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The chemical reactivity of the histidine-195 residue in lactate dehydrogenase thiomethylated at the cysteine-165 residue

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The specific thiomethylation of cysteine-165 (insertion of a methylthio group, CH_3–S–) in pig heart lactate dehydrogenase results in a decreased affinity for carbonyl ligands that is accompanied by a decreased nucleophilic reaction of histidine-195 with diethyl pyrocarbonate. The rate constants at 10°C for the modification of native and thiomethylated lactate dehydrogenase by diethyl pyrocarbonate were $173\,M^{-1}\cdot s^{-1}$ and $8.7\,M^{-1}\cdot s^{-1}$ respectively. It was found that 0.86 ± 0.07 histidine residue per subunit reacted with diethyl pyrocarbonate in thiomethylated lactate dehydrogenase. This reaction was not affected in the enzyme–NADH binary complex, but was diminished in the enzyme–NADH–oxamate ternary complex. In the enzyme–NADH complex the reaction of diethyl pyrocarbonate was controlled by two groups with pK_a 6.8 and 7.9. The decreased reactivity of histidine-195 was selective in thiomethylated lactate dehydrogenase, since the reactivity of arginine and/or lysine residues was enhanced.

Studies on the binding of pyruvate to lactate dehydrogenase have implicated protonated histidine-195 forming a hydrogen bond with the substrate carbonyl group (Woenckhaus *et al.*, 1969; Holbrook & Ingram, 1973; Holbrook & Gutfreund, 1973; Whitaker *et al.*, 1974; Bloxham *et al.*, 1975) and an arginine residue (arginine-171 or arginine-109) forming an ion-pair with the substrate carboxylate group (Holbrook *et al.*, 1975). Thiomethylation of cysteine-165 (insertion of a methylthio group, CH_3–S–) in lactate dehydrogenase produced a catalytically active enzyme that had a specifically diminished affinity for pyruvate and lactate (Bloxham & Wilton, 1977; Bloxham *et al.*, 1979). Since cysteine-165 is located some distance from histidine-195 (approx. 1.1 nm) in the crystal structure of the enzyme (Holbrook *et al.*, 1975), it was decided to determine whether thiomethylation of the cysteine residue induced a specific change in the properties of the catalytically important histidine-195 that might be related to the altered affinity for pyruvate. To accomplish this objective the effect of thiomethylation on the reaction of histidine-195 with diethyl pyrocarbonate was investigated. In the native enzyme this chemical reaction shows a unique rate enhancement (Holbrook & Ingram, 1973).

Materials and methods

Pig heart lactate dehydrogenase was obtained from Boehringer Corp. (London), London W.5, U.K. Thiomethylated lactate dehydrogenase was prepared by reaction of the enzyme with 10 mM-methyl methanethiosulphonate followed by four repeated $(NH_4)_2SO_4$ precipitations (Bloxham *et al.*, 1979). The thiomethyl-enzyme was stored as a suspension in $(NH_4)_2SO_4$ solution and was stable for several months at 4°C. The enzyme was collected by centrifugation at 12000 *g* for 20 min and dissolved in 0.1 M-sodium pyrophosphate buffer, pH 7, for use within 24 h.

For modification with diethyl pyrocarbonate (Sigma Chemical Co., Poole, Dorset, U.K.) the inhibitor was freshly diluted into anhydrous methanol and used immediately. The final concentration of methanol did not exceed 5% (v/v) in the enzyme reaction mixture. Diethyl pyrocarbonate was standardized by reaction with α-*N*-acetylhistidine (Holbrook & Ingram, 1973). After inhibition the modified enzyme was diluted a total of 2500-fold to assay activity so that the effect of inhibitor was negligible in the cuvette. The assay concentration of pyruvate with native and thiomethylated lactate dehydrogenase was 200 μM and 20 mM respectively, to overcome the different affinities for pyruvate (Bloxham & Wilton, 1977). Because of the very high rate of reaction of native enzyme with diethyl pyrocarbonate, this reaction was usually performed at 10°C. For direct comparison between native and thiomethylated lactate dehydrogenase the same temperature was always used; however, for many

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experiments modification of the thiomethyl-enzyme was performed at 20°C to obtain higher reaction rates.

Results

Diethyl pyrocarbonate modification of thiomethylated lactate dehydrogenase

Initially 50 μM (protomers) thiomethylated lactate dehydrogenase or native enzyme was treated with 1 mM-diethyl pyrocarbonate in 0.1 M-sodium pyrophosphate buffer at 10°C and the reaction was monitored by the increase in absorption at 240 nm. On the basis of a molar absorption coefficient of 3600 litre $\cdot$ mol^{-1} $\cdot$ cm^{-1} (Holbrook & Ingram, 1973), diethyl pyrocarbonate modified 0.98 $\pm$ 0.04 histidine residue per native subunit after 87 s, whereas the reaction of thiomethylated lactate dehydrogenase was considerably slower and only 0.86 $\pm$ 0.07 histidine residue per subunit reacted after 330 s. Neither modification was first-order (Fig. 1). Plots of log (activity) versus time were always slightly curved over a range of inhibitor concentration and reaction conditions (i.e. varied pH and temperature). Plots of the apparent $t_{\frac{1}{2}}^{-1}$ versus diethyl pyrocarbonate concentration (varied from 0.25 to 3 mM) were linear. This enabled calculation of the diethyl pyrocarbonate second-order reaction constant from slopes of these plots as 173 M^{-1} $\cdot$ s^{-1} and 8.7 M^{-1} $\cdot$ s^{-1} for native and thiomethylated lactate dehydrogenase respectively. The first value is similar to values (216 M^{-1} $\cdot$ s^{-1}) previously reported by Holbrook & Ingram (1973) and shows that the reactive group is activated in comparison with free histidine (24 M^{-1} $\cdot$ s^{-1} at 20°C). In contrast, the reaction rate constant with thiomethylated lactate dehydrogenase is nearer to that for free histidine and indicates the loss of some unique active-site property.

The $t_{\frac{1}{2}}$ values for inhibition of native and thiomethylated lactate dehydrogenase by 1 mM-diethyl pyrocarbonate at 10°C were 4 s and 85 s respectively, yielding apparent rate constants of 182 M^{-1} $\cdot$ s^{-1} and 7.4 M^{-1} $\cdot$ s^{-1}. These values are extremely close to the rate constants calculated from measurements of reaction by increase in absorbance at 240 nm. This indicates that enzyme inhibition and histidine modification must proceed concurrently for both enzymes. When the fraction of enzyme activity versus mol of ethoxycarbonylated histidine was plotted for native lactate dehydrogenase a straight line with a slope of -1 was obtained (Fig. 2), whereas with thiomethylated lactate dehydrogenase there was a slight break in the line after 50% inhibition and histidine modification was slightly greater than enzyme inhibition. This may reflect the fact that the low rate of diethyl pyrocarbonate

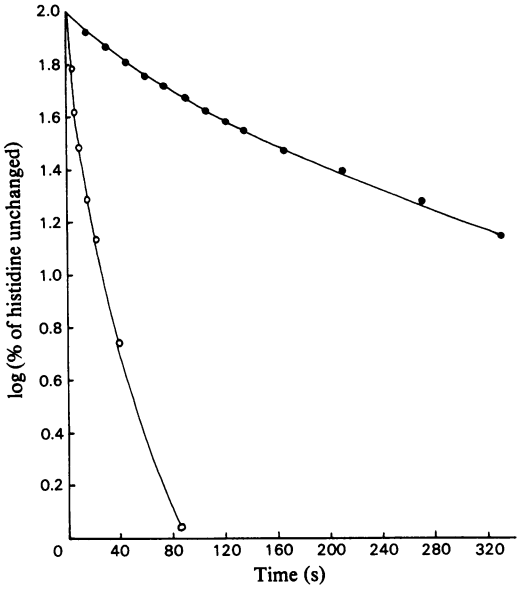

Fig. 1. *Comparison of reaction of native* (○) *and thiomethylated* (●) *lactate dehydrogenase with diethyl pyrocarbonate*

Lactate dehydrogenase (50 μM in protomers) was made to react at 10°C with 1 mM-diethyl pyrocarbonate in 0.1 M-sodium pyrophosphate buffer, pH 7.2, and the reaction was monitored by increased absorbance at 240 nm.

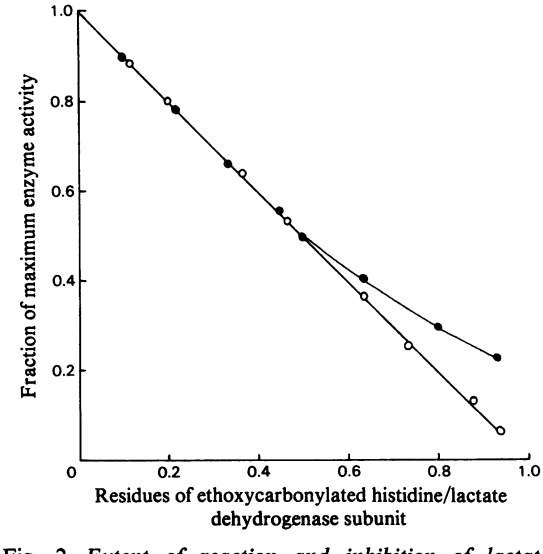

Fig. 2. *Extent of reaction and inhibition of lactate dehydrogenase by diethyl pyrocarbonate*

The reaction was performed exactly as described in Fig. 1 except that the activity of enzyme samples was recorded at the same time that 240 nm absorbance was measured.

reaction with histidine-195 enables alternative histidine residues to react in the later stages of enzyme modification.

The reaction of thiomethylated lactate dehydrogenase with diethyl pyrocarbonate showed protection by ligands that bind specifically to the enzyme active site (Fig. 3). Both NADH and oxamate, a non-reactive analogue of pyruvate (Novoa *et al.*, 1959), in the binary complex did not provide any protection against diethyl pyrocarbonate, whereas in the ternary complex the enzyme was virtually completely protected against inhibition. It is only in the ternary complex that full protection would be expected, since the protonated histidine-195 should be hydrogen-bonded to the pseudo carbonyl group of the oxamate amide. Much higher oxamate concentrations were required to provide protection with thiomethylated lactate dehydrogenase compared with native lactate dehydrogenase, which reflects the decreased affinity of the enzyme for the carbonyl substrate.

Reaction of thiomethylated lactate dehydrogenase with bromopyruvate

As discussed by Holbrook & Ingram (1973), there is little opportunity of proving that the residue that reacts with diethyl pyrocarbonate is histidine-195. However, Berghauser *et al.* (1971) have shown by peptide 'mapping' that bromopyruvate modifies exclusively histidine-195 in the enzyme–NAD^+ binary complex. Bromopyruvate was a substrate for thiomethylated lactate dehydrogenase in the presence of NADH with K_m 7mM, which compares with apparent K_m 12mM for pyruvate. However, the maximum specific activity was only 90μmol of NADH oxidized/min per mg of enzyme at 25°C, which compared with a maximum specific activity of 420μmol of NADH oxidized/min per mg of enzyme at 25°C with saturating pyruvate. Both

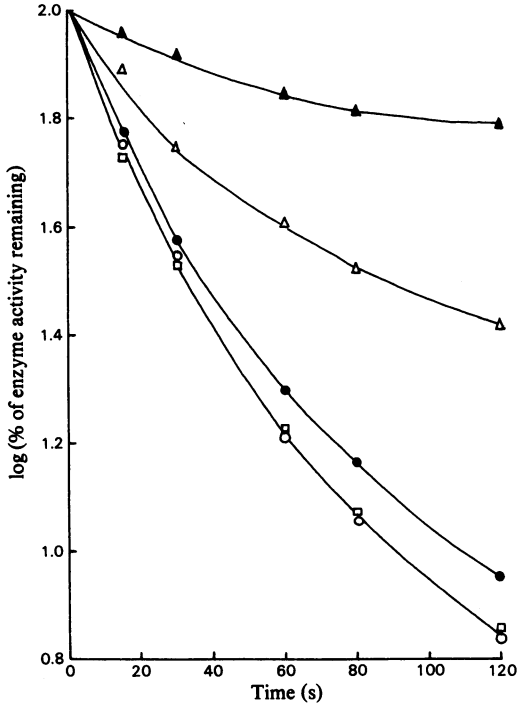

Fig. 3. *Ligand protection against inhibition of thiomethylated lactate dehydrogenase by diethyl pyrocarbonate*

Thiomethylated lactate dehydrogenase (40μM in subunits) in 0.1M-sodium pyrophosphate buffer, pH7.2, was made to react at 20°C with 2mM-diethyl pyrocarbonate. Ligand additions were: ○, none; □, 10mM-oxamate; ●, 280μM-NADH; △, 280μM-NADH and 5mM-oxamate; ▲, 280μM-NADH and 10mM-oxamate.

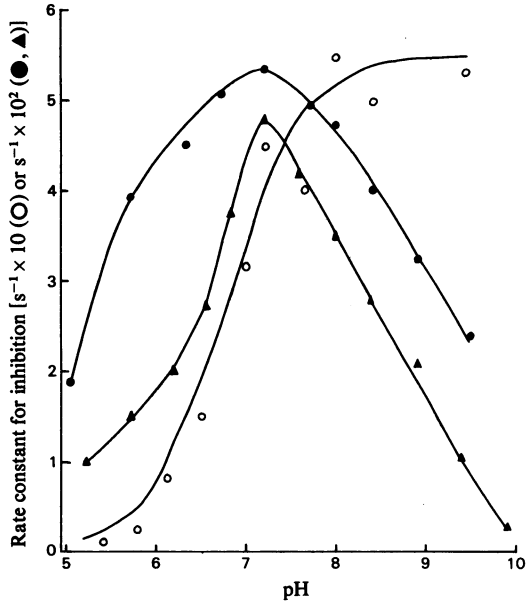

Fig. 4. *pH-dependence for the inactivation of native and thiomethylated lactate dehydrogenase*

The pseudo-first-order rate constant for the inactivation of both forms of lactate dehydrogenase (40μM in subunits) was measured at a variety of pH values with 0.1M-sodium pyrophosphate buffers. The native enzyme (○) was made to react with 0.4mM-diethyl pyrocarbonate at 0°C. The continuous line for the data was calculated for pK_a 6.8 and a maximum rate constant for inhibition of $55 \times 10^{-3} s^{-1}$. The thiomethylated enzyme was made to react with 2mM-diethyl pyrocarbonate at 20°C in the presence (▲) or absence (●) of 280μM-NADH.

native and thiomethylated lactate dehydrogenase (40 μM in subunits) in 50 mM-potassium phosphate buffer, pH 7.0, containing 17 mM-NAD^+ were made to react with 10 mM-bromopyruvate. Over 24 h the native enzyme lost 96% of enzyme activity in a time-dependent reaction, whereas the thiomethylenzyme lost only 11% of activity. Clearly this result confirms that it is the nucleophilic reactivity of histidine-195 in thiomethylated lactate dehydrogenase that is dramatically decreased.

pH-dependence of diethyl pyrocarbonate inhibition

The pH-dependence for the inhibition of native lactate dehydrogenase by diethyl pyrocarbonate (Fig. 4) fitted reasonably well to a single pK_a of 6.8 and corroborated earlier measurements (Holbrook & Ingram, 1973). For thiomethylated lactate dehydrogenase, rates of inhibition were lower at each pH value. Furthermore, for both the free enzyme and the enzyme–NADH binary complex, the pH–activity profile indicated a bell-shaped curve, with pH 7.2 as the optimum for inhibition. The decrease in rate of inhibition above pH 7.2 with thiomethylated lactate dehydrogenase was not due to unusual instability of ethoxycarbonylated histidine in the modified enzyme, since its $t_{\frac{1}{2}}$ for re-activation at pH 9.4 was 57 min, which is slightly slower than the same $t_{\frac{1}{2}}$ (26 min) for ethoxycarbonylated native lactate dehydrogenase.

Table 1. *Comparison of the inhibition of native and thiomethylated lactate dehydrogenase by arginine- and lysine-reactive reagents*

Native and thiomethylated lactate dehydrogenase (1 mg/ml) were made to react at 20°C with various concentrations of inhibitor over 5 h. The rate constant for inhibition was estimated from the slope of plots of log (activity) versus time. For phenylglyoxal and trypsin inhibition the reaction buffer was 0.1 M-sodium pyrophosphate buffer, pH 8.0, and for butanedione inhibition 0.25 M-sodium borate buffer, pH 8.0, was used. Both enzymes were stable in the absence of the inhibitor.

		$10^3 \times$ Rate constant for inhibition (min^{-1})	
Inhibitor	Concn.	Native enzyme	Thiomethyl-enzyme
Butanedione	40 mM	6.2 ± 0.3	20 ± 1.1
	100 mM	8.8 ± 0.5	29 ± 1.2
	200 mM	11.7 ± 0.45	35 ± 2.0
Phenylglyoxal	1.25 mM	1.1 ± 0.05	5.6 ± 0.45
	2.5 mM	2.3 ± 0.10	10 ± 0.7
	5.0 mM	3.6 ± 0.12	11.7 ± 1.0
Trypsin	50 μg/ml	4.2 ± 0.5	8.5 ± 0.7
	100 μg/ml	7 ± 0.5	16 ± 1.4
	200 μg/ml	21 ± 1.3	41 ± 3

Reaction of basic amino acid residues in thiomethylated lactate dehydrogenase

I was interested whether the decrease in chemical reactivity of histidine-195 was shared in a similar manner by other residues in the active site. The reactions of lactate dehydrogenase with phenylglyoxal, butanedione and trypsin (for lysine and arginine residues) (Berghauser & Falderbaum, 1971; Yang & Schwert, 1972; Jeckel *et al.*, 1973) have been reported previously, and all three of these reagents were tested with thiomethylated lactate dehydrogenase (Table 1). For all three methods of inhibition the loss of enzyme activity was time-dependent and followed pseudo-first-order kinetics. At extended time of reaction complete inhibition was given by all three treatments. In every case the rate of inhibition was higher for the thiomethylated enzyme, suggesting that arginine residues (or lysine residues in the tryptic modification) react more rapidly. This is the exact reverse to the reaction with histidine-195, and shows that the decreased activity of histidine-195 in lactate dehydrogenase must be a unique consequence of thiomethylation.

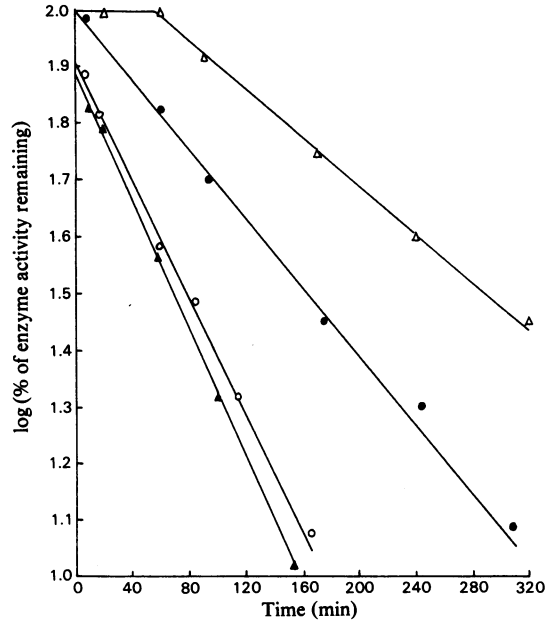

Fig. 5. *Ligand protection against inactivation of thiomethylated lactate dehydrogenase by phenylglyoxal*

Thiomethylated lactate dehydrogenase (40 μM in subunits) was made to react at 20°C in 0.1 M-sodium pyrophosphate buffer, pH 8.0, with 10 mM-phenylglyoxal in the presence of the following ligand concentrations: ○, none; ●, 280 μM-NADH; △, 280 μM-NADH and 10 mM-oxamate; ▲, 10 mM-oxamate.

The enhanced reactivity of basic residues in thiomethylated lactate dehydrogenase could reflect a general disruption of enzyme conformation, leading to an increase in general reactivity. However, thiomethylated lactate dehydrogenase was substantially protected from inhibition by phenylglyoxal and trypsin by NADH and oxamate (Fig. 5). Ligand protection is shown solely for phenylglyoxal inhibition, since the results obtained with trypsin were virtually identical. Oxamate alone provided negligible protection, whereas the enzyme–NADH binary complex showed marked protection. This contrasts with protection of histidine-195 from modification by diethyl pyrocarbonate, where slight protection was observed in the binary complex. The formation of a ternary complex between enzyme–NADH and oxamate rendered the enzyme even more resistant to inhibition by phenylglyoxal or trypsin.

Discussion

The present work proves that when cysteine-165 undergoes minimal modification by insertion of the thiomethyl group there is a dramatic decrease (approx. 23-fold) in the chemical reactivity of the essential histidine-195 residue accompanied by a much smaller increase (2–4-fold, depending on reagent) in the chemical reactivity of basic amino acid residues (i.e. arginine and/or lysine). Presumably these changes result from specific conformational alterations in the orientation of amino acid side chains within the protein. Crystallographic studies (Holbrook *et al.*, 1975) suggest that the modifying thiomethyl group will be situated between aspartate-168 and histidine-195, preventing movement of the histidine residue towards the substrate-binding site. Possibly this restriction accounts for the decreased chemical reactivity of the histidine residue in the modified enzyme. Presumably bulky or more polar groups than the thiomethyl group would produce a greater distortion, altering the properties of histidine-195 even further, and this would explain why the initial experiments on modification of cysteine-165 always generated a completely inactive enzyme (Fondy *et al.*, 1965; Holbrook & Pfleiderer, 1965; Holbrook, 1966). Consistent with observations on the thiomethyl-enzyme, the cysteine-165 thiocyanide derivative of lactate dehydrogenase also retains 40% of catalytic activity (Lodola *et al.*, 1978).

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