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A Detailed Investigation of the Properties of Lactate Dehydrogenase in which the 'Essential' Cysteine-165 is Modified by Thioalkylation

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The reaction of pig heart lactate dehydrogenase with methyl methanethiosulphonate resulted in the modification of one thiol group per protomer, and this was located at cysteine-165 in the enzyme sequence. On reduction, both the thiomethylation of cysteine-165 and any changes in kinetic properties of the enzyme were completely reversed. Cysteine-165 has been considered essential for catalytic activity; however, cysteine-165-thiomethylated dehydrogenase possessed full catalytic activity, although the affinity of the enzyme for carbonyl- or hydroxy-containing substrates was markedly decreased. The nicotinamide nucleotide-binding capacity was unaffected, as judged by the formation of fluorescent complexes with NADH. The enzyme-mediated activation of NAD^+, as judged by sulphite addition, was unaffected in thiomethylated lactate dehydrogenase. However, the affinity of oxamate for the enzyme–NADH complex was decreased by 100-fold and it was calculated that this constituted a net increase of 10.4 kJ/mol in the activation energy for binding. Thiomethylated lactate dehydrogenase was able to form an abortive adduct between NAD^+ and fluoropyruvate. However, the equilibrium constant for adduct formation between pyruvate and NAD^+ was too low to demonstrate this complex at reasonable pyruvate concentrations. A conformational change in the protein structure on selective thiomethylation was revealed by the decreased thermostability of the modified enzyme. The alteration of lactate dehydrogenase catalytic properties on modification depended on the bulk of the reagent used, since thioethylation resulted in an increase in K_m for pyruvate (13.5 ± 3.5 mM) and an 85% decrease in maximum catalytic activity. The implications of all these findings for the catalytic mechanism of lactate dehydrogenase are discussed.

Covalent modification of bovine, chicken (Di Sabato & Kaplan, 1963; Fondy *et al.*, 1965) and pig heart H_4 lactate dehydrogenase (Holbrook & Pfleiderer, 1965; Holbrook *et al.*, 1967) with either *p*-hydroxymercuribenzoate or *N*-alkylmaleimides revealed that the presence of an intact thiol group was essential to maintain normal catalytic activity. Subsequently, it was demonstrated that modification occurred at a single unique sequence corresponding to cysteine-165 in the dogfish muscle M_4 lactate dehydrogenase (Taylor *et al.*, 1973). The sequence appeared to be conserved in a variety of diverse dehydrogenases, including lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and alcohol dehydrogenase (Harris, 1964; Fondy *et al.*, 1965; Taylor *et al.*, 1973). This reinforced the concept that cysteine-165 played an essential central role in the catalytic mechanism of dehydrogenases.

More recently, information has accumulated that casts doubt on the essential nature of this group. X-ray crystallography of dogfish M_4 lactate dehydrogenase and its complexes has indicated that pyruvate probably hydrogen-bonds with histidine-195, and cysteine-165 is at least 1 nm from this site (Adams *et al.*, 1970*a*,*b*; Holbrook *et al.*, 1975). Furthermore a polypeptide chain is located between the two residues, making physical contact extremely difficult. Analysis of the sequence of lobster tail lactate dehydrogenase (Taylor & Oxley, 1976) showed that cysteine could be replaced by an alternative residue, namely threonine.

We have been using the reagent methyl methanethiosuphonate to thiomethylate proteins, in order to investigate the role of thiol groups in catalysis (Bloxham & Wilton, 1977; Bloxham *et al.*, 1978). Pig heart lactate dehydrogenase is one of the proteins that is susceptible to thiomethylation and this leads to an enzyme with altered catalytic properties compared with the native enzyme (Bloxham & Wilton, 1977). We have chosen to study this reaction further in the hope that this would increase the understanding of the catalytic role of the thiol group.

In the present work the exact site of the modification reaction at cysteine-165 is described and a detailed analysis of the changes in catalytic activity is made. The study is extended to investigate the properties of pig heart lactate dehydrogenase in which cysteine-165 is modified by the larger thioethyl group.

Materials and Methods

Chemical modification of lactate dehydrogenase

Pig heart lactate dehydrogenase was obtained as a crystalline suspension in $(NH_4)_2SO_4$ from the Boehringer–Mannheim Corp., Lewes, East Sussex, U.K. Suitable amounts of enzyme were then collected by centrifugation at 15000*g* for 20min at 4°C. The enzyme was dissolved in 50mM-potassium phosphate, pH7.0, at a protein concentration of 5mg/ml. Protein concentrations were determined by measuring the A_{280} of a sample in 10mM-Tris/HCl, pH8.0, containing 1mM-EDTA and 1% (w/v) sodium dodecyl sulphate, assuming a molar absorption coefficient of 2×10^5 litre·mol^{-1}·cm^{-1} (Holbrook & Ingram, 1973). Protomer concentrations were calculated by using a subunit mol.wt. of 36000 (Stinson & Holbrook, 1973).

The soluble enzyme was routinely allowed to react with 10mM-[^{3}H]methyl methanethiosulphonate for 2h at 18°C to complete the enzyme modification (Bloxham & Wilton, 1977). Excess reagent was removed by repeated precipitation of the enzyme by the addition of 370mg of enzyme-grade $(NH_4)_2SO_4$ per ml of solubilized enzyme. A minimum of three precipitations was necessary to remove the excess reagent.

The concentration of titratable thiol in the enzyme was determined by measuring the increase in A_{412} caused by the reaction of 6μM-lactate dehydrogenase (molarity in subunits) with 200μM-5,5′-dithiobis-(2-nitrobenzoate) in 50mM-potassium phosphate, pH8.0, containing 8M-urea. The molar absorption coefficient for the thionitrobenzoate anion was taken as 13200 litre·mol^{-1}·cm^{-1} (Ellman, 1959). Aqueous radioactive samples were counted in toluene containing 30% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in an Intertechnique ABAC-SL40 liquid-scintillation counter.

Reduction of thio[^{3}H]methylated lactate dehydrogenase

Thio[^{3}H]methylated lactate dehydrogenase was obtained after five precipitations from $(NH_4)_2SO_4$. The enzyme (5mg/ml) in 50mM-potassium phosphate, pH7.0, was reduced with 50mM-dithiothreitol for 4h at 20°C. The re-activated lactate dehydrogenase was then separated from contaminating radioactivity by dialysis against 25mM-potassium phosphate, pH7.0, for 18h at 0°C.

[^{3}H]Methyl methanethiosulphonate

The radioactive compound was synthesized by the condensation of [^{3}H]methyl iodide with potassium methylthiosulphonate (Bloxham *et al.*, 1978). It was finally obtained by vacuum-distillation. Its specific radioactivity was estimated by determining the ^{3}H present in a weighed sample. The method would obviously be inaccurate if the [^{3}H]methyl methanethiosulphonate was impure. However, both g.l.c. and n.m.r. indicated the product was at least 95% pure.

Conditions for analysis of peptides

Labelled protein was separated from excess reagents by exhaustive dialysis against water. NH_4HCO_3 was added to 50mM, and the protein was digested with 0.5% (w/w) diphenylcarbamoyl chloride-treated trypsin for 6h at 38°C. Trypsin was denatured at 90°C for 5min and removed by centrifugation at 2000*g* for 5min. The sample was freeze-dried and dissolved in water. Protein (0.1μmol, calculated on the basis of protomer molecular weight) was applied to the peptide 'map'. High-voltage electrophoresis was carried out at pH6.5 (pyridine/acetic acid/water, 25:1:225, by vol.) for 1.25h at 2500V. Descending chromatography was performed in the upper phase of butan-1-ol/acetic acid/water (4:1:5, by vol.) for 16h. Peptides were located by ninhydrin spray, and cut out, eluted with 1ml of 10% (v/v) acetic acid and counted for radioactivity.

For purified peptides *N*-terminal amino acid analysis was performed by the dansylation technique of Hartley (1970). Sequential degradation of peptides was achieved by the method of Bruton & Hartley (1972).

Spectral and fluorescence measurements

These were performed as described by Bloxham *et al.* (1975).

Conditions for the demonstration of adduct formation

Experiments to demonstrate enzyme-bound adduct formation between substrate and NAD^+ with either native or thiomethylated lactate dehydrogenase were carried out in a Cary 118 spectrophotometer, in split-compartment cells. In the blank, one contained enzyme and NAD^+ and the other cell contained either pyruvate or fluoropyruvate. Adduct formation was monitored by the increase in A_{325}.

To compare the equilibrium constant for the formation of adduct, the chemical reaction was carried out with pyruvate (0.1M) or fluoropyruvate (0.01M) and acetylpyridine–adenine dinucleotide (0.002M) in Na_2CO_3 buffer (0.1M) at pH10.0. The reaction was monitored spectrophotometrically and allowed to reach equilibrium. The equilibrium

constants for the formation of the adducts were calculated by using a molar absorption coefficient for the adduct of 6500 litre·mol^{-1}·cm^{-1} at 345nm (Di Sabato, 1970) and values of 5.9M^{-1} and 250M^{-1} were obtained for the two adducts. The value of 5.9M^{-1} for the pyruvate–acetylpyridine adduct compares favourably with the value of about 7M^{-1} reported previously by Di Sabato (1970). It should be noted that acetylpyridine–adenine dinucleotide was used to eliminate the complication shown by NAD^+ in the absence of enzyme of further reaction. This reaction involves the amide nitrogen of NAD^+ with the carbonyl group of pyruvate after initial adduct formation (Everse *et al.*, 1971).

The enzyme-bound NAD^+–sulphite addition complex was measured at 325nm in 100mM-potassium phosphate, pH7.0, containing enzyme (855μg/ml), NAD^+ (0.5mM) and Na_2SO_3 (17–170μM).

Chemical modification of pig heart lactate dehydrogenase by other alkyl thiosulphonates

The conditions for the modification and assay of pig heart lactate dehydrogenase have been described previously (Bloxham & Wilton, 1977); however, unlike methyl methanethiosulphonate, the other reagents were not soluble in water and were made up at 0.1M in aq. 50% (v/v) ethanol then diluted 10-fold into the incubation mixture.

Synthesis of ethyl methanethiosulphonate

Potassium methanethiosulphonate (3.0g; 0.02mol) was prepared as described by Bloxham *et al.* (1978) and dissolved in dry *NN*-dimethylformamide (15ml). Ethyl iodide (4.5g; excess) was added, and the reaction mixture was stirred at room temperature for 5h. Water (100ml) was then added to the reaction mixture and it was extracted with chloroform (3×50ml). The combined organic extracts were washed free of *NN*-dimethylformamide with more water and dried. The solvent was evaporated under reduced pressure to leave an oil, which was distilled under reduced pressure to give ethyl methanethiosulphonate as a colourless oil (65%), b.p. 97–99°C at 13.3Pa (literature b.p. 99–100°C; Smith *et al.*, 1975). The ^1H n.m.r. spectrum showed signals at δ 3.32 (3H,s), 3.18 (2H,q, $J = 7.5$Hz) and 1.42 (3H,t, $J = 7.5$Hz). The n.m.r. spectrum was recorded in [^2H]chloroform with tetramethylsilane as a reference.

Results and Discussion

Labelling of pig heart lactate dehydrogenase with [^3H]methyl methanethiosulphonate

We have previously shown that thiomethylation of pig heart lactate dehydrogenase resulted in a very specific change in the catalytic properties of the enzyme (Bloxham & Wilton, 1977). Thus the maximum catalytic activity, K_S^{NADH} and $K_S^{NAD^+}$ were all unchanged, but there was a marked increase in the K_m for pyruvate and lactate. To discuss this modification comprehensively there were two crucial omissions from our previous paper; first the exact extent of thiomethylation was not determined and second the sequence(s) containing the reactive group(s) were not determined. Therefore our first studies were concerned with resolving these two points.

Methyl methanethiosulphonate is readily synthesized by the oxidation of dimethyl disulphide with H_2O_2, but this has little advantage for the synthesis of radioactively labelled inhibitor, since the radioactive label cannot be restricted to the reactive thiomethyl group. Therefore the approach adopted to synthesize the radioactive inhibitor was to condense [^3H]methyl iodide with potassium methanethiosulphonate:

$$C^3H_3I + CH_3{-}SO_2{-}S{-}K \rightarrow KI + CH_3{-}SO_2{-}S{-}C^3H_3 \quad (1)$$

The tritiated reagent then reacts specifically with thiol groups to incorporate the radioactive label:

$$CH_3{-}SO_2{-}S{-}C^3H_3 + R{-}S^- \rightarrow CH_3SO_2^- + R{-}S{-}S{-}C^3H_3 \quad (2)$$

When lactate dehydrogenase was made to react with 10mM-[^3H]methyl methanethiosulphonate for 2h to generate the completely modified thiomethylated enzyme, the expected change in kinetic behaviour occurred, namely the enzyme was virtually inactive at concentrations of pyruvate that are optimal for the native enzyme; however, increasing the pyruvate concentration to 20mM ($K_m^{Pyr} = 12$mM for thiomethylated lactate dehydrogenase) regained a substantial proportion of catalytic activity (Table 1). The native enzyme was inhibited at 20mM-pyruvate as a result of the formation of an abortive complex (Fromm, 1961; Everse *et al.*, 1971; Arnold & Kaplan, 1974). This inhibition is not observed with the thiomethylated enzyme. The complete modification of lactate dehydrogenase coincided with the incorporation of 1.06 (mean value) thiomethyl groups per protomer of enzyme. This value was reasonably constant through three successive crystallizations once the excess radioactivity was removed.

The estimate of the incorporation of thiomethyl groups was also confirmed by the disappearance of thiol groups that titrate with 5,5′-dithiobis-(2-nitrobenzoate) (Table 1). Native pig heart lactate dehydrogenase contained 4.9 titratable thiol groups per protomer in 8M-urea. The number of free thiol groups declined to 3.7 per protomer in fully thiomethylated lactate dehydrogenase, corresponding to the modification of 1.2 thiol groups per protomer. The close agreement between the results for the

Table 1. *Modification of a single cysteine residue in lactate dehydrogenase by [^3H]methyl methanethiosulphonate*
The exact details for the estimation of modification with methyl methanethiosulphonate, protein concentration, the incorporation of radioactivity, the titration of thiol groups and the reduction of thiomethylated lactate dehydrogenase are described in the Materials and Methods section. For the limited-thiomethylation sample, the reaction time with [^{3}H]methylmethanethiosulphonate was decreased to 5 min. The numbers in parentheses indicate the number of experimental observations. Abbrevation: N.S., not significant.

Enzyme	$(NH_4)_2SO_4$ precipitations	Specific activity (μmol/min per mg) 200 μM-Pyruvate	Specific activity (μmol/min per mg) 20 mM-Pyruvate	Protomer concn. (μM)	Concn. of S-C^3H_3(μM)	S-C^3H_3 (mol/mol of protomer)	Titratable thiol groups/ protomer	Thiol groups modified by S-CH_3
Native (3)	1	350	56.2	258	—	—	4.9	—
Limited thiomethylated enzyme (4)	3	255	46	105	26.8	0.25	4.7	N.S.
Limited thiomethylated enzyme (2) after reduction	—	340	50	105	6.6	0.062	—	—
Thiomethylated enzyme (6)	3	3.6	259	113	114.6	1.014	3.6	1.3
Thiomethylated enzyme	4	3.6	245	109	119.3	1.094	3.65	1.25
Thiomethylated enzyme	5	3.4	260	109	116.4	1.067	3.7	1.20
Thiomethylated enzyme (4) after reduction	—	296	48	109	17	0.15	—	—

direct incorporation of [^{3}H]methyl methanethiosulphonate and for the disappearance of thiol groups clearly confirms that the estimate of [^{3}H]methyl methanethiosulphonate specific radioactivity was accurate (see the Materials and Methods section).

The proportional relation between the extent of enzyme inhibition and the incorporation of thiomethyl groups was confirmed by an experiment when the exposure to methyl methanethiosulphonate was decreased to a reaction time of 5 min plus a further reaction that occurred during the first $(NH_4)_2SO_4$ precipitation. Under these conditions (Table 1) the enzyme lost 28% of its enzyme activity and incorporated 0.25 thiomethyl group per protomer.

Reduction of thio[^3H]methylated lactate dehydrogenase

Thiomethylation of lactate dehydrogenase introduces a new disulphide bond into the enzyme, which can be reversed by reduction. Table 1 shows that thio[^{3}H]methylated lactate dehydrogenase was returned to the native form by reduction with 50 mM-dithiothreitol. Once the enzyme was exhaustively dialysed, then re-activation was accompanied by virtually complete loss of all the labelled thiomethyl groups. If the dithiothreitol-reduction step was omitted, then none of the radioactivity was lost from the protein on dialysis and the catalytic properties of thiomethylated lactate dehydrogenase were retained.

Further analysis of thio[^3H]methylated lactate dehydrogenase

The analysis of the reactive residue labelled with [^{3}H]methyl methanethiosulphonate would have been simple if the thio[^{3}H]methylated peptides could have been analysed directly. However, we found that tryptic digestion resulted in extensive loss of radioactivity. Alternatively peptide cleavage by pepsin yielded a rather complex series of peptides that were not analysed further. Instead we were forced to alternative analytical procedures to demonstrate the labelled site.

Conditions for the limited reaction of cysteine-165 in pig heart lactate dehydrogenase

To identify the reactive group exactly we needed to be able to make this group react with a second type of radioactive label, i.e. iodo[2-^{3}H]acetate or iodo[2-^{14}C]acetamide. We chose to define reaction conditions that would probably restrict the reaction of either of these to cysteine-165. Normally, cysteine residues of pig heart lactate dehydrogenase will not react with thiol reagents, such as iodoacetate, iodoacetamide or 5,5′-dithiobis-(2-nitrobenzoate)

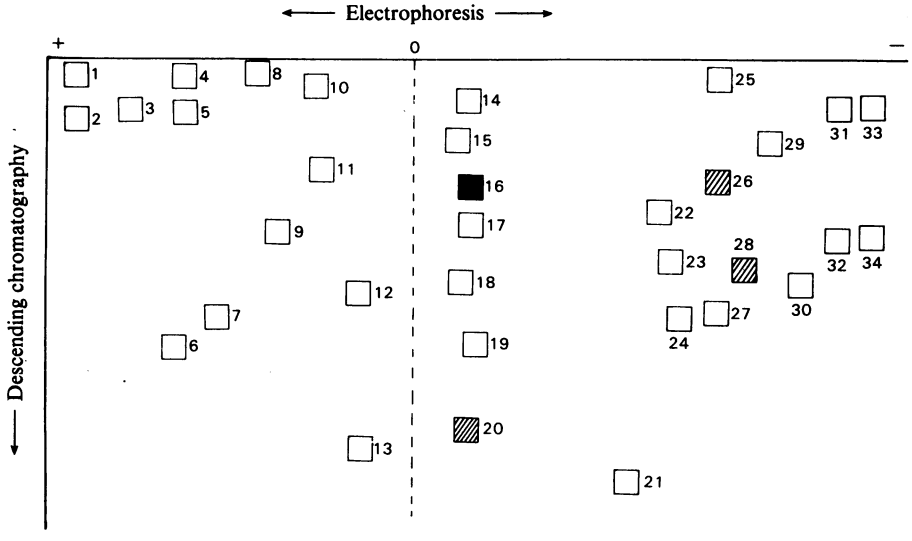
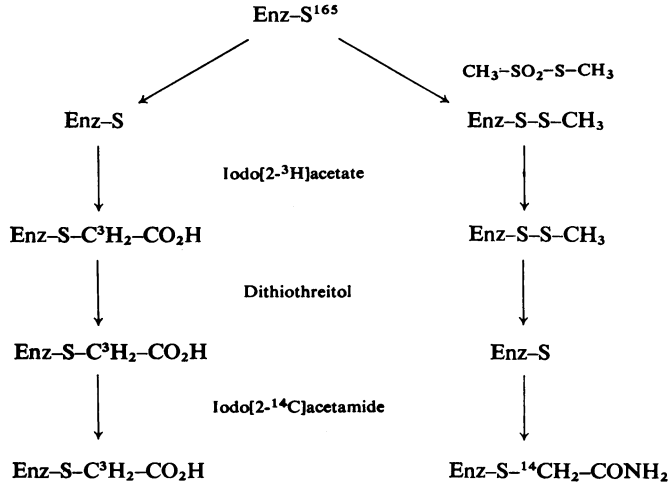


Fig. 1. *Tryptic-peptide 'map' of lactate dehydrogenase labelled by limiting reaction with iodo[2-^{14}C]acetamide in 4.5 M-urea*

Lactate dehydrogenase (125 μM in protomers) was reacted in 50 mM-potassium phosphate, pH 7.4, containing 4.5 M-urea and 6 mM-iodo[2-^{14}C]acetamide (sp. radioactivity 0.1 μCi/mol) for 2 h at 37°C. Tryptic-peptide 'mapping' was carried out as described in the Materials and Methods section. Peptide 16 contained 1.0 [^{14}C]carboxymethyl residue per protomer. Peptides 20, 26 and 28 contained traces of radioactivity.



Scheme 1. *Double-labelling experiment to demonstrate reactive cysteine in lactate dehydrogenase*

(Fondy *et al.*, 1965). However, as the concentration of urea is raised the rate of reaction increases progressively once 2 M-urea concentrations are exceeded, with a mid-point in the titration, with 5,5′-dithiobis-(2-nitrobenzoate) at 4.8 M-urea concentration. Under suitable conditions a concentration of urea can be selected that restricts chemical reaction to the most reactive cysteine, i.e. cysteine-165. The enzyme at 125 μM-subunit concentration was then made to react with iodo[2-^{14}C]acetamide in 4.5 M-urea for 2 h at 38°C, and a tryptic peptide-'map' of the labelled protein was analysed. This revealed 34 ninhydrin-positive spots (Fig. 1) compared with 33 peptides expected on the basis of arginine and lysine content

(Wachsmuth *et al.*, 1964). Of all these peptides only one contained a stoicheiometric amount of iodo-[2-^{14}C]acetamide (peptide 16). This peptide was purified in a separate 'mapping' experiment, and its amino acid composition agreed closely with that expected for the cysteine-165-containing peptide (Holbrook *et al.*, 1967; see below). Furthermore the *N*-terminal amino acid was valine as expected for the correct sequence of this peptide. Clearly restricting the urea concentration to 4.5M at 125μM-protein subunit has limited the accessibility of the protein thiol groups to the most reactive cysteine-165 residue. These conditions were used in the subsequent experiment.

Double-labelling of thiomethylated lactate dehydrogenase

The logic behind this experiment is illustrated in Scheme 1. Thiomethylated lactate dehydrogenase and native pig heart lactate dehydrogenase (50mg) were made to react with 6mM-iodo[2-^{3}H]acetic acid (sp. radioactivity 5μCi/μmol) in 10ml of 50mM-potassium phosphate, pH7.4, containing 4.5M-urea at 38°C for 2h. Most of the iodoacetic acid was removed by dialysis against urea-containing buffer. Removal of the masking thiomethyl group was achieved with 50mM-dithiothreitol for 3h at 38°C. After removing excess of dithiothreitol by dialysis against urea-containing buffer the newly exposed thiol group was allowed to react with 6mM-iodo-[2-^{14}C]acetamide (sp. radioactivity 0.5μCi/μmol) under conditions identical with those for the reaction with iodoacetic acid. The samples were then exhaustively dialysed against water, adjusted to 50mM with NH_4HCO_3 and digested with 0.5% (w/w) trypsin for 6h at 38°C. Denatured trypsin was removed by centrifugation at 2000*g* for 5min after being heated to 90°C for 5min, and then the samples were freeze-dried. Initially 20nmol peptide samples were analysed by high-voltage electrophoresis at pH6.5 for 1.75h at 2.5kV. Both samples showed the presence of 11 ninhydrin-positive peptide fractions, and, of these, fractions 4 and 5 contained the bulk of the radioactivity in both cases (Table 2). For native lactate dehydrogenase, peptide fraction 4 contained the bulk of the radioactivity. The peptide fraction was negatively charged and contained 1mol of carboxy[2-^{3}H]methyl residue per mol of protomer. The peptide charge and the type of radioactive molecule incoporated are consistent with the initial reaction of cysteine-165. In contrast, the thiomethylated enzyme gave peptide fraction 5 as the main radioactive peptide. This peptide fraction was neutral and contained 0.75mol of amido[2-^{14}C]carboxymethyl groups per mol of protomer. This peptide had the same electrophoretic mobility as the amidocarboxymethyl-peptide containing cysteine-165 identified in the previous section (Fig. 1). This labelling pattern was expected, since the thiol group was masked from reaction with iodo[2-^{3}H]-acetate, but was able to react with iodo[2-^{14}C]-acetamide after reduction. The presence of 0.34mol of carboxy[2-^{3}H]methyl-peptide fraction 4 is attributed to incomplete removal of unchanged iodo-[2-^{3}H]acetate by dialysis after the first reaction. The exact identity of the radioactive peptides was then established by purification. The peptides were initially subjected to chromatography on a column (54cm×1cm) of Dowex 50 (pyridinium form). The column was developed with a 300ml concave gradient

Table 2. *Peptide analysis of doubly labelled native and thiomethylated lactate dehydrogenase*

The preparation of carboxy[2-^{3}H]methyl, amido[2-^{14}C]carboxymethyl samples of thiomethylated lactate dehydrogenase and native pig heart lactate dehydrogenase is described in the text and Scheme 1. Each protein (20nmol; based on A_{280}) was analysed by high-voltage electrophoresis. Ninydrin-positive peptide bands were eluted with 10% (v/v) acetic acid and counted for radioactivity. The incorporation of 200000 and 20000 d.p.m. of ^{3}H and ^{14}C respectively corresponded to the incorporation of 1mol per mol of protomer.

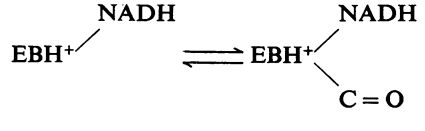
		Incorporation (μmol of ^{3}H or ^{14}C/μmol of protomer)			
		Native		Thiomethylated	
Peptide no.	Mobility (cm)	^{3}H	^{14}C	^{3}H	^{14}C
1	+19.5	—	—	—	—
2	+6.8	—	—	—	—
3	+5.3	0.001	—	0.00075	—
4	+3.5	1.015	—	0.34	—
5	−3.0	0.22	0.0085	0.18	0.75
6	−9.5	—	—	—	—
7	−11.0	0.0055	—	0.006	—
8	−11.8	—	—	—	—
9	13.3	—	—	—	—
10	−19.0	—	—	—	—
11	−21.2	—	—	—	—

between 0.2M-pyridine/acetic acid, pH3.1, and 2M-pyridine/acetic acid, pH5.0 (Schroeder, 1967). The ^3H- and ^{14}C-labelled peptides were eluted at 86.8±8ml and 118±12ml respectively. On high-voltage electrophoresis at pH6.5 for 1.5h at 2.5kV, the peptides migrated +3.1cm (^3H-labelled) and −2.5cm (^{14}C-labelled). Final purification was achieved by descending chromatography in the upper phase of butanol/acetic acid/water (4:1:1, by vol.) for 24h when the peptides moved 9.7cm (^3H-labelled) and 8.6cm (^{14}C-labelled). These peptides were judged to be pure. Amino acid analysis showed the composition of the ^3H-labelled peptide was Val (0.7)-Ile (0.61)-Gly (2.2)-Ser (1.98)-CmCys (0.8)-Asp (2.05)-Leu (0.88)-Ala (1.33)-Arg (0.77), and the ^{14}C-labelled peptide was Val (0.64)-Ile (0.62)-Gly (2.24)-Ser (1.70)-CmCys (0.75)-Asp (2.25)-Leu (1.23)-Ala (1.32)-Arg (0.88). Both of these analyses compare favourably with the expected amino acid analysis of the tryptic peptide containing cysteine-165, namely Val(1)-Ile(1)-Gly(2)-Ser(2)-Cys(1)-Asp(2)-Leu-(1)-Ala(1)-Arg(1) (Holbrook *et al.*, 1967). The identity of the two sets of peptides was confirmed by sequential degradation (Bruton & Hartley, 1972), which showed that for both the ^3H- and ^{14}C-labelled peptides the sequence of the first three amino acids was Val-Ile-Gly. The sequence of the tryptic peptide containing cysteine-165 is Val-Ile-Gly-Ser-Gly-Cys-Asn-Leu-Asp-Ser-Ala-Arg (Holbrook *et al.*, 1967).

Properties of thiomethylated lactate dehydrogenase from pig heart

NADH binding to thiomethylated lactate dehydrogenase. NADH forms a binary complex with lactate dehydrogenase that is characterized by an enhancement of fluorescence and a blue shift in the emission maximum (Winer *et al.*, 1959). Thiomethylated lactate dehydrogenase shows identical properties. Using 4μM-lactate dehydrogenase (protomers) in 50mM-potassium phosphate, pH8.0, K_S^{NADH} was estimated as 3.48±0.5μM for thiomethylated and native lactate dehydrogenase. In addition K_S^{NADH} was virtually pH-independent between pH6.0 and 9.0 for both enzyme preparations. This result has been established previously for native enzyme (Stinson & Holbrook, 1973). Collectively these results show that thiomethylation at cysteine-165 does not affect the NADH-binding component of the enzyme active site. This corroborates our earlier studies on the involvement of NADH in the catalytic reaction (Bloxham & Wilton, 1977).

Ternary complex-formation with oxamate. The binding of ligands to the enzyme–NADH complex can be followed by alteration of the fluorescence. Oxamate quenches the fluorescence of the enzyme–NADH complex by 87.8% (Novoa *et al.*, 1959; Winer & Schwert, 1959; Whitaker *et al.*, 1974) for both native and thiomethylated lactate dehydrogenase (Fig. 2). However, $K_S^{oxamate}$ was increased from 9.5±0.95μM to 960±50μM on thiomethylation of the enzyme. This represents a 100-fold increase in $K_S^{oxamate}$. Since oxamate does not participate in catalysis, this represents a measure solely of the influence of thiomethylation on ligand binding to the active site. An increase in dissociation constant would be achieved by increasing the off-constant, decreasing the on-constant or a combination of both for the interconversion



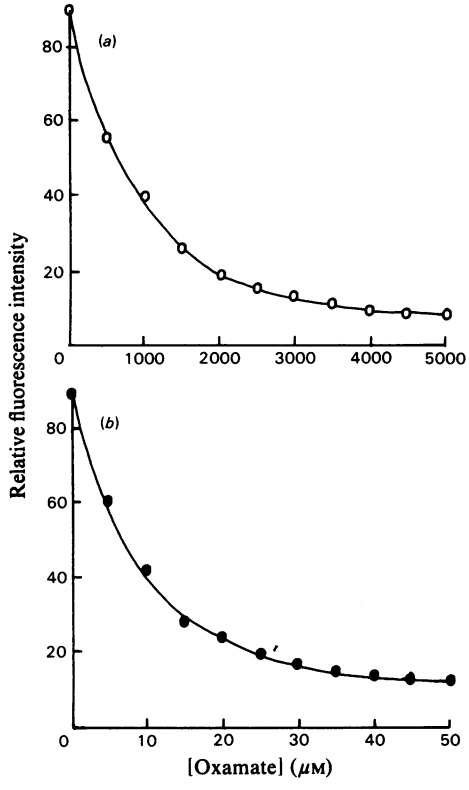


Fig. 2. *Titration of lactate dehydrogenase–NADH complexes with oxamate*

The binary complex between thiomethylated (*a*) and native lactate dehydrogenase (*b*) was formed by mixing 14μM-lactate dehydrogenase protomers with 4.2μM-NADH in 50mM-potassium phosphate, pH7.0. This resulted in a 3-fold enhancement of NADH fluorescence in both cases. The concentration of oxamate was then increased progressively by sequential additions from a 0.1M-stock solution.

(Holbrook & Gutfreund, 1973; C = O refers to a ligand containing the carbonyl functional group as in pyruvate or oxamate). Collectively an increase of 100 in $K_S^{oxamate}$ corresponds to a change of 10.4KJ/mol in the activation energy of these two processes (k_{off} decreases E_A; k_{on} increases E_A).

Reduction of the NADH–pyruvate transition-state analogue by thiomethylated lactate dehydrogenase. Thiomethylation of lactate dehydrogenase clearly distinguishes between the two halves of the active site, since nucleotide binding is unaffected and the affinity for the substrate containing a carbonyl or alcohol group is markedly decreased. Therefore it was decided to test the catalytic activity of lactate dehydrogenase, with a substrate where the two parts are covalently linked. Such a substrate is the NADH–pyruvate state analogue, 5-(2-oxalylethyl)–NADH, synthesized as described by Kapmeyer *et al.* (1976). We found that thiomethylated lactate dehydrogenase catalysed the reduction of 5-(2-oxalylethyl)–NADH. The apparent K_m and $V_{max.}$ for this single substrate were $18.9 \pm 1.5 \mu M$ and 2nmol of substrate reduced/min per mg in 50mM-potassium phosphate, pH7.4. The equivalent values for native lactate dehydrogenase in our hands were $26.5 \pm 0.65 \mu M$ and 10nmol of substrate reduced/min per mg. Clearly the catalytic reaction is decreased by 80% in the thiomethylated enzyme; however, the affinity for the enzyme is similar. This suggests that the NADH component of the analogue is the dominant feature in determining binding to the protein.

Assessment of the reactivity of the enzyme-bound NAD$^+$ by formation of the NAD$^+$–sulphite addition complex. NAD$^+$ forms an addition complex with the SO_3^{2-} and the equilibrium of this reaction is enhanced when the NAD$^+$ is bound to lactate dehydrogenase (Pfleiderer *et al.*, 1957; Holbrook *et al.*, 1975). When the equilibrium constant for the formation of the enzyme-bound NAD$^+$–sulphite complex was measured for the native enzyme and the thiomethylated enzyme they were found to be $8.6 \pm 5.9 \mu M$ for the native enzyme and $22 \pm 7.6 \mu M$ for the thiomethylated enzyme. This result establishes that the altered pyruvate-binding site does not significantly affect the reaction of the SO_3^{2-} with the bound NAD$^+$. This point is considered further in the general discussion.

Adduct formation between NAD$^+$ and pyruvate

Whereas the activity of native pig heart lactate dehydrogenase is inhibited by high concentrations of pyruvate, there is no detectable substrate inhibition by pyruvate with thiomethylated pig heart lactate dehydrogenases (Bloxham & Wilton, 1977). The phenomenon of substrate inhibition has been attributed to the formation of a tightly bound covalent adduct between NAD$^+$ and pyruvate at the active site of the lactate dehydrogenase, which may be readily characterized by an increase in A_{325} (Fromm, 1961; Everse & Kaplan, 1973). The lack of substrate inhibition is not surprising therefore in view of the 300-fold increase in K_m for pyruvate with the thiomethylated enzyme. However, it was important to establish that the modified lactate dehydrogenase was still able to catalyse adduct formation under suitable conditions. Table 3 shows the results of experiments carried out in an attempt to demonstrate adduct formation with both pyruvate and the substrate analogue fluoropyruvate (Eisman *et al.*, 1965). It was not possible to detect the formation of any 325nm-absorbing adduct by the modified lactate dehydrogenase and pyruvate as the substrate, whereas fluoropyruvate was able to form stoicheiometric amounts of adduct with both the native enzyme and the thiomethylated enzyme. It should be noted that the overall dissociation constant for the ternary complex with pyruvate is about 0.5mM, and therefore the greatly decreased affinity of the modified lactate dehydrogenase for pyruvate will require a concentration of pyruvate sufficiently high to make the experiment untenable by this procedure.

Table 3. *Adduct formation between pyruvate or fluoropyruvate and NAD$^+$ catalysed by native and thiomethylated lactate dehydrogenase*

Using the split-cell technique in a Cary 118 spectrometer the increase in A_{325} was measured in the reaction cells containing lactate dehydrogenase (313μg/ml), NAD$^+$ (0.25mM) and various concentrations of pyruvate or fluoropyruvate in 0.1M-potassium phosphate buffer, pH7.0 at 23°C. A value of 6250 litre·mol^{-1}·cm^{-1} was used as the molar absorption coefficient at 325nm for the enzyme-bound adducts (Eisman *et al.*, 1965).

	Adduct formed (mol/mol of active site)	
Substrate	Native lactate dehydrogenase	Thiomethylated lactate dehydrogenase
3.2mM-Pyruvate	0.86	Not detectable
1.6mM-Pyruvate	0.71	Not detectable
0.8mM-Pyruvate	0.56	Not detectable
0.4mM-Pyruvate	0.38	Not detectable
1.6mM-Fluoropyruvate	1.04	1.00

However, because of the inductive effect of the fluorine, the fluoropyruvate is more stable, and this is reflected in a much more favourable equilibrium constant for the reaction (see the Materials and Methods section).

Comparison of the thermal stability of native and thiomethylated lactate dehydrogenase

It had previously been observed that the modified lactate dehydrogenase was inactivated by dilution (Bloxham & Wilton, 1977) and therefore it was of interest to compare also the thermal stability of the native and modified enzymes. Denaturing conditions were chosen that were identical with those reported by Kapmeyer & Pfleiderer (1977) for studying the thermal stability of pig heart lactate dehydrogenase in which surface lysine residues had been modified. In that case the lysine residues had been hydrophobically modified to give an ε-(*N*-2,4-dinitrophenyl)aminohexanamidinate-containing enzyme, with an average incorporation of 38 groups per tetramer and a residual activity of 42%. Thiomethylated and native lactate dehydrogenase (1 mg/ml) were incubated at 55°C in 0.2M-glycine buffer, pH 8.0. This resulted in 90% inactivation of the thiomethylated enzyme in 45 min. This compares with 10 and 75% for the native and ε-(*N*-2,4-dinitrophenyl)aminohexanamidinate-containing enzymes respectively. Thiomethylated lactate dehydrogenase was also inactivated more rapidly compared with ε-(*N*-2,4-dinitrophenyl)aminohexanamidinate-containing lactate dehydrogenase. These results highlight the destabilization of the lactate dehydrogenase protomer produced by a minimal internal modification, whereas drastic surface modification has much less effect. The destabilization becomes more apparent when the essential thiol is modified by the larger thioethyl group (see below).

Glyoxalate and 2-oxobutyrate as substrates for the thiomethylated lactate dehydrogenase

Attempts to measure the K_m of thiomethylated lactate dehydrogenases for both glyoxalate and 2-oxobutyrate indicated values in excess of 100mM, as would be expected if both these substrates suffered the same 300-fold increase in K_m as does pyruvate. The K_m value for glyoxalate must of course be corrected for the 95% hydration of glyoxalate (Cooper & Redfield, 1975) to give the K_m value for the unhydrated carbonyl species.

pH-dependence for K_m^{Pyr} and $V_{max.}$ for thiomethylated lactate dehydrogenase

Assuming that pyruvate binds to the protonated form of histidine-195 (Holbrook & Gutfreund, 1973; Whitaker *et al.*, 1974; Bloxham *et al.*, 1975), then the apparent 'K_m for pyruvate should vary approximately according to the equation

$$K_m^{Pyr}(\text{app.}) = K_m^{Pyr}\,[1+(K_m/[H^+])]$$

(Bloxham *et al.*, 1975; strictly this holds for K_m^{Pyr}). From this equation, the apparent K_m^{Pyr} should increase by a factor of approx. 10 for each unit of pH increase above pK_a for histidine-195. Data for the pH-dependence of apparent K_m^{Pyr} and $V_{max.}$ are shown in Table 4. The apparent $V_{max.}$ was not statistically different except at low pH values. Above pH 7.0 K_m^{Pyr} increased markedly and was approx. 7-fold greater at pH 8.0. Since histidine-195 has a pK_a of 6.7–6.8 (Holbrook & Ingram, 1973; Holbrook & Stinson, 1973; Holbrook, 1973; Bloxham *et al.*, 1975), the data of Table 4 are reasonably consistent with this estimate and mean that the pK_a of the histidine is probably unaffected by thiomethylation.

Effect of thioethylation on the enzymic properties of lactate dehydrogenase

In the case of thiomethylation of pig heart lactate dehydrogenase the only significant effect on the catalytic properties was a 300-fold increase in the K_m for pyruvate with no apparent effect on the $V_{max.}$. However, it had previously been noted (Bloxham & Wilton, 1977) that the modified enzyme lost activity irreversibly on dilution, whereas we have described above how the thiomethylated enzyme was very much more sensitive to thermal denaturation. We therefore investigated the effect of modification of

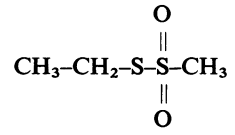
Table 4. *Influence of pH on kinetic constants for thiomethylated lactate dehydrogenase*

Thiomethylated lactate dehydrogenase (2μg/ml) was assayed in 50mM-sodium pyrophosphate solutions at several pH values in the presence of 0.15mM-NADH and variable pyruvate concentrations. K_m and $V_{max.}$ values were estimated by the method of Wilkinson (1961). Results are means ±S.D. for six observations.

pH	K_m^{Pyr}(mM)	$V_{max.}$ (μmol/min per mg at 20°C)
5.0	7.21±0.39	140±15
6.0	7.30±0.55	253±20
6.5	10.9±0.9	273±13
7.0	12.6±1.04	250±17
7.5	26.3±2.1	214±11
8.0	86.7±3.0	214±11

cysteine-165 with a series of larger more non-polar substituents on the properties of pig heart lactate dehydrogenase.

We describe here the effect of thioethylation of pig heart lactate dehydrogenase by ethyl methanethiosulphonate, which has the following structure:



Methanethiosulphonates containing larger alkyl thiol groups (trifluoroethyl and isopropyl) were also tested, but in these cases inactivated enzyme was denatured very rapidly, and this did not allow further analysis.

The initial rate of inactivation of lactate dehydrogenase by ethyl methanethiosulphonate was about three times faster than for methyl methanethiosulphonate, but, unlike thiomethylation (Bloxham & Wilton, 1977), no limiting value of enzyme activity was reached. Instead there was a continuous decline in enzyme activity with time, and protein precipitation commenced after about 1 h of treatment with the reagent. As in the case of the thiomethylated enzyme the activity of the thioethylated enzyme could be recovered by prolonged treatment with high concentrations of dithiothreitol. However, this recovery was progressively decreased after prolonged modification due to the commencement of protein precipitation.

Although the thioethylated lactate dehydrogenase was inherently unstable, the rate of denaturation was sufficiently low to allow some simple kinetic experiments to be carried out. For these experiments, thioethylation was limited to a 20 min reaction period. The $V_{max.}$ of the native enzyme was estimated by exhaustive reduction of the thioethylated enzyme with dithiothreitol. For the thioethylated enzyme at 0.14 mM-NADH the K_m for pyruvate was 13.5 ± 3.5 mM, and the $V_{max.}$ was about 15% of that obtained for the native enzyme.

It is interesting to note the insignificant change in K_m for pyruvate on changing from thiomethylated lactate dehydrogenase (12 mM) to thioethylated lactate dehydrogenase (13.5 mM) as compared with the 300-fold increase over that for the native enzyme (40 μM). One explanation for this result is that thioalkylation has prevented the ionization of cysteine-165, and hence has perturbed the pK of the histidine-195, with a resulting effect on the K_m for pyruvate. However, it might be expected that such a pK effect would decrease the K_m for lactate, whereas in fact the reverse is true (Bloxham & Wilton, 1977).

An alternative reason must therefore be sought for lower affinity of the substrates for lactate dehydrogenase in the modified enzyme. The most likely possibility is that the modification of cysteine-165 induces a conformational change in the enzyme to a new state perhaps because the cysteine can no longer ionize. An increase in the size of the thioalkyl group need not necessarily affect the nature of this new conformational state, either because this state is purely a result of the removal of the ionizable thiol group or because the extra bulk of the larger thioethyl group does not interact with the enzyme. The lower $V_{max.}$ for the thioethylated enzyme is interesting in that it suggests that the putative isomerization step, which is the rate-limiting step in the reduction of pyruvate (Whitaker *et al.*, 1974), might be adversely affected by the extra non-polar bulk of the thioethyl group.

Are the properties of the thioethylated lactate dehydrogenase due entirely to modification of the active site?

To provide confirmation that thioethylation was occurring only at cysteine-165 and that the properties described for this enzyme were a function of the modification of this cysteine only use was made of thiomethylated lactate dehydrogenase, in which we have established that only the active-site cysteine is modified. When native lactate dehydrogenase and thiomethylated lactate dehydrogenase were treated with ethyl methanethiosulphonate (Fig. 3), the activity of the thiomethylated lactate dehydrogenase was

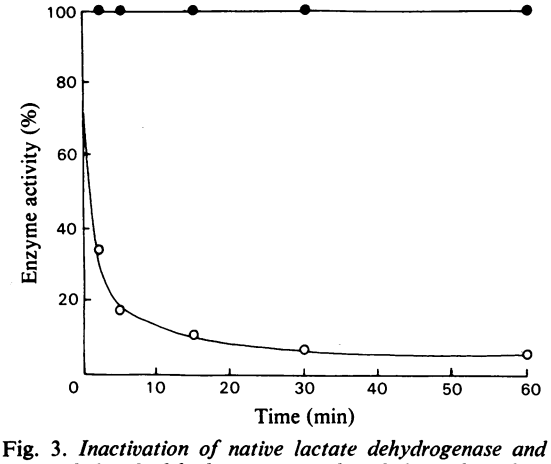


Fig. 3. *Inactivation of native lactate dehydrogenase and thiomethylated dehydrogenase with ethyl methanethiosulphonate*

Native lactate dehydrogenase (○; 1 mg/ml) and thiomethylated lactate dehydroganese (●; 1 mg/ml) were treated with 10 mM-ethyl methanethiosulphonate in 50 mM-potassium phosphate buffer, pH 7.5 at 23°C, then assayed for pyruvate reduction at the times shown. Enzyme activity is expressed as a percentage of the zero-time value.

unaffected, even after prolonged treatment with the reagent. On the other hand, the native enzyme not only lost activity but also started eventually to precipitate. Therefore none of the properties ascribed to the thioethylated lactate dehydrogenase are expressed when the single active-site cysteine-165 is protected by prior thiomethylation.

General discussion

The catalytic mechanism of lactate dehydrogenase is of particular interest because the interaction of the substrate carbonyl group or hydroxy group with the active-site histidine-195 is a major event in both the binding and activation of the susbtrate (Holbrook & Gutfreund, 1973; Whitaker *et al.*, 1974; Bloxham *et al.*, 1975). In the direction of pyruvate reduction, the hydrogen bond between the carbonyl oxygen and the protonated histidine results in a polarization and hence activation of the carbonyl group by the partial proton transfer from imidazole nitrogen to carbonyl oxygen. In the direction of lactate oxidation the hydrogen bond between the hydroxy hydrogen atom of the substrate and the nitrogen atom of the non-protonated imidazole gives rise to a partial proton transfer to the imidazole and facilitates the removal of the hydride ion (Akhtar & Wilton, 1973; Bloxham *et al.*, 1975). This intimate linking of binding and substrate activation make the properties of the thiomethylated dehydrogenase of particular interest because the greatly decreased binding or affinity of the substrate is not accompanied by any change in the overall rate of catalysis, $k_{cat.}$. Such a change in the properties of the enzyme would suggest that the modification did not directly alter the properties of histidine-195, because otherwise both K_m and $k_{cat.}$ might be expected to be adversely affected. In the present paper, we have provided some evidence that the pK_a of histidine-195 is not directly affected by thiomethylation and that the more likely explanation is that a conformational change has altered some other structural component of the pyruvate-binding site. This alteration of the binding site must be restricted, because the binding and the activation of the co-enzyme was not affected by the modification of cysteine-165.

Apart from the hydrogen-bonding of the substrate to histidine-195, the other possible interactions between the substrate and the enzyme are via the methyl and carboxy groups. Interaction via the methyl group must be minimal, because the enzyme reduces glyoxalate with a similar $k_{cat.}$ to that for pyruvate, and the K_m for both substrates is identical (Warren, 1970) when allowance is made for the 95% hydration of the carbonyl group of glyoxalate (Cooper & Redfield, 1975). It should be noted that with the thiomethylated enzyme the K_m for glyoxalate appeared to be increased in a parallel fashion to that of pyruvate, whereas it could be argued that a distorted methyl-binding site might more readily accommodate the binding of the smaller glyoxalate molecule.

The presence of the carboxy group in the substrate is essential for activity, because compounds such as acetaldehyde, acetone and methyl acetate are inactive as substrates (Neilands, 1954). This carboxy group should bind via an ion pair to a suitable cation on the enzyme. In the case of dogfish muscle lactate dehydrogenase, candidates for this cation are arginine-171 or possibly arginine-109 (Holbrook *et al.*, 1975). The latter residue (arginine-109) is of particular interest, becasue it forms part of the loop that undergoes a large (1.4nm) conformational change on formation of the ternary complex, and it is proposed that modification of cysteine-165 will prevent this arginine from taking up its correct position in the ternary complex (Holbrook *et al.*, 1975). The thiomethylation of cysteine-165 could prevent the correct location of arginine-109 and hence possibly arginine-171 in the ternary complex, with a resulting deleterious effect on the binding of the substrate carboxy group. The fact that the reactivity of SO_3^{2-} with enzyme-bound NAD^+ is identical with both the the native and thiomethylated enzymes is consistent with an altered binding site for the carboxy group of the substrate. Such an alteration would be more remote from the C-4 position of the nicotinamide ring than if the binding of the substrate carbonyl group had been affected and hence would still allow the approach of the SO_3^{2-}.

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