CONFORMATIONALLY IMPORTANT HISTIDINE RESIDUE IN GLUTAMATE DEHYDROGENASE FROM *NEUROSPORA CRASSA*

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1. Introduction

The NADP-specific glutamate dehydrogenase (GDH) [1] from *Neurospora crassa* [2] like the enzyme from *Candida utilis* [3] (EC 1.4.1.4.) exists as an equilibrium mixture of inactive or active conformations, the ratio of the 2 forms being dependant on the pH of the environment. Native wild-type enzyme is in an active form at pH > 7.8 and in an inactive form at pH < 7.0 in the absence of substrates. The p*K* of this transition is near pH 7.2 for both enzymes [2,3].

The amino acid sequence of the NADP-specific GDH of *N. crassa* has been reported to bear regions of high homology with the same enzyme from bovine liver and chicken liver [4]. Reactive amino acids such as lysine [5,6], arginine [7] and tyrosine [8] have been identified in both mammalian and the *Neurospora* enzymes with some protection against modification usually being given by the coenzyme [5–8]; it was suggested [7,8] that both the reactive arginine and tyrosine residues are in the nicotinamide-binding site of the enzyme. Here we report the presence of a group in the *Neurospora crassa* enzyme (NADP-specific) highly sensitive to diethylpyrocarbonate (DEP) at pH 6.0 which is usually indicative of the formation of a carboethoxyhistidine group. Evidence is presented to suggest that the histidine modified is conformationally important and that modification of this group perturbs the pH-sensitive conformational equilibrium which exists when the enzyme is in free solution at pH values close to neutrality [9].

2. Materials and methods

2.1. *Enzyme*

The growth conditions of the *Neurospora crassa* (STA-4) and the subsequent procedures for the isolation and storage of the purified GDH have been described [10]. The enzyme was assayed using 2.5 ml 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 M L-glutamate and 0.2 ml 0.2% $NADP^+$ solution. Usually 5–10 μl enzyme solution of 0.5–1.0 mg/ml were added to initiate the reactions at 21°C.

2.2. *Modification of the enzyme*

Diethylpyrocarbonate (50 μl), Sigma, London, were mixed with 5.0 ml absolute ethanol and 5 μl diluted reagent were added to 5 ml enzyme (0.5–1.0 mg/ml) pre-dialysed against 0.1 M sodium acetate buffer (pH 6.0) at 0–2°C. The reaction was monitored or allowed to proceed at 0°C. To terminate the reaction excess histidine (in solution) was added and after 10–15 min the incubation mixture was passed through a small Sephadex (G-10) column to remove free carboethoxyhistidine and excess histidine.

2.3. *Instrumentation*

Experimental data were obtained on a Cary 18 spectrophotometer and on a Perkin-Elmer M3 fluorimeter.

3. Results

Fig.1 displays typical data describing the loss of activity of the *Neurospora* GDH on incubation with DEP (for conditions see legend). No protection against modification is given by $NADP^+$, NADPH or 2-oxo-

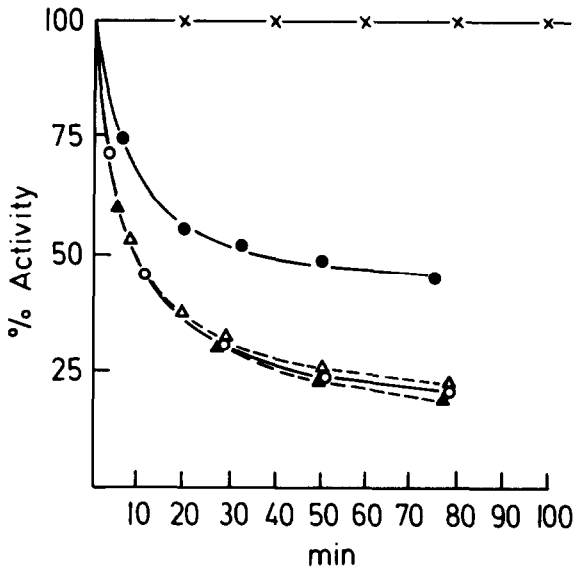

Fig.1. The relative rates of the reactions catalysed by GDH when incubated at pH 6.0 in 0.1 M sodium acetate buffer alone (x), plus 0.8 mM DEP (○), DEP + 100 μM $NADP^+$ (△, the same results are obtained with 100 μM NADPH or 300 μM NAD^+), DEP + 40 mM L-glutamate (●), DEP + 10 mM 2-oxoglutarate (▲). For assay conditions see section 2.

glutarate although L-glutamate could protect the enzyme. The amount of protection given by the amino acid was dependant on its concentration and on the concentration of the DEP. In fig.1 it is clear that complete inactivation of the enzyme does not take place under the conditions used in the experiment. However, if more DEP (sufficient to raise the final concentration to 1.5 mM) is added after 30 min incubation in the conditions indicated in fig.1, the residual activity of the enzyme disappeared altogether. The presence of L-glutamate could not completely protect the enzyme from inactivation by DEP.

It became obvious during the experiments that not only did the enzyme decrease in activity per se but that the time-dependant gain in activity induced by the transfer from incubation at pH 6.0 to the assay solution at pH 7.5 also became slower. The rate of the reactivation process was measured at 21°C by incubation of enzyme with 150 mM L-glutamate at pH 8.3 and then assaying the enzyme at various times in the standard assay solution (section 2). The results indicate that after modification has taken place, the normal substrate-pH-induced activation of enzyme proceeds at ~40% of the rate of native enzyme. Under these conditions native enzyme became active with a pseudo first-order rate of 1.3 min^{-1} and modified enzyme underwent similar activation at 0.5 min^{-1}. Furthermore for the activation to occur it was found necessary to expose the modified enzyme to more extreme conditions than the native enzyme; e.g., in the absence of $NADP^+$, glutamate had little effect on the modified enzyme at pH 7.3 whereas it greatly activates the native protein at this pH [10]. However, at pH ⩾7.8, the modified protein is activated by glutamate although pre-incubation of modified enzyme at pH 8.0 alone had virtually no effect on the initial rate of catalysis given by the enzyme.

This type of behaviour is typical of some of the *am* mutants [2,10] where single site amino acid replacements have been demonstrated to induce increased stability of an inactive (am^2, am^3, am^{19}) or an active (am^1) conformation of the enzyme.

Absorption spectra taken in the near UV region (270–300 nm) are identical for both DEP-reacted and native enzyme suggesting no modification or oxidation of tyrosine or tryptophan has taken place. However, hyperchromicity at 230, 240 and 255 nm in the DEP-reacted protein strongly suggests that histidine is being modified [11]. Using a molar absorption coefficient of 3200 cm^{-1} at 240 nm [11] it was estimated that 2–3 histidine residues/subunit were able to react with DEP although the loss of initial activity in an assay was associated with the first mole modified. This *Neurospora crassa* GDH does not exhibit the reversible association–dissociation phenomena [10] shown by the bovine liver enzyme and gel filtration studies using DEP-modified and native enzyme show that no dissociation of the hexameric enzyme occurs during or after the modification process even when 2–3 histidine residues/subunit are modified.

In wild-type and other forms of the enzyme from mutant strains of *N. crassa* there is a change in protein fluorescence intensity concomitant with the pH/substrate-linked conformational change [10]. Enzyme in the active conformation fluoresces with a higher intensity (100 fluorescence units) than enzyme in the inactive conformation (70 fluorescence units) and this fluorescence difference has been clearly shown to be conformational and not pH-dependant [10]. DEP-modified enzyme was found to have a similar fluorescence yield to native enzyme when both were at pH 6.0–6.6 whereas at pH 6.6–8.0 the modified protein failed to show the expected increase in fluorescence shown by native protein when the position of

its conformational equilibrium shifts to favour the active conformation. However, if either enzyme species originally at pH 6.0 were added to a system containing 150 mM L-glutamate at pH 8.2, a large increase in fluorescence yield from the modified enzyme and the native enzyme occurred although the rate of change of fluorescence yield from the two species proceeded at different rates. The rate of the process in the modified enzyme was 41% as fast as that which occurred in native enzyme (fig.2). The presence of L-glutamate at pH 7.3 had no effect on the fluorescence intensity of the modified protein although as expected the native enzyme showed an increase in fluorescence under these conditions. In both cases where a change in protein fluorescence took place it occurred at a similar rate as the increase in enzyme activity under the same conditions. The removal of the L-glutamate by gel-filtration from a solution of DEP-reacted enzyme causes the protein to reassume a low activity and low fluorescence state at pH 8.0 indicative of the inactive conformation of the enzyme. This indicates that the activation process leading to a higher activity and fluorescence state occurs in the modified protein and is not due to a catalysed cleavage of the normally, slowly released carboethoxy group from the modified histidine residue [11].

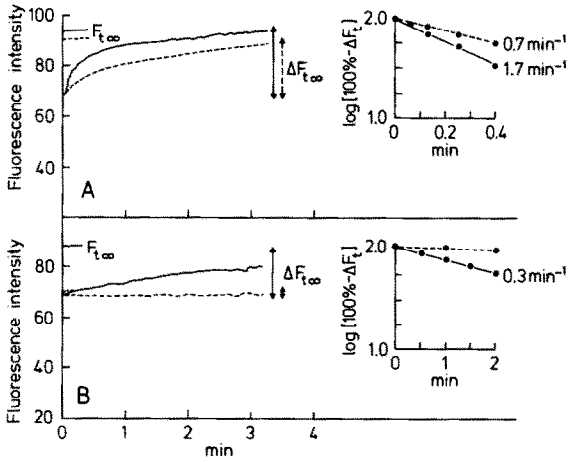

Fig.2. The slow increase in protein fluorescence from native (——) or DEP modified protein (– –) induced by the addition of 150 mM L-glutamate and simultaneous change in pH from 6.0 to pH 8.2 (A) or 7.3 (B). The insets are semi-logarithmic plots describing the rates of change of fluorescence expressed as a percentage (at time = t) of the maximum change $\Delta F_{t\infty}$. Native enzyme (●——●), modified enzyme (●– –●).

Experiments using the fluorescence emission from the nicotinamide moiety of NADPH demonstrated that the reduced-coenzyme binds to the DEP-reacted enzyme with the same affinity as to native enzyme at pH 6.0. The dissociation constant for the reaction between both modified and native enzyme and NADPH was 0.8 μM in 0.1 M sodium acetate buffer (pH 6.0 at 21°C). In both cases ~5.6 mol NADPH were bound/M_r 288 400 hexamer [6] (for experimental procedure see [13]) and the enchancement of the NADPH fluorescence was the same in both native and modified enzyme. Furthermore, the addition of L-glutamate to the binary complex, enzyme–NADPH, caused equal increases in fluorescence emission at 450 nm (correcting for changes in ionic strength) indicating the formation of stable abortive complexes [12].

4. Discussion

The failure of 2-oxoglutarate to protect the enzyme from modification and the ability of L-glutamate to partially prevent inactivation by DEP suggests that either glutamate alone forms a complex with enzyme which sterically hinders the DEP from the reactive residue, or that the amino group of the glutamate possibly decreases the effective concentration of the reagent. DEP suffers hydrolysis in aqueous solutions thus the residual enzyme activity seen in fig.1 by all curves is probably a result of the disappearance of reagent by hydrolysis. The fact that L-glutamate could not completely protect against inactivation by the DEP, especially at high DEP concentrations (⩾1.5 mM) is to be expected when consideration is taken of the reactivity of the reagent [11], i.e., second-site modifications not protected by L-glutamate may lead to irreversible inactivation by the reagent. More than one histidine has been shown to react under these conditions, although only the first group carboethoxylated is associated with the modification process leading to an inactivated enzyme able to be reactivated by glutamate at pH 8.3 and possessing a low fluorescence state at pH 8.0. The lack of protection exhibited by $NADP^+$ or NADPH throws doubt on the possibility of the reactive lysine residue 126 [5,6] being responsible for the loss of enzyme activity and preliminary results have shown that pyridoxal-5-phosphate can still be reduced onto the DEP-modified protein.

NADP or NADPH has also been shown to protect a reactive arginine and tyrosine present in this enzyme from reaction with 1,2-cyclohexamedisine and tetranitromethane respectively [7,8]. Both of these residues are thought to be part of the nicotinamide-binding domain in the active site and therefore any modification by DEP that occurs here would be expected to perturb either the enhancement of the fluorescence from the nicotinamide group of bound NADPH and/or the affinity of the NADPH for the enzyme.

The ability to activate the modified protein by high concentration of L-glutamate at high pH together with the results obtained from protein fluorescence studies lead us to propose that the active site of the enzyme is untouched and that the modification is in a residue essential to the maintenance of the active conformation of the enzyme. Interestingly, histidine is an amino acid not conserved in the primary structure of dehydrogenases and this probably is further indicative that the residue reactive to DEP is not in the active site where high homology or conservation of type of amino acid exists, in particular the more chemically reactive amino acids [3,4].

A single site replacement of histidine 142 by glutamine has given rise to an enzyme (am^2) with a stabilized inactive (low fluorescence) conformation [2] and studies using this enzyme are now in progress.

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References

[1] David, M., Rasched, I. and Sund, H. (1976) Eur. J. Biochem. 74, 379–385.
[2] Brett, M., Chambers, G. K., Holder, A. A., Fincham, J. R. S. and Wootton, J. C. (1976) J. Mol. Biol. 106, 1–22.
[3] Neumann, P., Markau, K. and Sund, H. (1976) Eur. J. Biochem. 65, 465–472.
[4] Wootton, J. C. (1974) Nature 252, 542–546.
[5] Gore, M. G., Wootton, J. C. and Fincham, J. R. S. (1973) Biochem. Soc. Trans. 1, 1278.
[6] Blumenthal, K. M. and Smith, E. L. (1973) J. Biol. Chem. 248, 6002–6008.
[7] Blumenthal, K. M. and Smith, E. L. (1975) J. Biol. Chem. 250, 6555–6559.
[8] Blumenthal, K. M. and Smith, E. L. (1975) J. Biol. Chem. 250, 6560–6563.
[9] West, D. J., Tuveson, R. W., Barratt, R. W. and Fincham, J. R. S. (1967) J. Biol. Chem. 242, 2134–2138.
[10] Ashby, B., Wootton, J. C. and Fincham, J. R. S. (1974) Biochem. J. 143, 317–329.
[11] Melchior, W. B. and Fahrney, D. (1970) Biochemistry 9, 251–258.
[12] Gore, M. G. and Greenwood, C. (1975) Biochem. Biophys. Res. Commun. 62, 997–1002.
[13] Holbrook, J. J., Yates, D. W., Reynolds, S. J., Evans, R. W., Greenwood, C. and Gore, M. G. (1972) Biochem. J. 128, 933–940.