Reactivity of the Essential Thiol Group of Lactate Dehydrogenase and Substrate Binding

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1. The preparation of a derivative of pig heart lactate dehydrogenase in which the essential thiol group has been converted into an *S*-sulpho group is described. The derivative has unchanged $s_{20,w}$ and is catalytically inactive. 2. The rate of reaction of the essential thiol group is controlled by a system with a $pK > 9$. 3. The essential thiol group is protected by NADH against reaction with maleimide. 4. Lactate dehydrogenase in which the essential thiol group has been converted into an *S*-sulpho group or alkylated with maleimide still binds one molecule of NADH/subunit but with a three- to four-fold diminished affinity. 5. The inhibited enzymes also bind one molecule of NAD^+ -sulphite complex/subunit but with affinity decreased 10^3 – 10^4 -fold. 6. The inhibited enzymes fail to bind C_2 and C_3 molecules to give the ternary complexes enzyme– NAD^+ –pyruvate, enzyme–NADH–oxamate and enzyme–NADH–oxalate. The 1:1:1 stoichiometry of the last-mentioned complex with the native enzyme was established by gel filtration. 7. Structures that account for these results are discussed.

Pig heart (H_4) lactate dehydrogenase (EC 1.1.1.27) contains an 'essential' thiol group as part of the sequence Val-Ile-Gly-Ser-Gly-Cys-Asn-Leu-Asp-Ser-Ala-Arg (Holbrook *et al.* 1967). The group is described as essential since its specific modification leads to a stoichiometric loss of the catalytic activity of the enzyme protein (Holbrook & Pfeleiderer, 1965; Holbrook & Jeckel, 1967). The inactivation is a result of neither a change in the state of aggregation of the enzyme nor an inability to bind NADH and NAD^+ -sulphite complex (Holbrook, 1966; Allison, 1968; Adams *et al.* 1970) nor a change in conformation sufficient to alter the optical-rotatory-dispersion spectrum (Jeckel, 1969). The present paper investigates a further possibility, that the loss of activity is due to the inability of the binary enzyme-nucleotide complex to bind substrate. The word nucleotide will denote NAD^+ and NADH; the words substrate and substrate analogue will denote pyruvate, lactate, oxalate, glyoxylate and oxamate.

EXPERIMENTAL

Pig heart lactate dehydrogenase was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., with a specific activity of 370 U/mg. The enzyme was treated with charcoal by the method of Wieland, Duesberg, Pfeleiderer, Stock & Sann (1962). 5,5'-Dithiobis-(2-nitrobenzoic acid) and maleimide were obtained from E.G.A.-Chemie, W. Berlin, Germany. The labelled compounds

$\text{Na}_2^{35}\text{SO}_3$ (8 mCi/mmol), [^{14}C]pyruvic acid (27.2 mCi/mmol) and [^{14}C]oxalic acid (50 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. NAD^+ and NADH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. The NADH was dissolved in water and freeze-dried to remove traces of solvents. The buffer used in all experiments, except where specifically mentioned, was 67 mM- KH_2PO_4 adjusted to pH 7.2 with 5 M-NaOH.

Specific enzyme activity. Enzyme activity was calculated from the initial rate of oxidation of 0.15 mM-NADH by 0.33 mM-pyruvate in buffer at 25°C. The rate was derived from the change in E_{340} recorded by a Hilger-Gilford reaction-kinetics spectrophotometer. Protein concentration of lactate dehydrogenase and its derivatives was determined either by the biuret method of Beisenherz *et al.* (1953) by using the factor 16.5 or by use of the factor 1.39 for the E_{280} of a 1% (w/v) solution.

Ultracentrifugation. The sedimentation of protein samples (5 mg/ml) that had been dialysed against 0.2 M- H_3PO_4 –0.08 M-NaCl adjusted to pH 7.0 was followed by using the schlieren optical system of a Spinco model E analytical ultracentrifuge. The sedimentation coefficient was derived from six photographs taken at 8 min intervals after the rotor had reached 60000 rev./min at 20°C. The values were corrected to give $s_{20,w}$ by using densities and viscosities determined by R. Cantwell (unpublished work) and assuming a partial specific volume of 0.745 ml/g.

5,5'-Dithiobis-(2-nitrobenzoate) modification. The rate of reaction of 5,5'-dithiobis-(2-nitrobenzoate) (2.86 mM) with lactate dehydrogenase (8.6 mg/ml) was followed by measurement of the rate of increase in E_{412} in 2 mm path-length cuvettes. The rate of extinction increase in blank

samples from which enzyme had been omitted was subtracted, and the apparent first-order rate constant for the reaction was evaluated by the method of Guggenheim (1926). The buffer consisted of 0.2M- $\text{Na}_4\text{P}_2\text{O}_7$ –0.2M- NaH_2PO_4 –1 mM-EDTA adjusted to the required pH with 10M-HCl or 5M-NaOH.

S-Sulpho-(lactate dehydrogenase). Compound I (see Scheme 1 in the Results section), in which the essential thiol group of lactate dehydrogenase forms an asymmetric disulphide with 5-thio-2-nitrobenzoate (3-carboxy-4-nitrobenzenethiol), was prepared by the method of Holbrook & Jeckel (1967). This is referred to as reaction *a*. During the preparation 0.98 mol of 5-thio-2-nitrobenzoate/mol of subunit was liberated and the preparation had a specific activity of 18 U/mg. For reaction *b*, 0.4 mCi of $\text{Na}_2^{35}\text{SO}_3$ in 4.36 μmol of Na_2SO_3 was mixed with a solution (10 ml) of compound I (53 mg) in buffer. After 30 min the yellow solution was filtered through a column (3 cm $\times$ 60 cm) of Sephadex G-50 equilibrated with buffer. The colourless *S*-sulpho-(lactate dehydrogenase) was eluted well before a peak of a yellow compound. In a trial experiment with unlabelled Na_2SO_3 , 0.145 mM-5-thio-2-nitrobenzoate was liberated from 0.16 mM-compound (I). The *S*-sulpho-enzyme contained 0.88 mol of sulphite/mol of subunit and had a specific enzyme activity of 18 U/mg, and was used in the experiments described here within 48 h of being prepared.

Maleimide-inhibited lactate dehydrogenase. This was prepared by a slight modification of the method of Holbrook (1966). Lactate dehydrogenase (12 mg/ml) and maleimide (10 mM) were incubated in buffer at 25°C. The half-time for the first-order rate of loss of enzyme activity was 24 min. At 1.86 h the solution was filtered through a column (3 cm $\times$ 60 cm) of Sephadex G-50 in buffer. The specific activity of the protein eluted in the first peak of u.v. absorption was 19 U/mg. By cautious addition of solid $(\text{NH}_4)_2\text{SO}_4$ to the solution, the inhibited enzyme was obtained as a precipitate that exhibited the sheen characteristic of micro-crystalline native enzyme.

Titration of ligand-binding sites. These titrations were carried out with a differential optical titrator. This device could be arranged either as a split-beam spectrophotometer, a split-beam differential fluorimeter or a single-beam fluorimeter with correction for fluctuations in the intensity of the exciting radiation. A split beam of monochromatic radiation was used to excite fluorescence in two 1 cm² quartz cuvettes. Fluorescence perpendicular to the incident beam was detected by two matched photomultipliers through Kodak 98 filters. In the differential mode, the signals from each photomultiplier were subtracted by a small analogue computer. Each cuvette was equipped with an overhead stirrer and ligand solution was added simultaneously to the contents (3 ml) of each cuvette at a rate of 5 $\mu\text{l/min}$. After calibrated amplification, the difference signal was displayed on a chart recorder and subsequently analysed in a digital computer. The instrument was initially adjusted by titrating ligand into the cuvettes and adjusting the gain of one channel so that there was no resultant difference signal. The equilibrium dissociation constant and the concentration of ligand-binding sites were evaluated from a graph of $1/(1-Q)$ against L_0/Q , where Q =fractional saturation of ligand binding sites=(difference in fluorescence)/(maximum difference signal) and L_0 =[total ligand in the cuvette]. As

a single-beam fluorimeter the instrument was used to measure the quench in enzyme-NADH fluorescence on addition of oxamate. In this case the second photomultiplier was used in a feedback circuit to correct for variations in the intensity of the exciting radiation and absorbance by the titrant. The instrument was also used as a split-beam spectrophotometer to measure the formation of the NAD^+ -sulphite complex by measuring changes in transmitted light at 325 nm. In this case the voltage analogue of the difference in the logarithm of the photomultiplier currents was recorded. Binding of NADH to *S*-sulpho-enzyme was measured by a simple fluorimeter similar to that described by Holbrook (1966). Discrete additions of nucleotide were made and the results and analysis are not as reliable as those obtained from continuous titration. The perturbations of fluorescence were first described by Winer & Schwert (1959).

Measurement of bound ligand by gel filtration. The technique introduced by Hummel & Dreyer (1962) was employed. A column (6 mm internal diam.) was filled to a height of 40 cm with Sephadex G-50 and equilibrated with buffer at a flow rate of 20 ml/h. The eluate from the column passed first through a scintillation counter adjusted to measure ^{14}C disintegrations by means of an NE808 flow head [Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh, U.K.] and then through the 3 mm round flow cell of a Uvicord I photometer (LKB Produkter AB, Stockholm, Sweden) in order to measure changes in transmitted light at 2537 Å.

Both the output from a rate-meter (time-constant 30 s) and that of the Uvicord were displayed on a two-channel recorder. In a typical experiment a stock solution containing NADH and ^{14}C oxalate was diluted with exactly 9 vol. of buffer and the dilute ligand solution allowed to flow through the column until the rate-meter and Uvicord signals indicated that equilibrium was attained. A solution (1.35 ml) of enzyme (8 mg/ml) was mixed with stock ligand solution (0.15 ml) and applied to the column. The elution of the protein was indicated by a decrease in transmission. Since both protein and NADH absorb at this wavelength, this change could not be used to determine the amount of NADH bound. The amount of ^{14}C oxalate bound to the enzyme was determined from the increase in radioactivity-counting rate as the protein was eluted. The NADH bound to the protein was calculated by collecting the eluate corresponding to the increased transmission at the elution volume characteristic of NADH. The difference in the concentrations of enzymically oxidizable NADH in this solution and a sample of the eluate when the column was at equilibrium was determined. The concentration of radioactive ligand should be 0.4 μCi of ^{14}C /ml to avoid large statistical fluctuations in the rate-meter output at this flow rate. Binding of NADH and oxalate was studied with $[\text{NADH}]=0.15$ mM and $[\text{oxalate}]=1.5$ mM. Binding of NAD^+ and pyruvate was studied with $[\text{NAD}^+]=1$ mM and $[\text{pyruvate}]=3$ mM. Since the rate-meter and photometer were connected in series, there was a lag of 1.0 ml between the two traces on the recorder.

RESULTS

The possible reactions involved in the synthesis of *S*-sulpho-lactate dehydrogenase are formulated in Scheme 1. Reaction *a* during the preparation of

Scheme 1. E-SH represents a lactate dehydrogenase subunit with its essential thiol group.

S-sulpho-enzyme should be formulated according to eqn. (1), since it is accompanied by the liberation of 1 mol of 5-thio-2-nitrobenzoate/mol of subunit during its preparation and since a further 1 mol of 5-thio-2-nitrobenzoate/mol of subunit was liberated when compound I was treated with excess of thiol (eqn. 4). Reaction *b* should be formulated as eqn. (2), since treatment of compound I with excess of sulphite yielded a yellow compound equivalent to 1 mol of 5-thio-2-nitrobenzoate/mol of subunit and was accompanied by the incorporation of 1 mol of sulphite/mol of subunit. The reaction formulated as eqn. (3) is excluded because of the incorporation of radioactivity into the protein and because reaction *b* did not result in the regeneration of the enzyme activity. As expected (Holbrook & Jeckel, 1967), the enzyme activity was completely regenerated when compound I was treated with excess of 2-mercaptoethanol, 2-thioglycollate, cysteine or dithiothreitol. The recovery of protein as compound III was 85% of theoretical. The quinonoid nature of the anion of compound II presumably explains both the favourable equilibrium of reaction 1 and the fact that reaction (2) is favoured compared with reaction (3). The sedimentation coefficient of the *S*-sulpho-enzyme was the same as that of the native enzyme ($s_{20,w}$ 6.9S). The reaction described by eqn. (2) should provide a general method of radioactively labelling thiols that have reacted with 5,5'-dithiobis-(2-nitrobenzoate).

The rate of reaction of the essential thiol group with modifying agents is very slow indeed. The

Fig. 1. Effect of pH on the apparent first-order liberation of 5-thio-2-nitrobenzoate from a reaction of lactate dehydrogenase and excess of 5,5'-dithiobis-(2-nitrobenzoate). Experimental details are given in the Experimental section.

second-order rate constant for the reaction with 5,5'-dithiobis-(2-nitrobenzoate) is $0.15 \text{ M}^{-1} \text{ s}^{-1}$, compared with 10^4 – $10^6 \text{ M}^{-1} \text{ s}^{-1}$ for the reaction of simple thiols and the essential thiol group of glyceraldehyde 3-phosphate dehydrogenase with this compound at pH 8.5 (Kirschner, 1968). The pH-dependence of the reaction (Fig. 1) shows that it depends on the ionization of a system with a $pK > 9$. Although this might be the pK of the essential thiol group, it might also be the pK of a protein side chain or side-chain system that controls access of 5,5'-dithiobis-(2-nitrobenzoate) to the thiol.

Fig. 2. Continuous fluorimetric titration of 0.27 mg of pig heart lactate dehydrogenase with NADH: A, in buffer (67 mM-phosphate, pH 7.2), amplified $\times 10$; B, in buffer containing 10 mM-sodium oxamate, amplified $\times 50$; C, in buffer containing 10 mM-sodium oxalate, amplified $\times 2$. All values have been corrected for changes in absorption of the exciting radiation (320 nm) and the emitted radiation (Kodak 98 filter) due to the added ligand. Correction for the small dilution of the original solution (3 ml) has also been made. The temperature was 20.5°C.

Fig. 3. Continuous fluorimetric titration of 0.93 mg of pig heart lactate dehydrogenase 95% inhibited by maleimide with NADH: A, in buffer; B, in buffer containing 10 mM-sodium oxamate; C, in buffer containing 10 mM-sodium oxalate. The amplification was the same for all titrations. Other conditions were as for Fig. 2.

Pig heart lactate dehydrogenase that is inhibited by maleimide still binds its full complement of NADH and NAD^+ -sulphite (Holbrook, 1966). This result was confirmed by using the differential

Fig. 4. Protection of lactate dehydrogenase by NADH from inhibition by maleimide. The enzyme (0.14 mM) in buffer was incubated at 25°C: Δ , alone; $\bullet$, with 2.0 mM-maleimide and 0.28 mM-NADH; $\square$, with 2.0 mM-maleimide and 0.049 mM-NADH; $\circ$, with 2.0 mM-maleimide. Samples of the incubation mixture were assayed for enzyme activity as described in the Experimental section.

fluorimetric titrator (Figs. 2A and 3A). The analysis of the curve is given in Table 1. The *S*-sulphoenzyme binds 3.6 mol of NADH/mol of tetramer with $K_{\text{E-NADH}}$ 3.3 μM .

Nucleotides protect the essential thiol group against modification. This is demonstrated by the results in Fig. 4 with NADH and maleimide. Excess of NADH gives essentially complete protection (at this concentration of enzyme all NADH-binding sites are saturated). The protection is seen to be stoichiometric, since 0.35 mol of NADH/mol of subunit lowers the rate of inhibition to 63% of its value when NADH is omitted.

The NAD^+ -sulphite-binding capacity of lactate dehydrogenase was originally determined by adding a single portion of concentrated sulphite solution to a mixture of the enzyme and NAD^+ (Holbrook, 1966). This demonstrated that the inhibited enzyme could bind as much NAD^+ -sulphite complex as did the native enzyme. Since sulphite has been reported to be a competitive inhibitor of lactate, this result was interpreted to mean that the substrate-binding site of the inhibited enzyme was also intact. Repetition of this experiment by a continuous titration (Fig. 5) confirmed the original finding, but demonstrated that sulphite is much less tightly

Fig. 5. Difference in E_{325} between solutions of 1 mM- NAD^+ , with and without lactate dehydrogenase, when titrated with 0.1 M- Na_2SO_3 . A, 2.5 mg of native enzyme; B, 2.7 mg of enzyme 95% inhibited by maleimide; C, 2.7 mg of *S*-sulpho-enzyme (5% residual activity). The volume was 3 ml.

bound to the inhibited enzymes than to the native enzyme. The affinity of the native enzyme for NAD^+ -sulphite is so great that the sulphite dissociation constant cannot be determined spectrophotometrically. To judge by the concentration of sulphite required to inhibit the enzyme by 50%, at a comparable NAD^+ concentration, the dissociation constant must be of the order of 0.1 μM . Modification of the essential thiol group has thus weakened sulphite binding by up to 10^4 -fold.

Binding of oxamate to the enzyme-NADH complex may be detected by the quenched fluorescence of the NADH in the ternary complex when compared with that of free NADH (Fig. 2B). A similar titration of enzyme 95% inhibited with maleimide shows an initial quench, due to the residual 5% active sites (Fig. 3B), but the remainder of the titration is quantitatively the same as if no oxamate were present (Fig. 3A).

The enzyme-NADH complexes derived from the native and inhibited enzymes were also titrated with oxamate (Fig. 6) and oxalate (Fig. 7). The analysis of these curves (Table 1) shows that oxalate binds to the residual active sites in the inhibited enzyme with the same affinity as to the native enzyme. It may be noted that the signal amplitudes of the inhibited preparations are decreased more than tenfold as compared with the native enzyme.

The binding of oxalate to the enzyme-NADH complex can be detected by the fivefold-enhanced fluorescence of NADH in the ternary complex as compared with that of NADH in the binary complex, and the over 20-fold enhancement compared with that of the free nucleotide. (The precise enhancement factors are dependent on the trans-

Fig. 6. Continuous titration with oxamate of lactate dehydrogenase plus NADH. A, 4 μM -Native enzyme + 20 μM -NADH; B, 4 μM -enzyme 95% inhibited by maleimide + 20 μM -NADH. The titrator was employed as a single-beam fluorimeter (see the Experimental section). Excitation was at 320 nm and emission through the Kodak 98 filter. The temperature was 20.0°C. The decreases in fluorescence from the inhibited and native enzymes were obtained at the same amplification. The values have been corrected for dilution.

Fig. 7. Continuous titration with oxalate of lactate dehydrogenase plus NADH. A, 4 μM -Native enzyme + 20 μM -NADH; B, 4 μM -enzyme 95% inhibited by maleimide + 20 μM -NADH. Other conditions were as for Fig. 6. The signal change is an increase in fluorescence.

mission of the analysing filter.) A titration of the native enzyme with NADH in oxalate is depicted in Fig. 2(C). Titration of enzyme 95% inhibited with maleimide under these conditions (Fig. 3C)

Table 1. *Analysis of the titration curves of Figs. 2, 3, 6 and 7 for equilibrium binding constants and for the concentration of ligand-binding sites of lactate dehydrogenase*

The 'inhibited' enzyme was 95% inhibited with maleimide. Concentrations of binding sites are expressed in mol/144 000 g of protein.

Reaction	Enzyme	In buffer (67 mM-phosphate, pH 7.2)	In buffer containing 10 mM-oxamate	In buffer containing 10 mM-oxalate
Enzyme + NADH	Native	$K = 0.7 \mu\text{M}$ Sites = 3.5	$K = 0.03 \mu\text{M}$ Sites = 4.13	$K = 0.1 \mu\text{M}$ Sites = 4.3
	Inhibited	$K = 2 \mu\text{M}$ Sites = 4.1	— Sites = 0.15* and 4.0†	— Sites = 0.2*
Enzyme-NADH + oxamate	Native	$K = 8 \mu\text{M}$		
	Inhibited	—		
Enzyme-NADH + oxalate	Native	$K = 0.34 \text{ mM}$		
	Inhibited	$K = 0.43 \text{ mM}$		

* For the residual active sites in the preparation.

† Total sites.

shows an initial large enhancement of fluorescence, due to the residual 5% active sites, followed by a curve similar to that obtained in the absence of oxalate (Fig. 3A).

The formation of a complex between the enzyme, NAD^+ and pyruvate was detected by an absorption band at 325 nm (Fromm, 1961; Winer, 1963; Gutfreund, Cantwell, McMurray, Criddle & Hathaway, 1968). This complex is slowly formed with the native enzyme (Fig. 8) but not detectably with the maleimide-inhibited enzyme.

In the gel-filtration binding experiment shown in Fig. 9 the native enzyme was eluted from the column with a peak of radioactivity due to bound oxalate (0.885 mol of oxalate/mol of subunit), but there was, to within the 'noise' level, no detectable peak of radioactive oxalate eluted with the maleimide-inhibited enzyme. Although only 0.885 mol of oxalate/mol of subunit was eluted with the native enzyme when the concentration of free oxalate was 1.5 mM, this is equivalent to 1 mol of oxalate/mol of subunit when adjusted for the binding constant of oxalate. The trough in NADH concentration corresponded to 0.98 mol of NADH bound/mol of native-enzyme subunit and to 0.95 mol of NADH bound/mol of inhibited-enzyme subunits.

With the same gel-filtration technique no detectable peak of $[^{14}\text{C}]$ pyruvate was eluted with the protein when the concentration of free pyruvate was 3 mM. This indicates that the binding constant to the apoenzyme must be greater than 15 mM. This is in agreement with the result of Takenaka & Schwert (1956) obtained in ultracentrifugation experiments. In the presence of 1 mM- NAD^+ a peak of radioactivity equivalent to 1.0 ± 0.3 mol of pyruvate/mol of subunit was eluted with the protein. The column technique, when coupled to flow-

Fig. 8. Time-course for the increase in E_{325} of mixtures of lactate dehydrogenase, pyruvate and NAD^+ . The 1 cm cuvettes contained sodium pyruvate (30 mM) and NAD^+ (6 mM) in buffer in the presence of: A, 2.76 mg of native enzyme; B, 2.78 mg of enzyme 95% inhibited by maleimide. The temperature was 23°C.

detectors, is a sensitive method of detecting ligand binding to proteins and has been employed (J. J. Holbrook, unpublished work) to detect binding of 2-oxoglutarate to apo(glutamate dehydrogenase).

DISCUSSION

Interest in the role of the essential thiol group of lactate dehydrogenase depends on two findings. First, the essential thiol group occurs in the same dodecapeptide irrespective of species or type of the enzyme (except for two conservative mutations, valine-isoleucine and serine-threonine). Both the number of non-essential thiol groups and the

Fig. 9. Record of radioactivity and transmission of 2537 Å radiation during the gel filtration of 0.296 μ mol of lactate dehydrogenase subunits 95% inhibited by maleimide or 0.195 μ mol of native lactate dehydrogenase subunits in the presence of 0.15 mM-NADH and 1.5 mM-sodium [14 C]oxalate in buffer, pH 7.2. The rate-meter signal from 1.5 mM- 14 C]oxalate was 8 vertical chart divisions. The flow rate was 20 ml/h.

sequence of amino acids in which they occur vary widely with the nature (M or H) of the enzyme and the species (for a review see Pfeleiderer, Woenkhaus, Jeckel & Mella, 1970). Secondly, the catalytic activity of the enzyme is stoichiometrically destroyed during specific modification of the essential thiol group by *N*(*N*-acetylsulphamoyl)-phenylmaleimide (Holbrook & Pfeleiderer, 1965). No other thiol group of the enzyme need be considered essential for the mechanism since Fondy *et al.* (1965) were able to prepare fully active frog M_4 lactate dehydrogenase in which all except the essential thiol groups were alkylated, and then demonstrated stoichiometric inactivation during modification of this last remaining thiol group with *p*-hydroxymercuribenzoate. The non-essential thiol groups are important in maintaining the structure of the enzyme extracted from other species.

Equilibrium (Takenaka & Schwert, 1956; Schwert, Miller & Peanasky, 1967) and transient kinetic (Heck, McMurray & Gutfreund, 1968) studies show that there is a compulsory order of binding of substrate and nucleotide to lactate dehydrogenase and that some of the steps in the turnover are:

where E is a lactate dehydrogenase subunit. The absence of pyruvate binding to the apoenzyme is

confirmed by the results presented in this paper, and the gel-filtration binding studies confirm the formulation of the active and abortive ternary complexes as 1:1:1 adducts of subunit, nucleotide and substrate.

Three possible consequences of the modification of the essential thiol group of the enzyme are now considered: (1) modification causes a change in the state of aggregation of the tetrameric enzyme and/or a large change in the conformation of the protein; (2) the modified enzyme cannot bind nucleotides to form the binary complexes; (3) the binary complexes cannot bind substrate to form the ternary complexes.

The molecular weight of lactate dehydrogenase inhibited by *N*(*N*-acetylsulphamoyl)phenylmaleimide is the same as that of the native enzyme (Holbrook, 1966), and the sedimentation coefficient of the *S*-sulpho-enzyme is the same as that of the native enzyme. The optical-rotary-dispersion spectra of inhibited preparations are the same as those of the native enzyme (Jeckel, 1969), and therefore possibility (1) is excluded. In addition, the maleimide enzyme has also been crystallized.

Possibility (2) was excluded by Holbrook (1966).

The unaltered binding of NADH to preparations of the enzyme with modified essential thiol groups was

confirmed by Allison (1968) and may also be deduced from the crystallographic experiments of Adams *et al.* (1970). Fluorescence enhancements and wavelength shifts are sensitive indications of the environment of the bound NADH, and these for the maleimide-inhibited enzyme are quantitatively identical with those for the native enzyme. The affinity of the inhibited enzyme for NADH is slightly less than that of the native enzyme. The essential thiol group must, however, be close to the NADH-binding site, since modification of this group with *N*(*N*-acetylsulphamoyl)phenylmaleimide (11 Å long) does interfere with NADH binding. The apparent anomaly that NADH protects the essential thiol group from alkylation but that NADH binds to the alkylated enzyme can be explained by a simple model. This model places the essential thiol group in a deep pocket whose entrance is closed by nicotinamide when NADH is bound. The pocket is sufficiently deep that small modifying agents that have reacted with the essential thiol group do not sterically interfere with NADH binding.

The results presented in this paper support possibility (3). Pyruvate, oxamate and oxalate do not cause the spectral and fluorescence perturbations with the inhibited enzymes that are characteristic of the formation of ternary complexes with the native enzyme. It might be objected that the ternary complexes are formed with the inhibited enzyme, but that the spectral perturbations are absent. The gel-filtration experiment with enzyme-NADH-oxalate, which does not depend on spectral perturbation, shows that this is not so. There is as yet no method to measure the formation of an active ternary complex of lactate dehydrogenase (e.g. enzyme-NADH-pyruvate). However, enzyme-NADH-oxalate may be considered as an active ternary product complex of the apparently irreversible conversion of glyoxylate and NAD⁺ into oxalate and NADH that is catalysed very efficiently by lactate dehydrogenase (Duncan & Tipton, 1969; Warren, 1970). The enzyme-NADH-oxamate complex is isoelectronic with enzyme-NADH-pyruvate and the properties of the two should be similar. Oxamate is also a competitive inhibitor of pyruvate. The enzyme-NAD⁺-pyruvate complex is the abortive ternary complex associated with the characteristic reaction catalysed by lactate dehydrogenase. On the basis of these results it is suggested that the enzyme in which the essential thiol group has been modified minimally is inactive because substrate cannot bind to the binary enzyme-nucleotide complex. It is likely to be difficult to decide whether the lack of substrate binding is due to direct steric hindrance between the modifying agent and the substrate-binding site or to an essential small conformational change neces-

sary for substrate binding being prevented by the modification.

At first sight the observation that ternary complexes of alkylated lactate dehydrogenase and NAD⁺ with small molecules, cyanide (Gerlach, Pfeleiderer & Holbrook, 1966) and sulphite are formed, whereas ternary complexes involving larger C₂ and C₃ substrates do not form at all, suggests steric hindrance in the alkylated enzyme. The alternative conformation-change hypothesis envisages that a small conformation change, associated with substrate binding, is prevented by the alkylation. The large changes in the quantum yields of NADH fluorescence on binding of substrate to the enzyme-NADH complex certainly indicate that the local environment of NADH is altered.

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