of ethoxyformyl histidine. Experimental details are given in the text.

that at pH 6.1. To investigate the difference in more detail the alkylation at pH 6.1 was performed before the reaction was done at pH 7.5.

Initially glutamate dehydrogenase was treated at pH 6.1 with ethoxyformic anhydride for 15 min as described above. The pH of the reaction mixture was then adjusted to pH 7.5 with $200\ \mu\text{l}$ of $0.1\ \text{M}$ -tris-HCl buffer, pH 7.5. The initial reaction at pH 6.1 resulted in the alkylation of three histidine residues with a corresponding loss of approx. 55% of the enzyme activity. On adjustment to pH 7.5 one further histidine became alkylated with a corresponding loss of the residual activity. The loss of activity was, moreover, directly proportional to the alkylation of one histidine residue (Fig. 4) indicating that this residue is intimately associated with the catalytic mechanism. The rate-constant for loss of enzyme activity and histidine modification was calculated to be $0.26\ \text{min}^{-1}$.

Removal of excess of reagent after the pH 6.1 treatment by gel filtration on a column ($2.5\ \text{cm} \times 25\ \text{cm}$) of Sephadex G-25 and subsequent adjustment of the treated enzyme to pH 7.5 did not cause any further decrease in enzyme activity. However, on addition of further alkylating agent (final concn. $20\ \mu\text{M}$) the previously observed effect at pH 7.5 was again seen.

Under the precise experimental conditions employed in the above experiments total loss of enzyme activity was never achieved. This is probably due to

Fig. 2. Loss of glutamate dehydrogenase activity as a function of the degree of acylation

Enzyme was used at a final concn. of $2.5\ \mu\text{M}$ and ethoxyformic anhydride at a final concn. of $20\ \mu\text{M}$. $\circ$, Acylation performed at pH 6.1; $\bullet$, acylation performed at pH 7.5. Experimental details are given in the text.

the rapid breakdown of ethoxyformic anhydride at pH 7.5 (half-life 2.4 min). Total loss of activity may be achieved, however, by the addition of further reagent to compensate for loss by hydrolysis.

The first of the histidine residues that undergoes reaction at pH 7.5 (Fig. 3) is presumably the same residue that undergoes reaction at pH 6.1 and whose modification appears responsible for the observed initial decrease in activity at these pH values. The other histidine residues undergoing reaction at pH 6.1 are not directly involved with the catalytic function, but the second residue undergoing reaction at pH 7.5 seems to perform an important role in this capacity.

Non-involvement of other amino acid residues in the alkylation of glutamate dehydrogenase

Since ethoxyformic anhydride is not totally selective in its mode of action, the possibility that the loss of enzyme activity observed at pH 7.5 reflected the alkylation of amino acid residues other than histidine was investigated.

The number of cysteine residues present in the enzyme preparation were assayed before and after

Fig. 3. Time-course of the inactivation and degree of acylation of glutamate dehydrogenase by ethoxyformic anhydride at pH 7.5

Enzyme was used at a final concn. of $2.5 \mu\text{M}$ and acylating agent at a final concn. of $20 \mu\text{M}$. $\circ$, Loss of enzyme activity; $\bullet$, formation of ethoxyformyl histidine. Experimental details are given in the text.

Fig. 4. Time-course of the inactivation and degree of acylation of glutamate dehydrogenase by ethoxyformic anhydride at pH 7.5 after the initial treatment of the enzyme with acylating agent at pH 6.1

Experimental details are given in the text.

alkylation by using 5,5'-dithiobis-(2-nitrobenzoate) as described by Bitensky *et al.* (1965). Assay mixtures (2.3 ml) were prepared by treating native or alkylated glutamate dehydrogenase (final concn. $7.6 \mu\text{M}$) with 5,5'-dithiobis-(2-nitrobenzoate) (final concn. 10 mM) in 0.1 M-tris-HCl buffer, pH 8.0.

A value of 6.4 cysteine residues/monomer was obtained for the unmodified enzyme preparation, which agrees well with the value obtained by amino acid analysis.

No significant alteration in this value was obtained after treatment of the enzyme as described above with ethoxyformic anhydride at pH 7.5.

The possibility of modifying tyrosine or tryptophan residues was examined by comparing the difference spectra obtained on treating the free amino acids (0.2 mM final concn.) with ethoxyformic anhydride (5 mM final concn.) with the difference spectra produced with either unmodified or glutamate dehydrogenase modified at pH 7.5.

Again, no evidence for the modification of either of these residues was obtained.

Modification of lysine-97 is known to cause a complete loss of glutamate dehydrogenase activity (Piszkiewicz *et al.*, 1970) and it was therefore suggested that this residue is of major importance in the catalytic function of the enzyme. The residue is particularly reactive towards pyridoxal phosphate, the interaction leading to characteristic changes in the absorption spectrum of the pyridoxal phosphate. This interaction was examined by incubating enzyme (final concn. $19 \mu\text{M}$) and pyridoxal phosphate (final concn. 0.5 mM) in the presence of 0.1 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 8.0 (total reaction volume 0.9 ml), at 25°C . Changes in E_{390} were recorded with a Hilger Gilford reaction kinetics spectrophotometer.

No changes in the spectral characteristics associated with the interaction of lysine-97 and pyridoxal phosphate were observed after the alkylation of glutamate dehydrogenase at pH 7.5, indicating that this residue at least was not modified in the ethoxyformic anhydride-treated enzyme.

Alkylation of glutamate dehydrogenase in the presence of substrates

The partial inactivation of glutamate dehydrogenase by ethoxyformic anhydride at pH 6.1 was not altered when the reaction was performed in the presence of either 10 mM-glutamate or 0.25 mM- NAD^+ . Further, no change in the degree of alkylation was observed in their presence. When comparable experiments were performed at pH 7.5 then a marked decrease in both the degree of alkylation and degree of inactivation occurred in the presence of glutamate (Fig. 5). NAD^+ (final concn. 0.25 mM) did not protect the enzyme against inactivation, though it did decrease the extent of alkylation, suggesting that the pro-

Fig. 5. Time-course of the inactivation and degree of acylation of glutamate dehydrogenase by ethoxyformic anhydride at pH 7.5 in the presence of glutamate (10 mM final concn.) or NAD^+ (0.25 mM final concn.)

○, Loss of enzyme activity without additions; □, loss of enzyme activity in the presence of 0.25 mM- NAD^+ ; △, loss of enzyme activity in the presence of 10 mM-glutamate; ●, degree of acylation without additions; ■, degree of acylation in the presence of 0.25 mM- NAD^+ ; ▲, degree of acylation in the presence of 10 mM-glutamate. Experimental details are given in the text.

tected residue was not involved in any catalytic function. When the enzyme was initially pretreated at pH 6.1 as described above and then readjusted to pH 7.5, the single histidine residue that undergoes reaction at the latter pH under these conditions was markedly protected by the presence of glutamate at a final concentration of 10 mM.

Effect of histidine modification on the normal response of glutamate dehydrogenase in the presence of GTP and ADP

Further information on the role of histidine residues in glutamate dehydrogenase was afforded by studying the response of modified enzyme in the presence of GTP and ADP, well-known effectors of enzyme activity.

The normal activating effect of ADP was unaffected by treating the enzyme with alkylating agent at either pH 6.1 or 7.5 under the standard experimental conditions.

The responses obtained when GTP was employed as the effector molecule are illustrated in Figs. 6 and 7.

After treatment of glutamate dehydrogenase at

Fig. 6. Effect of acylation of glutamate dehydrogenase at pH 6.1 by ethoxyformic anhydride on the response of the effector GTP

Enzyme was used at a final concn. of $2.5 \mu\text{M}$ and acylating agent at a final concn. of $20 \mu\text{M}$. ○, Enzyme activity in the absence of GTP; ▲, enzyme activity in the presence of GTP; ●, formation of ethoxyformyl-histidine in the presence of GTP. Experimental details are given in the text.

Fig. 7. Effect of acylation of glutamate dehydrogenase at pH 7.5 by ethoxyformic anhydride on the response to the effector GTP

Enzyme was used at a final concn. of $2.5 \mu\text{M}$ and acylating agent at a final concn. of $20 \mu\text{M}$. ○, Normal enzyme activity; ▲, enzyme activity in the presence of GTP; ●, formation of ethoxyformylhistidine in the presence of GTP. Experimental details are given in the text.

pH 6.1, the normal inhibitory effect of GTP was modified such that when two histidine residues had been alkylated, no further response was observed and the progress curve simply parallels the activity of the enzyme in the absence of GTP.

Treatment at pH 7.5 again resulted in the loss of the normal response to GTP. This time, however, little effect was observed after one histidine residue had been modified, the progress curve after this point again paralleling that obtained in the absence of GTP.

Discussion

The photo-oxidation of glutamate dehydrogenase in the presence of Rose Bengal leads to the oxidation of four histidine residues/monomer and an almost complete loss of activity. Three of the residues are rapidly oxidized without a corresponding decrease in enzyme activity and it seems reasonable to conclude that they are situated on the surface of the enzyme molecule. The loss of activity on photo-oxidation can apparently be attributed to the modification of a single histidine residue.

Further evidence for the participation of histidine residues in the normal functioning of glutamate dehydrogenase is afforded by the results obtained from the alkylation of the enzyme with ethoxyformic anhydride. Under the experimental conditions used the reagent reacted exclusively with histidine residues. At pH 6.1 the modification of three histidine residues took place with a corresponding loss of approximately half the original enzyme activity. The residues undergoing modification appear to be functionally distinct and tentative suggestions can be put forward for their probable roles in the mode of action of the enzyme. The partial but rapid initial loss of enzyme activity at pH 6.1 seems to be due to the alkylation of a single histidine residue, modification of the other two residues having little further effect on the residual activity. Neither glutamate nor NAD^+ is effective in protecting it against alkylation. A possible explanation for this observation is that a conformational change occurs as a result of this modification accompanied by a decrease in the maximum velocity of the enzyme-catalysed reaction.

The two histidine residues that undergo slower alkylation at pH 6.1 are not involved in the catalytic activity of the enzyme. One of them, however, may be associated with the GTP-regulatory site, perhaps together with the more rapidly reacting residue. Alkylation of the third histidine residue does not further affect any of the properties of the enzyme thus far examined and speculation about its function must await further experimental results.

The alkylation of glutamate dehydrogenase at pH 7.5 with ethoxyformic anhydride leads to the modification of two histidine residues. However, if

an initial alkylation at pH 6.1 is performed before that at pH 7.5, then only one residue is modified at the latter pH. The modification of this residue at pH 7.5 leads to a further substantial decrease in enzyme activity. Total loss of enzyme activity has not been observed under the experimental conditions used, probably because ethoxyformic anhydride is rapidly destroyed at pH 7.5. Increasing the concentration of the reagent to compensate for this degradation does result in complete loss of enzyme activity.

The presence of glutamate, but not NAD^+ , effectively retards the modification of the latter residue at pH 7.5, suggesting its involvement either in the catalytic action of the enzyme or alternatively in the binding of the amino acid substrate. The fact that this residue preferentially reacts under alkaline conditions would tend to imply that it is involved in the catalytic process rather than binding. It should be noted, however, that the presence of glutamate in the reaction medium could stabilize the protein against secondary changes occurring because of alkylation, which incidentally leads to the loss of enzyme activity, and before histidine can definitely be implicated in the catalytic mechanism of glutamate dehydrogenase, this should be investigated.

The higher degree of alkylation recorded at pH 6.1 is somewhat unexpected, based on the normal pK value for the imidazole group. Normally a greater degree of alkylation would be expected to occur at pH 7.5. The histidine residues that undergo preferential reaction at either pH value are clearly distinguishable by virtue of their effects on the enzyme and they are presumably sited differently in the protein molecule. A simple explanation for the observed reactivities could thus be proposed on the basis of the particular environment associated with the respective sites. Those histidine residues preferentially reacting at the lower pH would thus be expected to be found in largely hydrophobic or positively charged regions.

In the above context some speculation as to the location of the histidine residues might be possible. On the basis of the available evidence it is presumed that one of the histidine residues undergoing reaction at pH 7.5 is also alkylated at pH 6.1, and modification moreover is associated with the loss of the GTP response at both pH values. In this respect the presence of a histidine residue (histidine-413) adjacent to a tyrosine residue (tyrosine-421) (Landon *et al.*, 1971), whose modification by tetranitromethane also results in the loss of the normal GTP response (Piszkiwicz *et al.*, 1971), should be noted. These results may indicate that part of the primary sequence around residues 412 and 413 are associated with the GTP-binding site. Moreover, this region of the protein is largely hydrophobic (Landon *et al.*, 1971). However, Freedman & Radda (1969) have suggested that the modification of a specific lysine residue also

results in the loss of the normal GTP response. Goldin & Frieden (1971) showed that either lysine-425 or lysine-428 was the lysine residue probably undergoing modification in the experiments of Freedman & Radda (1969) and again a histidine residue (histidine-429) is found in the vicinity (Landon *et al.*, 1971). Clearly, to substantiate these suggestions it is necessary to determine the sequences around the histidine residues in the modified enzyme.

Baker (1967) has suggested that an imidazole group may be present at the active site of glutamate dehydrogenase, its function being to donate a proton or to accept it from the substrate. The chemical evidence given in the present paper supports this suggestion. It is noteworthy that both Greville & Mildvan (1962) and Rogers (1971) have reported the presence of a group in the enzyme-substrate complex with a pK value of between 7.2 and 7.8, which could well indicate the involvement of a histidine residue in the catalytic function.

At present little can be said about the effect of modification on the responses of the enzyme to GTP. The response at pH 6.1 is similar to that reported by Freedman & Radda (1969), who suggested that it may be due to a change in the ability of the enzyme to undergo a conformational change or alternatively may reflect an alteration in the ability of the modified enzyme to bind GTP. Preliminary investigations (R. Bailey-Wood & N. Tudball, unpublished work) suggest that an alteration in GTP binding does occur after modification at pH 6.1.

At pH 7.5, modification produces a pronounced activation in the presence of GTP. The reason for this is not immediately apparent; however, it is well known that GTP stimulates the enzyme when monocarboxylic acids are used as substrates. If the same catalytic site is responsible for the observed activity of glutamate dehydrogenase towards its many substrates then it seems reasonable to suggest that a conformational change occurs on modification similar to that occurring when monocarboxylic acids interact with the enzyme subsequently causing the observed GTP effect. The effects of GTP on enzyme activity, however, cannot be considered in isolation and the effects of modification on cofactor binding must also be considered before worthwhile conclusions can be made. Meaningful explanations of the observations must thus await the results of experiments particularly designed to answer these questions.

At present it is not possible to make unequivocal statements about the function of the many histidine residues in glutamate dehydrogenase. Tentative suggestions are that eight of the residues/monomer are located on or near the surface of the enzyme and that five of these may have a purely structural func-

tion. Of the other three residues, one seems to be associated directly or indirectly with the catalytic function of the enzyme whereas another may be associated with the GTP site, either as a structural agent or as binding agent. The role of the third residue is at present unclear but it may be associated with a regulatory site.

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