Histidine Residues and the Enzyme Activity of Pig Heart Supernatant Malate Dehydrogenase

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(Received 25 February 1974)

1. Supernatant pig heart malate dehydrogenase is completely inhibited by reaction with diethyl pyrocarbonate at pH 6.5, when 0.58 ± 0.1 residue of ethoxycarbonylhistidine is formed per NADH-binding site. 2. Oxaloacetate and hydroxymalonate protect the enzyme from inhibition in the absence of coenzyme. 3. Limited ethoxycarbonylation does not alter the binding of NADH to the enzyme but prevents the enzyme-NADH complex from interacting with hydroxymalonate in a ternary complex.

Supernatant malate dehydrogenase and lactate dehydrogenase have very similar tertiary structures (Hill *et al.*, 1972; Adams *et al.*, 1972). Histidine-195 plays a central role in the activity of lactate dehydrogenase as the acceptor and donor of the proton involved in the reaction (Holbrook & Gutfreund, 1973). A proton is also a reactant for malate dehydrogenase, and the work reported here is an attempt to discover if this enzyme also has a histidine residue with the same function as histidine-195 of lactate dehydrogenase. Anderton & Rabin (1970) found that a histidine residue was involved in the activity of the mitochondrial malate dehydrogenase, although it is not known if this form of malate dehydrogenase is a structural homologue of lactate dehydrogenase.

The method of Gerding & Wolfe (1969) was used to prepare the supernatant enzyme from pig heart. The negative absorptions (steps 4-7) were not necessary. In our hands the hydroxyapatite column chromatography did not reproducibly give a purification and was omitted. Enzyme activity was expressed as units of μmol of NADH produced/min in an incubation mixture containing at 25°C 5 mM-NAD⁺, 0.1 M-L-malate and 0.1 M-glycine adjusted to pH 10 with 5 M-NaOH. Protein was determined from absorbance measurements at 280 nm by using $E_{1\text{cm}}^{1\%} = 9.3$ given by Gerding & Wolfe (1969). The specific enzyme activity of our preparations was 77 units/mg; this is only 70% of the 108 units/mg reported by Gerding & Wolfe (1969) for the pure enzyme. The presence of impurity could also be deduced from a determination of the NADH-binding capacity of the preparation by using the continuous micro-fluorimetric titration as reported by Holbrook & Wolfe (1972). Only 1.4 mol of NADH was bound per mol of protein, assuming that the protein molecule consists of two subunits of mol.wt. 35000. Before the experiments reported here the crystalline enzyme suspension was centrifuged

and the crystals were dissolved in 20 mM-H₃PO₄ adjusted to pH 6.5 with 5 M-NaOH and then filtered through a column of Sephadex G-50 equilibrated in the same buffer. If (NH₄)₂SO₄ was not completely removed it competed effectively for the diethyl pyrocarbonate. This latter reagent was obtained from Sigma (London) Chemical Co., Kingston-on-Thames, U.K., and the batch used in these experiments was 2.6 M when assayed as described by Holbrook & Ingram (1973).

Preliminary experiments established that enzyme was most rapidly inhibited in a buffer prepared by adjusting a solution of 20 mM-H₃PO₄ with 5 M-NaOH to pH 6.5. This buffer was used for all the experiments reported in this paper. The rate of inhibition was half its maximum value at pH 6 and 7.5. The enzyme could be protected from inhibition by diethyl pyrocarbonate if substrates and coenzymes were present in the incubation mixture. For example, the rate of loss of enzyme activity ($k_{\text{obs}} = 2 \text{ min}^{-1}$; samples assayed every 15 s) when 7 μM -enzyme (calculated from a relative subunit mass of 35000) was incubated with 0.78 mM-inhibitor was decreased 10-fold by the inclusion of either 3 mM-oxaloacetate or 25 μM -NADH in the incubation mixture. In another experiment with 28 μM -enzyme and 0.5 mM-inhibitor the rate of inhibition was decreased 10-fold by 3.3 mM-hydroxymalonate or 0.4 mM-NADH and no inhibition was detected over 20 min if the hydroxymalonate and NADH were both present. Oxaloacetate did not react with the inhibitor. There is as yet no definitive steady-state kinetic investigation of the mechanism of the supernatant enzyme. The form of the initial velocity equations is consistent with a sequential Bi Bi mechanism (A. Lodola, unpublished work), but it is not known whether there is a compulsory, preferred or random order of addition of substrate and coenzyme. The above results suggest that a preferred or random order should be considered, although the

protection by oxaloacetate in the absence of NADH does not necessarily mean that this substrate binds at the active site.

Inhibition after reaction with diethyl pyrocarbonate is due to the modification of histidine residues. Although the inhibitor will react with other side chains it was possible to reactivate the inhibited enzyme with hydroxylamine. For example $4\mu\text{M}$ -enzyme was 98% inhibited after incubation with the inhibitor (2.6mM) for 10min and then diluted into 0.7M-hydroxylamine hydrochloride, which had been adjusted to pH6.5 with 5M-NaOH. When assayed after 10min the enzyme activity was $100 \pm 5\%$ of that of an untreated control. Hydroxylamine regenerates histidine from ethoxycarbonylhistidine, but is without effect on the ethoxycarbonyl derivatives of tyrosine and lysine (Melchior & Fahrney, 1970). Cysteine residues are not essential for the activity of this enzyme (Devenyi *et al.*, 1966). When inhibited enzyme was freed from excess of inhibitor by filtration through a column of Sephadex G-50 the enzyme activity of the protein peak slowly reappeared. The initial rate of regain of activity was approx. 2%/h. This is much slower than with lactate dehydrogenase or glutamate dehydrogenase (Holbrook & Ingram, 1973; Wallis & Holbrook, 1973).

The formation of ethoxycarbonylhistidine in an enzyme can be monitored from the absorbance peak at 240nm. In samples of enzyme that had not been more than 90% inhibited this was the only absorbance peak visible in a spectrum of the inhibited enzyme

measured against that of an uninhibited control. With greater degrees of inhibition a negative absorbance peak at 280nm appeared (ethoxycarbonyltyrosine; Mùhlrad *et al.*, 1967). The rate of appearance of ethoxycarbonylhistidine was approx. $40\text{M}^{-1}\cdot\text{s}^{-1}$ at pH6.5, and this is similar to the rate of reaction of free imidazoles at the same pH (Holbrook & Ingram, 1973). The rate of reaction was not quite fast enough for it to be possible to add 1 equivalent of inhibitor to 1 equivalent of enzyme and observe 100% inhibition with the formation of 1 equivalent of ethoxycarbonylhistidine. The closest that this ideal has been approached is in experiments similar to that shown in Fig. 1. In that case $86\mu\text{M}$ -inhibitor reacted with $72\mu\text{M}$ -active enzyme to yield $36\mu\text{M}$ -ethoxycarbonylhistidine and 68% inhibition after 18min. Thus there is sufficient inhibitor unaccounted for to allow the reaction of up to 0.5mol of another amino acid side chain/mol of NADH-binding sites. Such a calculation is the worst case and does not take into account the loss of inhibitor by hydrolysis during the 18min incubation.

The results described in detail in the legend to Fig. 1 show that a sample of enzyme (3.6mg/ml) containing $72\mu\text{M}$ -NADH-binding sites is completely inhibited by the reaction of 0.58 ± 0.1 histidine residue/NADH-binding site. It seems unlikely that this unusual ratio arises from the use of only 70%-active enzyme, since the results are referred to NADH-binding sites. The reactivation of the enzyme in hydroxylamine indicates that it is the histidine modification that causes

Fig. 1. Inhibition of supernatant malate dehydrogenase by ethoxycarbonylation of histidine residues in the enzyme

Fig. 1(a) is a record of the increase in absorbance at 250nm in a solution containing enzyme (3.6mg/ml, 70% active, $72\mu\text{M}$ in sites that can bind NADH) monitored on a double-beam spectrophotometer with a recorder output sensitivity of 0.15 absorbance unit full scale during reaction with $86\mu\text{M}$ -diethyl pyrocarbonate at pH 6.5. After 19 min the inhibitor concentration was increased to $133\mu\text{M}$. During this and similar reactions samples of the incubation mixture were assayed for enzyme activity, and Fig. 1(b) shows the relation between the residual enzyme activity and the concentration of ethoxycarbonylhistidine formed on the enzyme: 1 mM-ethoxycarbonylhistidine has an absorbance of 1.9 in a 1 cm light-path at 250nm (Holbrook & Ingram, 1973). The two broken lines enclose the results and indicate that the enzyme is inhibited by formation of 0.58 ± 0.1 mol of ethoxycarbonylhistidine/mol of NADH-binding sites.

Fig. 2. Nucleotide binding by ethoxycarbonylated malate dehydrogenase

Samples of enzyme (165 μM in NADH-binding sites) were incubated with concentrations of diethyl pyrocarbonate between 130 μM and 2.6 mM for 5 min. At exactly the same moment one portion of the incubation mixture was diluted 1:100 (v/v) and immediately assayed, while another portion was diluted 35-fold into 10 mM-hydroxymalonate in 20 mM- H_3PO_4 (which had been adjusted to pH 6.5 with 5 M-NaOH) and was titrated micro-fluorimetrically with NADH. The traces in Fig. 2(a) show the increases in NADH fluorescence (excited at 320 nm, emission at about 430 nm via a Kodak Wratten no. 98 filter) after subtraction of the fluorescence of NADH added to a solution without enzyme. The apparatus was described by Holbrook (1972) and the application to malate dehydrogenase by Holbrook & Wolfe (1972). The continuous lines are (from the top) for fully active enzyme and for samples that had been increasingly inhibited by diethyl pyrocarbonate. Fully active enzyme (----) and 98%-inhibited enzyme (-.-.-.-) were also titrated with NADH in the absence of hydroxymalonate. Fig. 2(b) shows the end point of the fluorescence titration as a function of the degree of inhibition by diethyl pyrocarbonate. The line is drawn to show how fluorescence is predicted to decrease if inhibition blocked only hydroxymalonate binding and did not change NADH binding.

the loss of activity. The difficulties which prevent the identification of histidine residues modified by diethyl pyrocarbonate were discussed by Holbrook & Ingram (1973). With the amino acid sequence as yet not completely determined there is only indirect evidence that the two subunits in the dimeric molecule are identical (Devenyi *et al.*, 1966), and it should not be concluded that only one of the two subunits is enzymically active. An alternative explanation might be that the histidine residue that is modified is at the interface between the two subunits and that modification of one of the two symmetrically related histidines at this interface prevents a conformational change in both subunits which is essential for enzyme activity. There have been some indications that the two subunits in the crystal may not be identical as regards NAD^+ binding (Hill *et al.*, 1972). Further experiments are required before the results can be interpreted in molecular terms.

The results shown in Fig. 2 suggest that, up to about 80% inhibition, the modification of histidine residues

destroys the ability of the enzyme-NADH complex to interact with hydroxymalonate. Only during the latter stages of the inhibition, where the specificity of the reaction for histidine is not certain, is the ability of the enzyme to form a complex with NADH destroyed. This result is consistent with there being a histidine residue at the substrate-binding site of malate dehydrogenase. The analogy to lactate dehydrogenase is not complete, however, since the reactivity of the malate dehydrogenase histidine residue is much more affected by NADH than is the reactivity of the lactate dehydrogenase histidine. In view of the unusual ratio noted in Fig. 1(b) it would be interesting to determine whether there are one or two hydroxymalonate sites on the dimeric enzyme. However, this experiment needs unavailably high concentrations of enzyme and probably also the use of labelled substrate analogue.

A. L. is supported by an S.R.C. studentship. The fluorimeter was constructed with a grant-in-aid from The Royal Society (London).

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