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Inactivation and Modification of Lactate Oxidase with Fluorodinitrobenzene

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1. Dinitrophenylation of 2±0.2mol of residues/mol of enzyme-bound FMN resulted in the complete inactivation of the flavoenzyme L-lactate oxidase. 2. Hydrolysates of the inactivated enzyme contained 1mol each of N^{im}-Dnp-histidine (abbreviation: Dnp-, 2,4-dinitrophenyl-; N^{im} indicates that either of the N atoms in the imidazole ring is substituted) and ε-Dnp-lysine/mol of FMN. 3. Competitive inhibitors decreased the extent of inactivation to a 10% loss of activity, and dinitrophenylation was decreased from 2 to approx. 0.5mol/mol of FMN. Only N^{im}-Dnp-histidine was detected in the hydrolysates. 4. Although the dinitrophenylated enzyme did not possess enzyme activity, L-lactate reduced approx. 50% of the enzyme-bound flavin slowly (0.6min^{-1}), and approx. 50% of the flavin in the modified enzyme formed a complex with bisulphite. 6. The modified enzyme (2mol of Dnp/mol of FMN) was unable to bind substrate analogues and competitive inhibitors.

Numerous studies on the reaction mechanisms of the flavoprotein oxidases D-amino acid oxidase (EC 1.4.99.1) and L-lactate oxidase (lactate 2-mono-oxygenase, EC 1.13.12.4) have provided strong evidence for a reaction mechanism that involves a heterolytic C–H bond cleavage by abstraction of a proton and transfer of two electrons from the transient carbanion intermediate through the formation of a covalent intermediate (Walsh *et al.*, 1971, 1973; Porter *et al.*, 1972; Averill *et al.*, 1975; Hersh & Schuman-Jorns, 1975; Ghisla & Massey, 1975*a*,*b*; Ghisla *et al.*, 1976: Schonbrunn *et al.*, 1976). These studies have emphasized the need for more detailed information on the amino acid residues within the active sites of these enzymes. Direct information is limited, but the binding of substrates and competitive inhibitors indicated the presence of positively charged active-site residues in both enzymes (Massey & Ganther, 1965; Lockridge *et al.*, 1972; Ghisla & Massey, 1975*a*). Furthermore, it has been suggested that a positively charged residue near the flavin group in oxidases contributes to the stabilization of the red anionic semiquinone derivative, and the addition of bisulphite to position N-5 of the oxidized flavin and the reactivity with O_2 (Massey *et al.*, 1969). Treatment of D-amino acid oxidase in the presence of substrate with $NaBH_4$ resulted in enzyme inactivation and the covalent modification of a lysine residue (Hellerman & Coffey, 1967). It was, however, subsequently shown that the inactivation could be accounted for by the formation of 3,4-dihydro-FAD (Muller *et al.*, 1969).

We have presented evidence for an essential histidine residue in lactate oxidase (Choong *et al.*, 1977). Modification of two histidine residues in lactate oxidase with diethyl pyrocarbonate resulted in complete enzyme inactivation. Ethoxycarbonylation did not prevent the binding of substrate or competitive inhibitors, but the substrate-directed reduction of the flavin was abolished. It was therefore suggested that a histidine residue could participate in the proton abstraction step in catalysis.

The earlier work has been extended in a study of the inactivation of lactate oxidase by 1-fluoro-2,4-dinitrobenzene as described in the present paper.

Abbreviations used: Dnp-, 2,4-dinitrophenyl-; Mes, 4-morpholine-ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Materials and Methods

The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: N^{α}-acetyl-L-histidine, 1-fluoro-2,4-dinitrobenzene, ε-Dnp-lysine, 2,6-dichlorophenol-indophenol, phenazine methosulphate and cytochrome *c*. 1-Fluoro-2,4-dinitro[U-^{14}C]benzene (sp. radioactivity 17mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of analytical grade.

Buffers

Buffers were prepared from the Tables compiled by Dawson & Elliott (1959), except those listed

below: Mes and Hepes (Sigma) were adjusted to the final pH with 0.1M-NaOH and then diluted to the final concentration.

Spectrophotometry

Absorption spectra were recorded with a Cary 118 spectrophotometer, and absorbance measurements at fixed wavelength were carried out with a Unicam SP. 800 spectrophotometer fitted with a Gilford photomultiplier. Anaerobic studies were carried out as described previously (Choong *et al.*, 1977).

Enzyme

L-Lactate oxidase from *Mycobacterium smegmatis* was prepared as described by Sullivan *et al.* (1977). The assay for L-lactate oxidase activity, protein determinations, units of enzyme activity, specific activity and the measurement of enzyme-bound FMN concentration were as described previously (Choong *et al.*, 1975; Sullivan *et al.*, 1977).

Lactate-acceptor reductase assays

Assays were carried out under anaerobic conditions. Each assay contained 50μmol of sodium phosphate buffer, pH 7.0, 100μmol of L-lactate, pH 7.0, 50–100μg of enzyme and one of the following acceptors: 2,6-dichlorophenol-indophenol (0.1μmol), 2,6-dichlorophenol-indophenol (0.1μmol) plus phenazine methosulphate (0.1μmol), $K_3Fe(CN)_6$ (0.45μmol) or cytochrome *c* (0.05μmol). The total volume was 3.0ml and L-lactate was added last from the side arm. Absorbance was measured for 5min at the following wavelengths: 2,6-dichlorophenol-indophenol (600nm), ferricyanide (410nm) and cytochrome *c* (550nm).

Dinitrophenylation

1-Fluoro-2,4-dinitrobenzene was diluted to the desired concentration (approx. 0.1M in ethanol) and assayed by the method of Murdock *et al.* (1966), by using 14.8×10^3 litre·mol^{-1}·cm^{-1} as the molar absorption coefficient for the Dnp group at 360nm. 1-Fluoro-2,4-dinitro[U-^{14}C]benzene (sp. radioactivity 17mCi/mmol) was diluted with the unlabelled compound to a specific radioactivity of 0.15 or 0.82μCi/μmol. The concentration was determined as described above and the radioactivity measured in 10ml of toluene/Triton X-100 scintillant (Lockridge *et al.*, 1972).

Lactate oxidase (1–4mg/ml in 50mM-Mes/NaOH buffer, pH 6.0) was treated with specified concentrations of 1-fluoro-2,4-dinitrobenzene (1–2mM) at 25°C for the times indicated (see Fig. 1). The concentration of ethanol never exceeded 2% (v/v). Incubations were protected from light. Controls used throughout this work consisted of incubations of the enzyme with ethanol at the same concentration used in the 1-fluoro-2,4-dinitrobenzene incubations. After modification, enzyme solutions were either diluted 20-fold and assayed immediately for activity or dialysed in the dark for 2–3 days with several changes of the dialysis buffer. Enzyme activity was, within experimental error, the same before and after dialysis. Dialysis buffer was either 20mM-Mes/NaOH, pH 6.0, or 20mM-sodium phosphate, pH 6.0, as indicated.

Measurement of radioactivity

Radioactivity and ^{14}C incorporation were measured as described by Choong *et al.* (1977).

Protein hydrolysis

Lactate oxidase (2–5mg of protein) modified with 1-fluoro-2,4-dinitro[U-^{14}C]benzene was treated with ice-cold trichloroacetic acid (final concentration 10%, w/v) and then centrifuged for 5min at 10000*g*. The pellet was washed five times with trichloroacetic acid by this procedure and then carefully transferred to a hydrolysis tube as a pellet. HCl (6M; 0.4ml) was added and the tubes were sealed *in vacuo* after repeated evacuation and flushing with O_2-free N_2. The samples were hydrolysed at 110°C for 20–24h, evaporated to dryness and dissolved in water.

Electrophoresis and t.l.c.

Electrophoresis was carried out on Whatman 3MM paper (12.5cm × 80cm) with a flat-bed Shandon high-voltage apparatus. The solvent was 1.5M-formic acid, pH 1.9 (Rosen & Rosen, 1966), and the Dnp-amino acids were separated with an applied voltage of 3000V for 1h. Silica-gel t.l.c. plates (0.5cm thick) were used for chromatography. The solvent was chloroform/methanol (3:1, v/v) and the development time was 2.5h.

Synthesis and characterization of N^{im}-Dnp-L-histidine

N^{im}-Dnp-L-histidine (where N^{im} indicates that either of the N atoms in the imidazole ring is substituted) was synthesized from N^{α}-acetyl-L-histidine by the method of Sanger (1945). Crystallization was not successful. The reaction mixture (yellow–brown) was extracted with ether. The aqueous phase was judged to be a 98%-pure solution of N^{im}-Dnp-histidine by the criteria listed below. T.l.c. and high-voltage paper electrophoresis in the systems described above indicated the presence

of only one Dnp derivative, with the expected mobility for N^{im}-Dnp-histidine.

The preparation and the compound separated by chromatography reacted with ninhydrin reagent (Elliott, 1959), but showed no reaction with diazotized sulphonic acid (Elliott, 1959). When the developed chromatography plates or electrophoretograms were exposed to room light for 3–4 days the spot darkened, as described by Margoliash (1955). Finally, the Dnp derivative was stable to incubation in 6M-HCl at 110°C for 24h (Porter, 1950), but was degraded by exposure to 20% (v/v) NH_3 at 110°C for 2h (Lowther, 1951).

Results

Kinetic analysis of the inactivation by 1-fluoro-2,4-dinitrobenzene

Fig. 1 shows that the inactivation of lactate oxidase with various concentrations of 1-fluoro-2,4-dinitrobenzene (4–40-fold molar excess with respect to enzyme-bound FMN) was pseudo-first-order over the entire concentration range of the reagent. A plot of $\log(1/t_{\frac{1}{2}})$ (the reciprocal of the half-time of inactivation) against log [1-fluoro-2,4-dinitrobenzene] (Levy *et al.*, 1963) had a slope of 2 (Fig. 1 insert), which indicated that the reaction of 2mol of 1-fluoro-2,4-dinitrobenzene/mol of FMN was responsible for the irreversible inactivation. A possible interpretation for this analysis is presented in the Discussion.

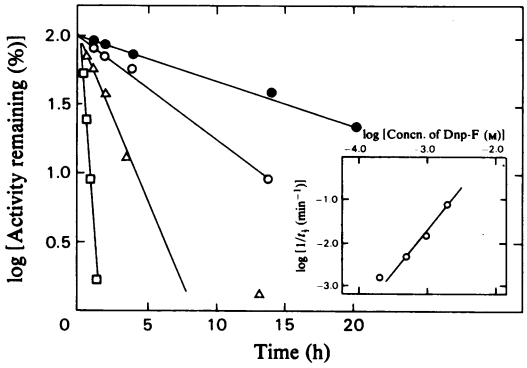

Fig. 1. *Inactivation of lactate oxidase by 1-fluoro-2,4-dinitrobenzene*

Each incubation contained, in a total volume of 0.15ml, 50mM-Mes/NaOH buffer, pH 6.0, lactate oxidase (50μM-enzyme-bound FMN) and 1-fluoro-2,4-dinitrobenzene (Dnp-F) as indicated: ●, 0.2mM; ○, 0.5mM; △, 1.0mM; □, 2.0mM. Samples were incubated in the dark at 25°C and at the times indicated portions (5 or 10μl) were diluted 21-fold in cold water and assayed immediately for enzyme activity. The insert shows the log of the reciprocal half-time of inactivation ($t_{\frac{1}{2}}$) plotted against log [Dnp-F].

Rates of inactivation with 1mM-1-fluoro-2,4-dinitrobenzene were determined over the range pH 5.4–8.0 in 50mM-Mes/NaOH buffer (pH 5.4–7.1) or 50mM-Hepes/NaOH (pH 7.5–8.0). The pseudo-first-order rate constant increased only 2-fold between pH 5.4 and 6.5 (0.004 to 0.008min^{-1}), but increased 10-fold between pH 6.5 and 8.0, to a final value of 0.075min^{-1}.

Stoicheiometry of the inactivation

To confirm the stoicheiometry of 2mol of 1-fluoro-2,4-dinitrobenzene/mol of FMN indicated by the kinetic analysis an experiment was carried out in which both the enzyme inactivation and the incorporation from 1-fluoro-2,4-dinitro[U-^{14}C]benzene were measured with respect to time. As shown in Fig. 2(*a*), there was close agreement between these two progress curves. Complete inactivation was obtained after 4h and the extent of ^{14}C incorporation was the same between 4 and 6h. Further incorporation was, however, detected in samples measured after 6h. A plot of percentage of enzyme activity remaining against mol of [^{14}C]Dnp group incorporated/mol of FMN (Fig. 2*b*) was linear, and intercepted the axis (zero activity) at 1.95mol of Dnp group incorporated/mol of FMN. It was established in this experiment that all of the radioactivity incorporated was with the apoprotein and not the FMN.

Effect of substrate and competitive inhibitors

The competitive inhibitors phosphate, nitrate and α-hydroxymalonate (Lockridge *et al.*, 1972) decreased the rate of inactivation. As shown in Fig. 3, the pseudo-first-order rate constant with 1.65mM-1-fluoro-2,4-dinitrobenzene was decreased from 0.0135min^{-1} in the absence of ligands to 0.002min^{-1} and 0.005min^{-1} by nitrate and α-hydroxymalonate respectively. Under these conditions the extent of incorporation after 3h with the unprotected enzyme varied from 1.8 to 2.3mol of Dnp group/mol of FMN for zero residual activity. In the presence of nitrate or α-hydroxymalonate, residual activity after 3h was 90 and 95% respectively of the original value; incorporation of [^{14}C]Dnp varied in different experiments from 0.45 to 0.70mol/mol of FMN. In the course of the present work it was also established that 2-methyl-lactate was a competitive inhibitor of lactate oxidase. The K_i in the standard assay system (Choong *et al.*, 1975) was 4mM. This substrate analogue also protected the enzyme against 1-fluoro-2,4-dinitrobenzene and decreased the incorporation of Dnp to approx. 0.5mol of Dnp group/mol of FMN.

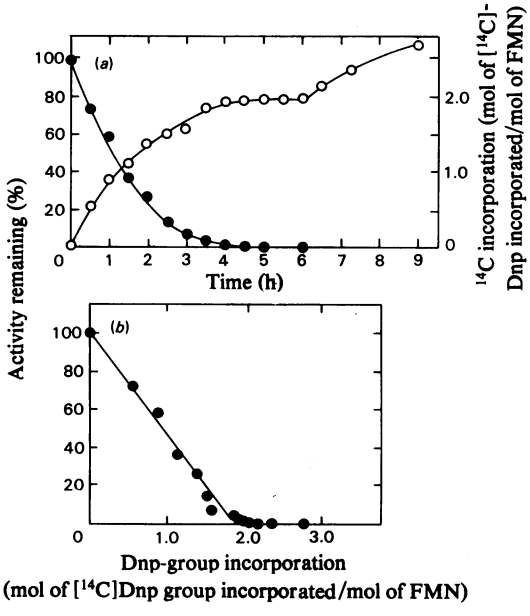

Fig. 2. *Stoicheiometry of the 1-fluoro-2,4-dinitrobenzene modification*

Lactate oxidase (48 μM-FMN) in 50mM-Mes/NaOH buffer, pH 6.0, was incubated with 1-fluoro-2,4-dinitro[U-^{14}C]benzene (1.65mM, and 2.04×10^5 c.p.m./μmol). The total volume was 0.8ml. At the times indicated 5 μl and 50 μl samples were taken for enzyme assays and measurement of the ^{14}C incorporation. Radioactivity was determined as described in the Materials and Methods section. The activity of the original enzyme was 1150 units/mg of protein (where 1 unit of activity is defined as the amount of enzyme required to convert 1 μmol of substrate/min). (*a*) Plots of the percentage of enzyme activity remaining (●) and ^{14}C incorporation (mol of Dnp group/mol of FMN; ○) against time; (*b*) plot of percentage of activity remaining against [^{14}C]Dnp-group incorporated (mol of Dnp group/mol of FMN).

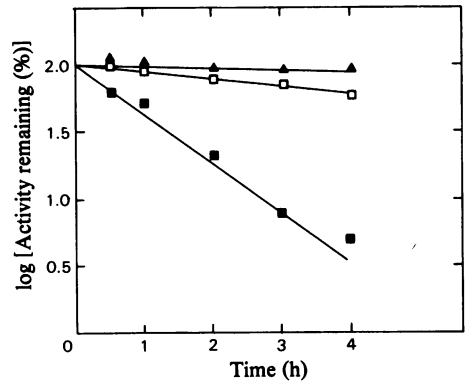

Fig. 3. *Effect of competitive inhibitors on inactivation by 1-fluoro-2,4-dinitrobenzene and Dnp incorporation*

(*a*) Solutions of lactate oxidase (48 μM-FMN) in 50mM-Mes/NaOH buffer, pH 6.0, were incubated at 25°C for 5min under the following conditions: (■), no additions; (□), with 38mM-$NaNO_3$; (▲), with 38mM-α-hydroxymalonate. At zero time 1-fluoro-2,4-dinitro[U-^{14}C]benzene was added to give a final concentration of 1.5mM and 2.04×10^5 c.p.m/μmol. The final volume was 0.62ml. At the times indicated 5 μl samples were diluted and assayed immediately for enzyme activity. The specific rates of inactivation were: 0.0135min^{-1} (■); 0.0022min^{-1} (□); 0.0005min^{-1}. (▲).

Identification of the Dnp-amino acid derivatives in the modified enzyme

Lactate oxidase was inactivated by treatment with 1-fluoro-2,4-dinitro[U^{14}C]benzene, and the protein was hydrolysed as described in the Materials and Methods section. Only two Dnp derivatives were detected in the hydrolysate by t.l.c. The derivatives co-chromatographed with N^{im}-Dnp-histidine and ε-Dnp-lysine (Fig. 4*a*). Yields calculated from the radioactivity were 1.05 and 0.95mol of Dnp-amino acid/mol of FMN respectively. High-voltage electrophoresis gave the same result: only two Dnp derivatives were detected and the yields were as given above. This experiment was also carried out with either 2-methyl-lactate or α-hydroxymalonate included in the incubation, both giving a loss of enzyme activity of approx. 5%. Incorporation of the [^{14}C]Dnp group was decreased from 1.8 to 0.5mol of Dnp/mol of FMN. All the radioactivity was located in the N^{im}-Dnp-histidine, and negligible ε-Dnp-lysine was present in these hydrolysates. Fig. 4(*b*) shows the analysis of the incubation with 2-methyl-lactate: that with α-hydroxymalonate gave essentially the same result.

Properties of the Dnp-enzyme

Modification of the enzyme with 1-fluoro-2,4-dinitrobenzene did not alter the flavin, as judged by the spectrum in the region 430–550nm and the observation mentioned above that the radioactivity from 1-fluoro-2,4-dinitro[U-^{14}C]benzene was located excusively with the apoprotein. The increased absorption from 300 to 430nm was attributed to the Dnp residues (Fig. 5).

Dinitrophenylation did not affect the structure of lactate oxidase, as judged by discontinuous gel electrophoresis: only one band, with the same relative mobility as the native enzyme (Choong *et al.*, 1975), was present in the gels.

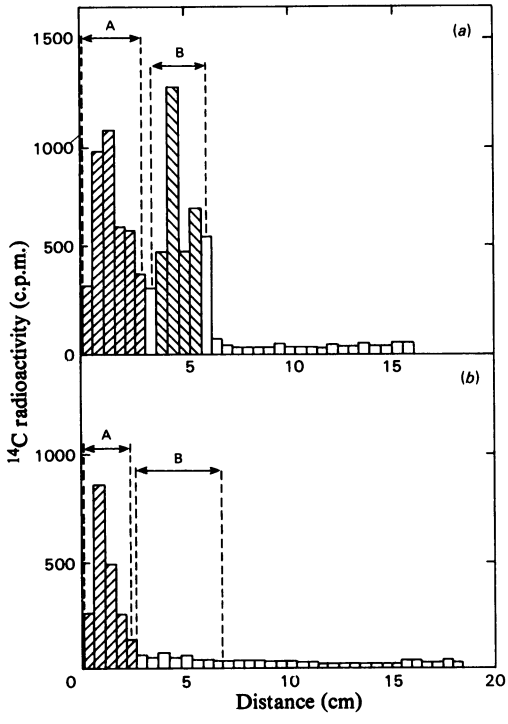

Fig. 4. *Separation and identification of the Dnp-amino acids*

(*a*) Lactate oxidase (4.1 mg/ml) in 50 mM-Mes/NaOH buffer, pH 6.0, was treated with 1 mM-1-fluoro-2,4-dinitro[U-^{14}C]benzene (1.95×10^6 c.p.m./μmol) for 10h at 25°C in the dark. The total volume was 4.28 ml. Apoprotein was recovered and hydrolysed as described in the Materials and Methods section. A portion of the hydrolysate, containing the equivalent of 0.16 mg of original protein, was analysed by t.l.c. as described in the Materials and Methods section. Standard N^{im}-Dnp-histidine (A) and ε-Dnp-lysine (B) chromatographed as indicated. Radioactivity was located by scraping 0.5 cm sections of the silica-gel layer into vials containing scintillant. (*b*) Conditions were as described above except that 46 mM-2-methyl-lactate was added to the original incubation 10 min before 1-fluoro-2,4-dinitrobenzene.

Although the modified enzyme exhibited no activity with molecular O_2 as the electron acceptor, experiments were carried out to check for lactate-acceptor reductase activity. No activity was detected with ferricyanide, cytochrome *c*, 2,6-dichlorophenol-indophenol or phenazine methosulphate plus 2,6-dichlorophenol-indophenol.

Somewhat unexpectedly, modified lactate oxidase (2.1 mol of Dnp/mol of FMN) was reduced, albeit slowly, by L-lactate. The reduction of the enzyme-bound FMN followed pseudo-first-order kinetics: a rate constant of 0.6 min^{-1} was obtained by analysing the change in A_{450}. It was established from several experiments that 45–55% of the total FMN was reduced (Fig. 5). The enzyme was rapidly reoxidized on the re-admission of air. Complete reduction of the flavin was observed with dithionite.

Modified enzyme also formed a complex with bisulphite (Fig. 6). The complex had an absorption maximum around 320 nm and the spectra of the complex and the oxidized enzyme were isosbestic at 338 nm. These features agree with the previous description of the flavoprotein–bisulphite complex (Massey *et al.*, 1969). Although the spectrum of native oxidized lactate oxidase was completely bleached by a 2-fold molar excess of bisulphite, only 50% of the flavin in the modified enzyme was complexed by the addition of a 25-fold molar excess of bisulphite (Fig. 6). A dissociation constant for the bisulphite-modified enzyme reaction was calculated on the basis that 50% of the total flavin underwent reaction. The value obtained, 7 μM, is close to the previously reported value of 1.2 μM at pH 6.0.

The modified enzyme with 2 mol of Dnp group/mol of FMN was unable to bind the competitive inhibitors oxalate (Ghisla & Massey, 1975*b*) and α-hydroxymalonate (Lockridge *et al.*, 1972), as judged by the failure of the compounds to elicit any perturbations to the flavin spectrum, as is characteristic with the native enzyme. Furthermore, the modified enzyme was completely insensitive to oxalate in the presence of light. When the native enzyme was irradiated in the presence of oxalate the flavin underwent a rapid photoreduction, as described by Ghisla & Massey (1975*b*).

Discussion

The kinetic analysis of inactivation by 1-fluoro-2,4-dinitrobenzene (Fig. 1) and the stoicheiometry of [^{14}C]Dnp incorporation (Fig. 2) indicated that inactivation resulted from the modification of 2 residues/molecule of FMN. The kinetic analysis used in this work has been used frequently to estimate the number of moles of the modifier that react with an enzyme during inactivation (Scrutton & Utter, 1965; Keech & Farrant, 1968; Choong *et al.*, 1977). Inactivation of lactate oxidase by 1-fluoro-2,4-dinitrobenzene could involve two consecutive reactions; $E+I \rightarrow EI$, and $EI+I \rightarrow EI_2$, where E is the native enzyme, I is the modifier and EI and EI_2 are modified forms of the enzyme. This would account for the apparent third-order kinetics (Fig. 1) and the linear plot of enzyme activity against incorporation (Fig. 2*b*) provided that (i) EI retained enzyme activity and (ii) the second reaction was fast compared with the first reaction.

Protein hydrolysates of the labelled protein contained equimolar amounts of N^{im}-Dnp-histidine

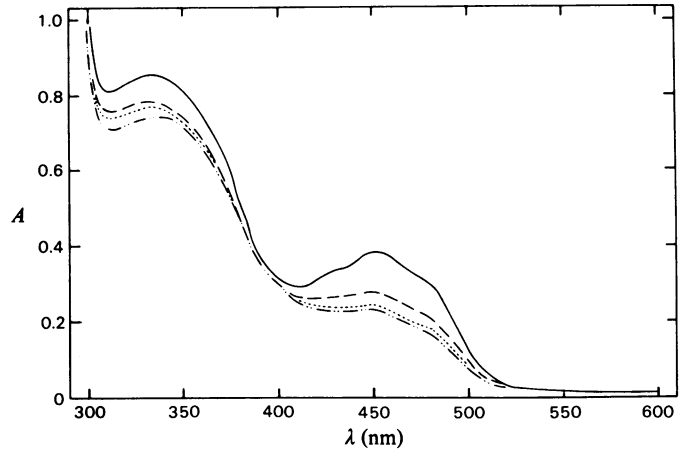

Fig. 5. *Reduction of modified lactate oxidase with* L-*lactate*

Modified enzyme, prepared as described in the legend to Fig. 4(*a*), was dialysed extensively against 50mM-sodium phosphate buffer, pH6.0. The protein concentration was 1.8mg/ml. The spectra, recorded under anaerobic conditions, are: ——, modified enzyme before the addition of L-lactate; — —, 15min, · · · ·, 1h; —·—, 2 and 4h after the addition of 0.1ml of 0.25M-L-lactate from the side arm of the anaerobic cuvette. Spectra were corrected for dilution and the initial volume was 1.1ml.

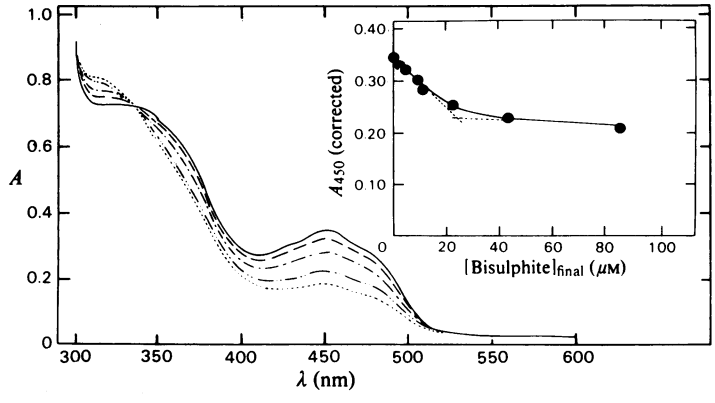

Fig. 6. *Titration of the modified enzyme with bisulphite*

The modified enzyme was prepared as described in the legend of Fig. 4(*a*) and dialysed extensively against 50mM-sodium phosphate buffer, pH6.0. The enzyme solution used (0.9ml) was 42.5μM with respect to enzyme-bound FMN. Portions (5 or 10μl) of freshly prepared sodium bisulphite in the same buffer were added to the cuvette as indicated. ——, Original spectrum. Spectra after the addition of $NaHSO_3$ to the following final concentrations: — — —, 4.4μM; —·—, 11μM; —· · —, 43.3μM; · · · ·, 171μM and 1.03mM. The spectra have been corrected for dilution. The insert shows the plot of A_{450}, corrected for dilution, against $NaHSO_3$ concentration, which was used to determine the dissociation constant. A_{450} values at concentrations up to 1.03mM, not shown in the plot, were the same as the value at 85μM.

and ε-Dnp-lysine. The total incorporation varied within the range 1.8–2.3mol of Dnp/mol of FMN for complete inactivation. It is possible that the second onset of incorporation, which followed complete inactivation (Fig. 2*a*), reflected secondary changes in the protein structure. Further studies are required to establish the basis for the second onset of incorporation, but the gross structure of the inactivated enzyme was unaffected as judged by gel electrophoresis.

Almost complete protection against 1-fluoro-2,4-dinitrobenzene (over 90% of activity remaining) was obtained with added competitive inhibitors nitrate, α-hydroxymalonate and 2-methyl-lactate. Incorporation in the presence of inhibitors decreased from 2.0 to 0.45–0.7mol of Dnp group/mol of FMN. The hydrolysates did not contain ε-Dnp-lysine and all of the label was with the N^{im}-Dnp-histidine (Fig. 4*b*). This suggests that the lysine residue is essential for catalytic activity, but that the histidine

residue is not an essential residue. In a study on the modification of lactate oxidase with diethyl pyrocarbonate (Choong *et al.*, 1977) it was shown that the enzyme contains 2 reactive histidine residues/FMN molecule and that one of these was essential for activity. Substrate and competitive inhibitors partially protected both histidine residues against diethyl pyrocarbonate. It is possible that 1-fluoro-2,4-dinitrobenzene reacts with one of these residues, but further studies would be required to clearly establish this proposal.

The essential lysine residue, positively charged, may be involved in binding substrate and competitive-inhibitor anions to the active site. Binding of anions to the active site of the native enzyme results in significant perturbations to the flavin spectrum (Lockridge *et al.*, 1972; Ghisla & Massey, 1975*a*,*b*). Oxalate and α-hydroxymalonate did not bind to the dinitrophenylated enzyme, as judged by the failure of these anions to elicit any perturbation of the spectrum. Also the modified enzyme did not undergo photoreduction with oxalate. Ghisla & Massey (1975*b*) have shown that photoreduction is preceded by binding of oxalate to the active site.

Two of the most intrigu pingroperties of the modified enzyme were the slow reduction of 50% of the enzyme-bound FMN by lactate and the binding of bisulphite with 50% of the total flavin. The rate constant for reduction, $0.6 min^{-1}$, is much smaller than any of the rate constants of the steps in the reduction of the native enzyme (Lockridge *et al.*, 1972). This reaction could be accounted for by a random rather than the directed alignment of the substrate. It is more difficult to account for the extent of flavin reduction and bisulphite binding. In both cases the possibility that insufficient reagent was added to the system was discounted by repeated experiments. The amount of L-lactate used represented a 60-fold molar excess, and the dissociation constant for the bisulphite binding to half of the total flavin was, within experimental error, the same as the previously reported value for the native enzyme (Massey *et al.*, 1969). These reactions suggest that the modified enzyme contains two populations of catalytic sites. The two types of catalytic sites could have been produced directly by the dinitrophenylation, or they could have arisen as a result of a secondary change in protein structure. In either case the modification of one-half of the active sites with respect to slow substrate reduction and bisulphite binding is of interest, because we have shown that native lactate oxidase contains eight subunits (Sullivan *et al.*, 1977). It should also be noted that Muller & Massey (1969) proposed that a positively charged residue at the active site of flavoprotein oxidases was responsible for the primary binding of bisulphite and the facilitated nucleophilic attack on position N-5 of the flavin. Either a histidine or a lysine residue could fulfil this role.

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