Modification of Pig Heart Lactate Dehydrogenase with Methyl Methanethiosulphonate to Produce an Enzyme with Altered Catalytic Activity

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Methyl methanethiosulphonate was used to produce a modification of the essential thiol group in lactate dehydrogenase which leaves the enzyme catalytically active. Methyl methanethiosulphonate produced a progressive inhibition of enzyme activity, with 2mm-pyruvate and 0.14mm-NADH as substrates, which ceased once the enzyme had lost 70–90% of its activity. In contrast, with 10mm-lactate and 0.4mm-NAD+ as substrates the enzyme was virtually completely inhibited. The observed inhibition was critically dependent on the chosen substrate concentration, since methanethiolation with methyl methanethiosulphonate resulted in a large decrease in affinity for pyruvate. At 0.14mm-NADH, methanethiolation increased the apparent K\text{m}^{PP} from 40μM for the control enzyme to 12mm for the modified enzyme. Steady-state kinetics showed that there was not a statistically significant change in either K\text{m}^{NADH} or K\text{m}^{NADH}. At saturating NADH and pyruvate concentrations, the V\text{max} was virtually unaffected for the methanethiolated enzyme. However, a decrease in V\text{max} was observed when the modified enzyme was incubated in dilute solution. The modification of lactate dehydrogenase by methyl methanethiosulphonate involved the active site, since inhibition was completely prevented by substrate-analogue pairs such as NADH and oxamate or NAD+ and oxalate. The formation of complexes between methanethiolated lactate dehydrogenase and substrates or substrate analogues can also be shown by re-activation experiments. The methanethiolated enzyme was re-activated in a time-dependent reaction by dithiothreitol and this was prevented by oxamate, by NADH and by NADH plus oxamate in increasing order of effectiveness. The results of this work are interpreted in terms of a role for the essential thiol group in the binding of substrates.

Lactate dehydrogenase (l-lactate–NAD+ oxidoreductase, EC 1.1.1.27) from a number of sources has been extensively studied by kinetic, chemical-modification, crystallographic and sequence techniques [Holbrook et al. (1975) and references cited therein]. The three-dimensional X-ray structure has clearly established the presence of an active-site cleft that is enclosed by a peptide loop during catalysis (Smiley et al., 1971) and this must produce the classical micro-environment long advocated by enzymologists. We were particularly interested in introducing reagents into this site which would allow the enzyme to retain catalytic activity and eventually serve as reporter groups of events within the ternary complex.

An obvious candidate for alkylation by suitable electrophilic reagents is the essential thiol group that is characteristic of a number of dehydrogenases such as lactate dehydrogenase (Fondy et al., 1965; Holbrook & Pfeiderer, 1965; Holbrook, 1966; Holbrook et al., 1975). In lactate dehydrogenase the X-ray-crystallographic structure has indicated that cysteine-165 is within the general area of the active-site cleft, but apparently not involved in the catalytic process (Adams et al., 1970; Holbrook et al., 1975). On the other hand, modification by a number of reagents leads to a complete loss of enzyme activity (Neilands, 1954; Velick, 1958). Kenyon and his colleagues (Smith et al., 1975; Nishimura et al., 1975) have developed the use of methyl methanethiosulphonate as a reagent which by formation of an enzyme-S–S–CH_3 derivative produces a minimal modification of enzyme thiol groups. This may allow residual activity, which is eliminated by the presence of charged or more bulky modifying reagents. Further, the use of 13C-labelled reagents or of similar reagents containing fluorine might allow these compounds to be used as n.m.r. (nuclear-magnetic-resonance) probes of the active site.

The present work demonstrates that methyl methanethiosulphonate is a reversible alkylation
inhibitor which is active-site-directed. The major change observed in the catalytic properties of the enzyme is a large decrease in the affinity of the enzyme for pyruvate.

**Materials and Methods**

Methyl methanethiosulphonate was synthesized by \( \text{H}_2\text{O}_2 \) oxidation of dimethyl disulphide (Smith et al., 1975). For use in alkylation reactions the compound was prepared as a 0.1 m solution in 50 mM-potassium phosphate, pH7.

Pig heart lactate dehydrogenase [Boehringer Corp. (London) Ltd., London W.5, U.K.] was used throughout this work. In the direction of pyruvate reduction the enzyme was assayed as a routine in 50 mM-potassium phosphate, pH7, containing 2 mM-pyruvate and 0.14 mM-NADH to determine maximum activity. In the reverse direction the buffer was 100 mM-potassium phosphate, pH9.4, containing 10 mM-lactate and 0.4 mM-NAD\(^+\). Where appropriate, kinetic constants are presented ±S.E.M. and were determined by the method of Cleland (1967). For methanethiolation experiments, lactate dehydrogenase (1 mg of protein/ml) in 50 mM-potassium phosphate, pH7.4, was treated with the selected concentration of methyl methanethiosulphonate at 18°C. At appropriate times, 20 µl samples were removed, diluted 50-fold with the buffer to stop the alkylation reaction and a sample was removed to assay its catalytic capacity.

Protection experiments were performed with either oxamate or oxalate acting as inert analogues of pyruvate or lactate respectively (Novoa et al., 1959; Winer & Schwert, 1959). The inert analogues were chosen rather than substrates to ensure that there could be no change in composition of the protecting ligands during the course of the protection experiment.

All chemicals used in this work were obtained from either British Drug Houses, Poole, Dorset, U.K., or Koch-Light Laboratories, Colnbrook, Bucks., U.K.

**Results**

**Inactivation of lactate dehydrogenase**

When lactate dehydrogenase was incubated with 10 mM-methyl methanethiosulphonate at pH7.4, there was a time-dependent decrease in catalytic activity. This loss of activity was observed with either NADH and pyruvate or NAD\(^+\) and lactate as substrates (Fig. 1). With the chosen concentration of lactate (10 mM) and NAD\(^+\) (0.4 mM) the inhibition was virtually complete (98–99%), whereas with pyruvate (2 mM) and NADH (0.14 mM) the maximum inhibition observed in a number of experiments was 70–90%. Further inhibition was difficult to obtain. This effect might represent an equilibrium process dependent on the concentration of inhibitor. Fig. 2 shows that this was not the case, since varying the concentration of methyl methanethiosulphonate resulted in alteration of the rate of alkylation; however, it did not substantially alter the final residual enzyme activity. The residual enzyme activity was maintained even when the alkylation period was extended up to 24 h duration. A further explanation of the cessation of inactivation is that the inhibitor might decay during the alkylation reaction. This was eliminated by adding further methyl methanethiosulphonate once the initial alkylation phase was complete. This did not result in an increase in inactivation.

The retention of residual enzyme activity after modification by methyl methanethiosulphonate could also be explained by the following scheme:

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E + I \overset{k_1}{\longrightarrow} EI \overset{k_{+2}}{\longrightarrow} EI^* \overset{k_{-2}}{\longrightarrow} E + I
\]

The key feature of this mechanism is the formation of a reversible covalent complex leading to an equilibrium mixture between EI\(^*\) (covalent complex) and EI (active on dilution). For this model, if the inhibitor is removed there should be recovery of enzymic activity. This was tested by inhibiting lactate dehydrogenase with 10 mM-methyl methanethiosulphonate until 85% of its activity in the direction

![Fig. 1. Difference in methyl methanethiosulphonate inhibition of lactate dehydrogenase depending on the direction of enzyme assay](image-url)
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Fig. 2. Effect of methyl methanethiosulphonate concentration on the rate of inactivation and the final residual activity of lactate dehydrogenase

Lactate dehydrogenase was treated with methyl methanethiosulphonate and its activity was measured by NADH oxidation as described in the Materials and Methods section. The concentrations of methyl methanethiosulphonate used were as follows: ■, 20mM; ▲, 10mM; ●, 5mM; ▲, 2.5mM; ○, 1mM. Over a 24h incubation at 18°C there was no detectable loss of activity in control incubations. Enzyme activity is expressed as percentage of zero-time value.

Fig. 3. Replot data for the inactivation of lactate dehydrogenase by methyl methanethiosulphonate

The first-order rate constants ($k_{app}$) for inactivation at several concentrations of methyl methanethiosulphonate were estimated from the initial loss of lactate dehydrogenase activity using more extensive data similar to that shown in Fig. 2. $K_i$ and $k_{i+2}$ were estimated as 6.74±0.91 mM and 0.157±0.008 min$^{-1}$ respectively (Kitz & Wilson, 1962).

of pyruvate reduction was lost. The solution was then diluted 100-fold with buffer and the activity followed for 8h. During this period there was no re-activation, demonstrating that a reversible covalent complex is not formed ($k_{-a} = 0$). This experiment was confirmed by exhaustive dialysis, which also failed to give any re-activation.

The presence of an initial binding phase in the alkylation reaction by methyl methanethiosulphonate was confirmed by a double-reciprocal plot of the first-order rate constants ($k_{app}$) for inactivation versus methyl methanethiosulphonate concentration. This gave a straight line (Fig. 3), indicating saturation kinetics (Kitz & Wilson, 1962). From the results of this experiment $K_i$ was estimated as 6.74±0.91 mM and $k_{i+2}$ as 0.157 min$^{-1}$.

**Methanethiolation of lactate dehydrogenase results in an increase in $K_{m}'$**

For experiments involving irreversible modification of an enzyme, the assays are performed with saturating concentrations of substrates to eliminate changes in affinity for substrates playing a part in the inhibition of the enzyme. We thought these criteria were satisfied in the previous experiments for NADH and pyruvate ($K_{m}'$ approx. 40μM; assay concentration 2mM), but were aware that this was not strictly true for lactate ($K_{m}^{Lac}$ approx. 2mM; assay concentration 10mM). This might contribute to the differential modification observed when either lactate and NAD$^+$ or pyruvate and NADH were used as substrates (Fig. 1). Therefore we decided to look for changes in the binding properties of the methanethiolated enzyme for pyruvate. Lactate dehydrogenase was methanethiolated with 10mM-methyl methanethiosulphonate and then assayed at a fixed concentration of NADH in the presence of different concentrations of pyruvate (Fig. 4). Methanethiolation resulted in an increase in $K_{m}'$ (app.) from 40μM for the control enzyme to 12mM for the methanethiolated enzyme. The $V_{max}$ for the methanethiolated enzyme at saturating pyruvate concentration was virtually unaffected. This means that under the conditions used in Fig. 4 any change in catalytic activity is the result of an increase in $K_{m}'$. For unmodified lactate dehydrogenase, high pyruvate concentrations result in an inhibition of enzyme activity, presumably owing to formation of the abortive enzyme-NAD$^+$-pyruvate complex (Everse et al., 1971; Arnold & Kaplan, 1974). This did not occur with methanethiolated enzyme, indicating that the abortive complex does not occur.

Previous experiments by Holbrook & Stinson (1970), using S-sulpholactate dehydrogenase (modified at cysteine-165), showed that although the enzyme was catalytically inert, NADH was still bound normally. We wished to examine whether a
similar result for NADH binding was obtained with the methanethiolated enzyme. For this experiment lactate dehydrogenase was modified with methyl methanethiosulphonate and kinetic analysis was performed at pH 8.0 (50 mM-potassium phosphate). This pH was chosen because $K_m^{Pr}$ increases, so that kinetic experiments may be performed more easily. However, it must be borne in mind that complexities in reaction mechanism may be introduced at this pH (Boland & Gutfreund, 1975). Double-reciprocal plots of initial velocity versus NADH concentration at various pyruvate concentrations intersected and kinetic constants were estimated from both slope and intercept plots (Cleland, 1967). For the control enzyme $K_m^{NADH}$ and $K_m^{NAD}$ were estimated as $5.42 \pm 1.48 \mu M$ and $4.77 \pm 0.72 \mu M$ respectively, and these compared with $3.3 \pm 0.79 \mu M$ and $3.2 \pm 1.22 \mu M$ for the same kinetic constants for methanethiolated lactate dehydrogenase. The paired $t$ test showed no significant statistical variation between the two sets of constants. Under the same experimental conditions there was an increase in $K_m^{Pr}$. This result indicates that the binding of NADH was not affected by methanethiolation and the major change is an increase in $K_m^{Pr}$.

An additional feature of the previous experiment was that under the conditions used there was a decrease in $V_{max}$ on methanethiolation, which seemed to contradict the result presented above (Fig. 4). This result can be attributed to a conformational change produced by the high dilution of the enzyme required to produce reasonable rates at NADH concentrations around $K_m^{NADH}$. This was shown by incubating lactate dehydrogenase (1 mg/ml) with 10 mM-methyl methanethiosulphonate until the maximum inhibition was obtained. The enzyme was then diluted to a variety of protein concentrations. On further incubation (Fig. 5), the specific activity of the diluted methanethiolated enzyme declined progressively with time and the decrease in specific activity was proportional to the dilution of the enzyme. Methanethiolated lactate dehydrogenase incubated at a protein concentration of 1 mg/ml did not show a fall in specific activity.

**Activity of thiomethylated lactate dehydrogenase with lactate and NAD$^+$ as substrates**

Steady-state kinetic experiments with lactate and NAD$^+$ as substrates for methanethiolated lactate dehydrogenase have proved difficult to perform in similar detail, primarily owing to the low affinity for substrates. However, in 0.1 M-potassium phosphate, pH 9.2, at a single concentration of NAD$^+$ (2.5 mM) the apparent $K_m^{Lac}$ for unmodified lactate dehydrogenase was $1.37 \pm 0.29$ mM and for methanethiolated lactate dehydrogenase the apparent $K_m^{Lac}$ was $97 \pm 10.3$ mM. This represents approximately a 70-fold increase in $K_m^{Lac}$.

To measure the effect of methanethiolation on the affinity for NAD$^+$ it was not practical to use saturating lactate concentrations (approx. 1 mM) for methanethiolated lactate dehydrogenase. An alternative approach was used in which lactate concent-

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**Fig. 4.** Comparison of the effect of pyruvate concentration on the rate of reaction catalysed by normal and methanethiolated lactate dehydrogenase

Lactate dehydrogenase was assayed in the direction of pyruvate reduction as described in the Materials and Methods section, except that the concentrations of pyruvate were used. ○, Normal enzyme (0.4 μg/ml); ●, enzyme (1 mg/ml) modified by 10 mM-methyl methanethiosulphonate in 50 mM-potassium phosphate buffer, pH 7.4, for 2 h and then diluted and assayed at 0.4 μg of protein/ml.

**Fig. 5.** Effect of dilution on the activity of normal and methanethiolated lactate dehydrogenase

Various dilutions of the normal and modified enzyme were incubated at 25°C, then assayed for pyruvate reduction at the times shown. ○, Normal enzyme at 4 μg/ml and methanethiolated enzyme at 1 mg/ml; △, methanethiolated enzyme at 40 μg/ml; ●, methanethiolated enzyme at 20 μg/ml; E, methanethiolated enzyme at 4 μg/ml.
tration was fixed at a fraction of the value measured for the apparent $K_m^{Lac}$. For 0.5 $K_m^{Lac}$ the concentrations of lactate are 50 mm for methanethiolated enzyme and 0.7 mm for unmodified enzyme. With these concentrations of lactate the apparent $K_m^{NAD^+}$ values were 0.41±0.03 mm and 0.25±0.03 mm for the modified and control enzyme respectively. The difference in these values is obviously small in comparison with the change in apparent $K_m^{Lac}$. Again this indicates that modification has only a small effect on binding of the nicotinamide nucleotide.

**Active-site character of modification reaction**

So far it has been assumed that the methanethiolation reaction with methyl methanethiosulphonate is active-site-directed. However, there is no reason to suppose that methyl methanethiosulphonate has any special structural characteristics which enable it selectively to modify solely the active-site thiol group. Therefore the loss of catalytic activity could merely reflect the multiple modification of the enzyme. To investigate this possibility, the action of ligands that bind specifically at the active site on the inhibition by methyl methanethiosulphonate was examined. Fig. 6 shows the results of these experiments. Binary enzyme–NADH and enzyme–NAD$^+$ complexes were partially protected against inactivation; however, neither oxamate nor oxalate provided any protection against inhibition by methyl methanethiosulphonate. These results are consistent with the compulsory order of binding to lactate dehydrogenase, which involves binding of the nicotinamidenucleotidebefore the pyruvate (oxamate) or lactate (oxalate) analogue (Novoa et al., 1959; Winer & Schwert, 1959). For the ternary complexes enzyme–NADH–oxamate or enzyme–NAD$^+$–oxalate, there was complete protection against alkylation by methyl methanethiosulphonate. Overall these results show that the loss of activity on alkylation by methyl methanethiosulphonate is the result of an active-site modification. Further, the fact that protection is maximal in the ternary complex is consistent with the idea that the locus of modification is in the active site.

If methyl methanethiosulphonate modifies lactate dehydrogenase so that the active-site thiol group is modified, then the addition of a second reagent which also alkylates this thiol group should not result in any further change in enzyme activity. This experiment was performed by using N-ethylmaleimide as the second reagent for thiol modification (Holbrook, 1966). Whereas N-ethylmaleimide resulted in the complete inactivation of the control enzyme, the methanethiolated enzyme showed only a 15% decrease in enzyme activity. This result is consistent with methyl methanethiosulphonate causing the modification of all the active sites of the lactate dehydrogenase.

A further feature which is consistent with the modification of the active-site region of the protein comes from study of the pH-dependence of the alkylation reaction (Fig. 7), which showed that the

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**Fig. 6. Protection against inactivation of lactate dehydrogenase by methyl methanethiosulphonate by using active-site-directed ligands**

Lactate dehydrogenase (1 mg/ml) was treated with 10 mm-methyl methanethiosulphonate in 50 mm-potassium phosphate, pH 7.4, containing a variety of ligands which are known to bind to the enzyme active site. The composition of protecting ligands in (a) was: ○, none; △, 2.5 mm-oxamate; ●, 0.125 mm-NADH; ▲, 2.5 mm-oxamate and 0.125 mm-NADH. The composition in (b) was: ○, none; △, 2.5 mm-oxalate; ●, 0.5 mm-NAD$^+$; ▲, 2.5 mm-oxalate and 0.5 mm-NAD$^+$.
Lactate dehydrogenase (1 mg/ml) was treated with 10 mm-methyl methanethiosulphonate in 50 mm-potassium phosphate solutions at several pH values. The apparent rate constant for inactivation ($k_{app}$) was estimated for each pH value from the slope of plots of log(activity [% remaining]) versus time. The pH-dependence of $k_{app}$ is given by the equation

$$k_{app} = \frac{k^* \cdot K_A}{[H^+] + K_A}$$

where $k^*$ is the pH-independent rate constant for inhibition. $k^*$ and $P_K$ were estimated as 0.090±0.012 min$^{-1}$ and 7.28±0.3 from a plot of ($k_{app}$)$^{-1}$ versus [H$^+$]. The solid line shows the theoretical distribution by using these values for $k^*$ and $P_K$.

$p_K$ for the alkylation reaction was 7.28±0.30. This value corresponds closely to estimates for the binding of other ligands to the active site of lactate dehydrogenase: oxamate, $p_K$6.9 (Holbrook & Stinson, 1973); pyruvate, $p_K$7.4–7.7 [data of Schwert et al. (1967) analysed by Boland & Gutfreund (1975)]; epoxybutyric acid, $p_K$6.8 (Blokhom et al., 1975); diethyl pyrocarbonate modification of histidine-195, $p_K$6.8 (Holbrook & Ingram, 1973). Generally all of these $P_K$ values have been considered to represent the functional $p_K$ of the active-site histidine residue; however, the observation that a virtually identical $p_K$ value was obtained for modification of a cysteine group at the active site may indicate that these $P_K$ values might constitute an overall value for the $p_K$ of the enzyme active site.

Re-activation of the modified enzyme

The reaction between methyl methanethiosulphonate and lactate dehydrogenase should lead to the generation of a new disulphide bond within the protein. If this bond is accessible to the external environment then the addition of a reducing agent should lead to the regeneration of catalytically active enzyme.

Initially it was extremely difficult to re-activate modified lactate dehydrogenase. The direct addition of a tenfold (0.1 M) excess of reducing agent to the inhibited enzyme was virtually ineffective. Further, dialysis followed by short-term exposure to thiols was only marginally effective. Eventually we decided that our failure to achieve re-activation was the result of our expectation that the formation of the disulphide bond between methyl methanethiosulphonate and lactate dehydrogenase would be reversed as easily as in those cases where an aromatic residue is involved in the disulphide link (see, e.g., Bloxham et al., 1973). We therefore decided on a more drastic procedure which would markedly improve the chances of the reduction procedure. In this technique, immediately the modification reaction was complete, the mixture was diluted fiftyfold into a re-activation buffer containing high concentrations of reducing agents [up to 700-fold (0.14 M) excess over the methyl methanethiosulphonate] and the re-activation followed over 6 h. Table 1 shows that under these conditions it was possible to obtain substantial re-activation of the enzyme. The most effective reducing reagent was dithiothreitol, followed by mercaptoethanol. Tributylphosphine was tested, since it is reported to reduce disulphide bonds in non-polar environments (Nishimura et al., 1975). With methanethiolated lactate dehydrogenase it was only marginally effective, and indicated that the difficulties obtained in re-activating the enzyme were not due to the location of the disulphide bond in a non-polar environment, but rather they were due to inaccessibility to the reducing agent. Finally mercaptoacetic acid was tried in the hope that the possession of a carboxyl group might enable it to enter the active site more readily. This does not seem to be the case, since mercaptoacetic acid was inferior.

Table 1. Re-activation of methanethiolated lactate dehydrogenase by reducing agents

<table>
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<tr>
<th>Reducing agent</th>
<th>Recovery of lactate dehydrogenase activity (%)</th>
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<tr>
<td>Tributylphosphine</td>
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<tr>
<td>Mercaptoacetic acid</td>
<td>14 mm</td>
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<tr>
<td>Mercaptoethanol</td>
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<td></td>
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<td>Dithiothreitol</td>
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to dithiothreitol and had a similar potency to mercaptoethanol.

Having established that a large excess of reducing agent is required efficiently to regenerate active lactate dehydrogenase from the methanethiolated enzyme, the time-course for the re-activation process was measured (Fig. 8). With 2mm-pyruvate and 0.14mm-NADH as substrates, the time-course for re-activation was apparently biphasic, consisting of an initial slow phase, followed by a second more rapid phase (Fig. 8a,b,c). In contrast, when lactate and NADH were used as substrates, then the re-activation process appeared to be first-order (Fig. 8d). The only difference between these systems is that with pyruvate and NADH as substrates the enzyme has residual activity (approx. 20%), whereas with lactate and NAD+ the enzyme was completely inactive. This raised the possibility that the rate profile for re-activation was a function of the starting activity of the methanethiolated enzyme. This proposal was confirmed by carrying out the assays in a re-activation experiment with much lower pyruvate concentrations (200μM). At this concentration of pyruvate the methanethiolated enzyme was virtually completely inactive in comparison with the control enzyme, and the re-activation procedure now showed pseudo-first-order kinetics. This result indicates that the thiol groups modified by methyl methanethiosulphonate are equivalent, at least with regard to their reaction with a reducing agent.

Formation of binary and ternary complexes with methanethiolated lactate dehydrogenase: demonstration of protection against re-activation

The retention of catalytic activity in methanethiolated lactate dehydrogenase demonstrates that the enzyme must form complexes with its substrates. The formation of complexes can also be demonstrated by an approach which is a variation of the substrate-dependent protection against inactivation by active-site-directed inhibitors. The rationale behind this

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**Fig. 8. Time-dependence of the re-activation of methanethiolated lactate dehydrogenase by dithiothreitol**

Methanethiolated lactate dehydrogenase (20μg/ml; 85% inhibited) was reduced with dithiothreitol in 50mM-potassium phosphate buffer, pH7.4. The recovery of activity is presented in the form of a pseudo-first-order plot where \( \frac{dA}{dt} \) is the increase in activity to give completely re-activated enzyme and \( A \) is the recovery of activity at the time of sampling. The concentrations of dithiothreitol were: □, 100mM; ◦, 20mM; Δ, 10mM. In the absence of dithiothreitol there was no recovery of activity. In a separate experiment (●) with 100mM-dithiothreitol, the recovery of enzyme activity was assayed in the direction of lactate oxidation.

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**Fig. 9. Protection against re-activation of methanethiolated lactate dehydrogenase by active-site-directed ligands**

The experiment measures the time-dependent recovery of lactate dehydrogenase activity. Methanethiolated enzyme (20μg/ml) was measured with 20mM-dithiothreitol in 50mM-potassium phosphate buffer, pH7.4, in the presence of a variety of ligands which bind to the enzyme active site. The concentrations chosen in (a) were: ○, none; △, 2.5mM-oxamate; ●, 0.2mM-NADH; Δ, 2.5mM-oxamate and 0.2mM-NADH. The concentrations in (b) were: ○, none; Δ, 2.5mM-oxalate; ●, 0.5mM-NAD+; Δ, 2.5mM-oxalate and 0.5mM-NAD+.
approach is as follows. If the methanethiolated lactate dehydrogenase active site is functional (but at a lower rate), then it should form complexes with substrates. In this event the active-site loop will close (Smiley et al., 1971), rendering the active site inaccessible to the external reducing agent, and this will protect against re-activation.

This concept was evaluated by testing the effect of NADH, oxamate, NADH plus oxamate, oxalate, NAD+ and NAD+ plus oxalate on the re-activation of methanethiolated lactate dehydrogenase. The experiments with NADH and oxamate provided an excellent verification that the methanethiolated enzyme forms active-site-directed complexes with its substrates or substrate analogues. Thus oxamate provided marginal protection against re-activation, NADH was fairly efficient and NADH plus oxamate provided complete protection (Fig. 9a). This protection pattern is consistent with the compulsory binding order for lactate dehydrogenase (NADH first, followed by oxamate), and is obviously identical with the protection pattern for inhibition with methyl methanesulphonate (Fig. 6a). Fig. 9(b) also indicates that NAD+ and oxalate must still form complexes with the active site, since they do offer substantial protection against re-activation. Whereas NAD+ and oxalate separately offered only marginal protection against re-activation, NAD+ plus oxalate was much more effective. This difference in nature of protection by the two substrate pairs may be related to the fact that NAD+ binds only poorly to lactate dehydrogenase, in contrast with NADH (Holbrook & Stinson, 1973), and oxalate enhances the binding of NAD+ to the enzyme (Kolb & Weber, 1975).

Discussion

The principal aim of this work was to develop the use of a reagent that could modify lactate dehydrogenase such that a substantial proportion of the enzyme's normal catalytic function was retained. Subsequently the modifying reagent might then be used as a probe for conformational changes occurring at the active site during catalysis.

The development by Kenyon and his colleagues (Smith et al., 1975; Nishimura et al., 1975) of methyl methanesulphonate as a modifying reagent seems particularly useful for this purpose. Thus methanethiolation introduces a relatively small group into the protein (–S–CH₃) without necessarily altering the charge of groups within the active site. The present work clearly demonstrates that methanethiolation of lactate dehydrogenase appears to achieve the primary goal. The observed properties of methanethiolated lactate dehydrogenase may be summarized as follows. First, there is a substantial increase in \( K_{\text{m}}^{\text{pyruvate}} \) without a decrease in the affinity for NADH. Secondly, with saturating concentrations of NADH and pyruvate the maximum catalytic activity (\( V_{\text{max}} \)) is unaffected. Thirdly, at dilute protein concentrations the methanethiolated enzyme shows a conformational transition which results in the loss of activity. With NAD+ and lactate as substrates, it is far more difficult to demonstrate that the modified enzyme is catalytically active, owing to the very high values for \( K_{\text{m}}^{\text{a}} \); however, the formation of functional complexes at the active site has been shown with oxalate and NAD+ by using a novel technique of protecting the enzyme against re-activation by a reducing agent.

A difficulty in the unambiguous interpretation of the current results is that we have not identified precisely which cysteine thiol group is modified by methyl methanethiosulphonate. Obviously this is an important aspect, which requires investigation. Preliminary ethanethiolation experiments with methyl [2-\(^3\)H]ethanethiosulphonate

\[
\text{CH}_3\text{CH}_2\text{S-S-CH}_3
\]

have established that this type of reagent does lead to the incorporation of radioactivity into the protein. However, the analysis involves considerable problems because of random disulphide rearrangements, which occur with the modified protein. For the present discussion, we presume that cysteine-165 is modified by methyl methanethiosulphonate. Thus cysteine-165 is near the enzyme active site (Adams et al., 1970) and is modified by a number of reagents which cause loss of enzyme activity (Fondy et al., 1963; Holbrook & Pfleiderer, 1965; Holbrook, 1966). We presume that modification by methyl methanethiosulphonate is active-site-directed, since modification is prevented by substrates and inhibitors which bind at the active site.

The retention of catalytic activity in methanethiolated lactate dehydrogenase shows that the modified cysteine residue does not participate in an essential covalent step in the catalytic mechanism. It may be contrasted with histidine-195, which participates in proton transfer and whose modification renders the enzyme completely inactive (Holbrook & Ingram, 1973). On this basis it seems most probable that the modified cysteine residue participates in a binding process in the catalytic mechanism involving pyruvate rather than NADH. This concept is in accord with the observations of Holbrook & Stinson (1970), who showed that lactate dehydrogenase with cysteine-165 sulphonated bound NADH, although it was catalytically defective. How cysteine-165 might participate in the binding of pyruvate is unknown; however, this residue is within
0.5nm of histidine-195 (Adams et al., 1970) and it is possible that the state of the cysteine residue may influence the ability of histidine-195 to enter into the protonated form. An increase in $K_m^{pr}$ would result if methanethiolation prevented histidine-195 from assuming a protonated state, since it is known that $K_m^{pr}$ is a function of the $pK_a$ of histidine-195 (Holbrook & Gutfreund, 1973; Whitaker et al., 1974; Bloxham et al., 1975). Alternatively methanethiolation of cysteine-165 could simply decrease the ability of the pyruvate to bind at the active site, because of steric hindrance.

In conclusion, we have shown that treatment with methyl methanethiosulphonate leads to reversible modification of lactate dehydrogenase, to produce an enzyme with considerable residual catalytic capacity.

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References