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Modification of Lactate Oxidase with Diethyl Pyrocarbonate

EVIDENCE FOR AN ACTIVE-SITE HISTIDINE RESIDUE

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1. Diethyl pyrocarbonate inactivated L-lactate oxidase from *Mycobacterium smegmatis*. 2. Two histidine residues underwent ethoxycarbonylation when the enzyme was treated with sufficient reagent to abolish more than 90% of the enzyme activity, but analyses of the inactivation showed that the modification of one histidine residue was sufficient to cause the loss of enzyme activity. The rates of enzyme inactivation and histidine modification were the same. 3. Substrate and competitive inhibitors decreased the maximum extent of inactivation to a 50% loss of enzyme activity and modification was decreased from 1.9 to 0.75–1.2 histidine residues modified/molecule of FMN. 4. Treatment of the enzyme with diethyl [^{14}C]pyrocarbonate (labelled in the carbonyl groups) confirmed that only histidine residues were modified under the conditions used and that deacylation of the ethoxycarbonylhistidine residues by hydroxylamine was concomitant with the removal of the ^{14}C label and the re-activation of the enzyme. 5. No evidence was found for modification of tryptophan, tyrosine or cysteine residues, and no difference was detected between the conformation and subunit structure of the modified and native enzyme. 6. Modification of the enzyme with diethyl pyrocarbonate did not alter the following properties: the binding of competitive inhibitors, bisulphite and substrate or the chemical reduction of the flavin group to the semiquinone or fully reduced states. The normal reduction of the flavin by lactate was, however, abolished.

Recent studies on the flavoprotein oxidases D-amino acid oxidase (EC 1.4.99.1) and lactate oxidase (EC 1.13.12.4) suggest that catalysis may involve abstraction of the hydrogen from the α-carbon atom as a proton and electron transfer, in a flavin–carbanion complex, via N-5 of the isoalloxazine ring. This mechanism is supported by: experiments with β-chloro-substituted substrates (Walsh *et al.*, 1971, 1973*a*, *b*), studies with the oxidases reconstituted with 5-deaza-flavin nucleotides (Hersh & Schuman-Jorns, 1975; Averill *et al.*, 1975; Fisher *et al.*, 1976) and the mechanism of inactivation of lactate oxidase (lactate 2-mono-oxygenase, EC 1.13.12.4) by the substrate 2-hydroxybut-3-ynoate (Ghisla *et al.*, 1976; Schonbrunn *et al.*, 1976).

Although it was postulated that proton abstraction requires a basic residue in the active site (Walsh *et al.*, 1971, 1973*a*; Hersh & Schuman-Jorns, 1975), there is limited information on the active-site residues in these enzymes. The presence of positively charged residues at or near the active site of both enzymes is indicated by anion binding (Massey & Ganther, 1965; Lockridge *et al.*, 1972; Ghisla & Massey, 1975). Hellerman & Coffey (1967) have shown the presence of a reactive lysine residue at or near the active site of D-amino acid oxidase.

In the present paper we describe studies on the inactivation of lactate oxidase by diethyl pyrocarbonate, which results in the specific modification of 1–2 histidine residues. Diethyl pyrocarbonate has been used widely as a histidine-acylating reagent, e.g. for ribonuclease and chymotrypsin (Melchior & Fahrney, 1970), actin (Hegyi *et al.*, 1974), thermolysin (Burstein *et al.*, 1974), octopine dehydrogenase (Huc *et al.*, 1971), malate dehydrogenase (Holbrook & Ingram, 1973) and pyruvate kinase (Dann & Britton, 1974). Several flavoproteins, including β-cyclopiazonate oxidocyclase (Steenkamp *et al.*, 1974), arginine 2-mono-oxygenase (EC 1.13.12.1) and D- and L-amino acid oxidases (Thomé-Beau *et al.*, 1971), were inactivated by diethyl pyrocarbonate. Acylation of 1–2 histidine residues/molecule of FAD in arginine 2-mono-oxygenase and 3 histidine residues/molecule of FAD in β-cyclopiazonate oxidocyclase resulted in complete inactivation, but the amino acid oxidases contain many reactive histidine residues.

Materials and Methods

Diethyl pyrocarbonate (ethoxyformic anhydride) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Diethyl [^{14}C]pyrocarbonate (labelled in the carbonyl residue; 9.46 mCi/mmol) was a gift from the Institute of Isotopes of the Hungarian Academy of Science, Budapest, Hungary. Guanidine hydro-

chloride, dithioerythritol, 5,5′-dithiobis-(2-nitrobenzoic acid) and the N^{α}-acetyl derivatives of L-histidine, cysteine, lysine and tyrosine were also from Sigma. All other chemicals were of analytical grade.

Buffers

Buffers were prepared from the tables compiled by Dawson & Elliott (1959) except those listed below. Mes (4-morpholine-ethanesulphonic acid; Sigma) was adjusted to the final pH with 0.1M-NaOH and then diluted to the final concentration. Disc-gel-electrophoresis buffer was as described by Davis (1964).

Spectrophotometry

Absorption spectra were recorded with a Cary 118 spectrophotometer, and absorption measurements at fixed wavelengths were carried out with a Zeiss PMQII spectrophotometer or a Unicam SP.800 spectrophotometer.

Fluorimetry

A Perkin–Elmer model MPF-3L spectrophotofluorimeter was used in fluorescence measurements.

Ultracentrifuge studies

Sedimentation-velocity analyses were made with a Spinco model E ultracentrifuge by using a standard 12.0mm cell with quartz windows. Sedimentation coefficients were calculated by the method of Schachman (1957).

Enzyme

L-Lactate oxidase from *Mycobacterium smegmatis* was prepared as described by Sullivan *et al.* (1977), and stored in 1.0M-sodium acetate/acetic acid buffer, pH5.7, at 4°C in the dark. The assays 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).

Ethoxycarbonylation studies

Diethyl pyrocarbonate was diluted to the desired concentration (approx. 0.5M in ethanol), and stored at −10°C. Stock solutions were used for up to 1 week; the half-life of diethyl pyrocarbonate under these conditions was 8 days as detemined in the system described below.

A molar absorption coefficient of 3.9×10^{3} litre·mol^{-1}·cm^{-1} for N^{α}-ethoxycarbonylhistidine derivatives at 242nm was used throughout this work. This value was established by treating various concentrations of N^{α}-acetylhistidine (0–0.3mM) in 20mM-Mes/NaOH buffer, pH6.0, with 5mM-diethyl pyrocarbonate (final concentration in the assay). Reactions were followed to completion at 242nm. The concentrations of stock solutions of diethyl pyrocarbonate were determined by adding portions containing approx. 0.05mol of diethyl pyrocarbonate to 10mM-N^{α}-acetylhistidine in 20mM-Mes/NaOH, pH6.0.

Lactate oxidase, 1–3mg/ml in 20mM-Mes/NaOH buffer, pH6.0, was treated with portions of a stock solution of diethyl pyrocarbonate and incubated at 25°C for the times indicated. The concentration of ethanol never exceeded 2% (v/v). Controls used throughout this work consist of incubations of the enzyme with ethanol at the same concentration as in incubations with diethyl pyrocarbonate. Enzyme modification was stopped, almost immediately as judged by enzyme activity, either by diluting the reaction mixture 10-fold with buffer or by the addition of imidazole/HCl, pH7.0, to a final concentration of 10–20mM.

Deacylation of the modified enzyme was carried out by diluting samples with an equal volume of 1.0M-hydroxylamine in 0.1M-Tris/HCl buffer, pH7.0, containing 0.5mM-dithioerythritol and incubation at 4°C for 1h. Hydroxylamine was omitted from controls.

Determination of amino acids and amino acid derivatives

Thiol groups in the native and modified enzyme were determined with 5,5′-dithiobis-(2-nitrobenzoic acid) by the method of Ellman (1959). Sodium dodecyl sulphate was added to give a final concentration of 0.1% (w/v). Tyrosine and tryptophan were determined by the spectrophotometric method of Bredderman (1974), by using 340μg samples of the enzyme in 0.8ml of 6.5M-guanidine hydrochloride, pH6.5.

Amino acid analysis was performed in a JOEL JLC-6AH analyser. Samples (0.4ml) containing approx. 10nmol of each amino acid in 0.2M-sodium citrate buffer, pH2.2, were applied to the short column (8mm×95mm) and eluted at 66°C with 0.35M-sodium citrate, pH5.28.

Measurement of radioactivity

Radioactivity was measured with a Packard Tri-Carb liquid-scintillation model 3003 spectrometer. Protein solutions (0.1–0.4ml) were added to 10ml of Bray's (1960) scintillant. Enzyme samples denatured either by incubation at 100°C for 3min or by ethanol (90%, v/v, final concn.) were collected on membranes (0.45μm pore size; Millipore Corp., Bedford, MA, U.S.A.), washed with water or 90% (v/v) ethanol and counted for radioactivity in 10ml of toluene/Triton

X-100 scintillant (Lockridge *et al.*, 1972). The efficiency was 60%.

Results

Kinetics of inactivation

Plots of logarithm of enzyme activity against time for diethyl pyrocarbonate inactivation at various concentrations of modifier were linear down to 30% of the initial enzyme activity (Fig. 1). This indicated that the inactivation process approximates to first-order kinetics with respect to time at any fixed concentration of diethyl pyrocarbonate. The same data replotted and presented in Fig. 1 insert show that the inactivation process was first-order with respect to modifier concentration. Thus from the slope of the plot shown in Fig. 1 insert a value of $75\,M^{-1}\cdot s^{-1}$ was derived for the second-order rate constant of inactivation of lactate oxidase with diethyl pyrocarbonate. This value is less than the second-order rate constant of $216\,M^{-1}\cdot s^{-1}$ for the reaction of an essential histidine residue of pig heart lactate dehydrogenase (Holbrook & Ingram, 1973), with diethyl pyrocarbonate, but is greater than the rate constant of $20\,M^{-1}\cdot s^{-1}$ for the reaction of free histidine, imidazole or N^{α}-acetylhistidine with diethyl pyrocarbonate as reported by Holbrook & Ingram (1973).

The rate of ethoxycarbonylation of histidine residues in the enzyme was determined spectrophotometrically with various concentrations of diethyl pyrocarbonate in the range 0.021–0.25 mM and under the same conditions used to measure the kinetics of inactivation. The overall absorption change was biphasic, and the initial rapid phase, which represented approx. 70% of the total change, was first-order, with a rate constant of $0.18\,min^{-1}$ at 0.16 mM-diethyl pyrocarbonate. The pseudo-first-order rate constant for inactivation at the same concentration of diethyl pyrocarbonate was $0.20\,min^{-1}$.

The rate of enzyme inactivation was also determined over the range pH 4.0–8.0. As expected, the reciprocal of the half-time of inactivation, $1/t_{\frac{1}{2}}$, increased with pH. A plot of $1/t_{\frac{1}{2}}$ versus pH was similar to that obtained by Holbrook & Ingram (1973) for the reaction of diethyl pyrocarbonate with an essential histidine residue in pig heart lactate dehydrogenase. This suggested that diethyl pyrocarbonate

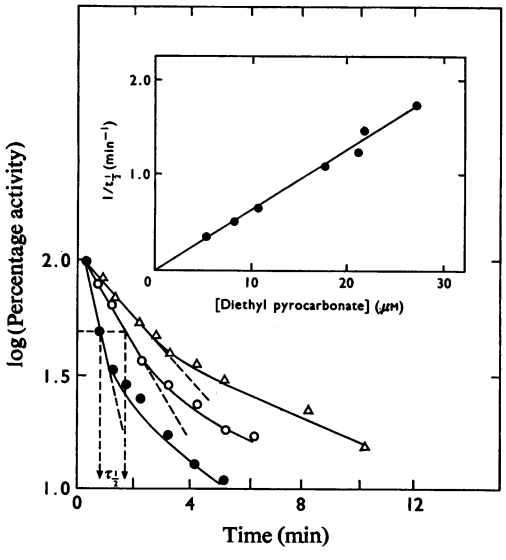

Fig. 1. *Analysis of the kinetics of inactivation*
Each incubation mixture contained lactate oxidase (22 μM-enzyme-bound FMN) and specified initial concentrations of diethyl pyrocarbonate in the range 55–272 μM. The total volume of each incubation was 0.2 ml and the temperature 25°C. Samples (0.01 ml) were taken at various times and assayed for enzyme activity. Analyses are shown at three concentrations of diethyl pyrocarbonate: △, 82 μM, ○, 109 μM; ●, 272 μM. Insert: data were replotted in the form $1/t_{\frac{1}{2}}$ versus diethyl pyrocarbonate concentration.

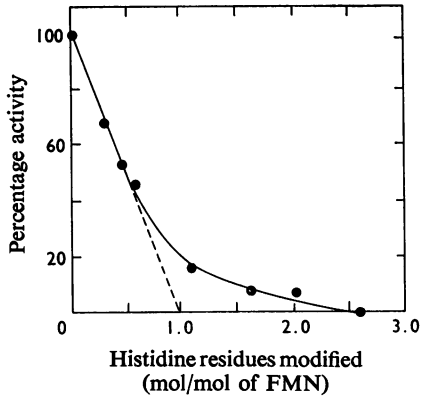

Fig. 2. *Relationship between enzyme inactivation and histidine modification*
Solutions of lactate oxidase, 21.7 μM with respect to enzyme-bound FMN, were treated with diethyl pyrocarbonate at 25°C for 15 min. The modification of histidine residues was completed in this time as judged by A_{242} change. The final volume of each incubation was 0.3 ml. The experimental points represent different initial concentrations of diethyl pyrocarbonate (μM) as follows: 100%, 0; 69%, 26.0; 54%, 40.0; 46%, 41.5; 17%, 82; 9%, 124; 0%, 246. Specific activity at 0 min was 1200 units/mg of protein [for definition of enzyme units see Choong *et al.* (1975)].

Table 1. *Stoicheiometry of enzyme modification with diethyl [^{14}C]pyrocarbonate*

Lactate oxidase (0.122 μmol of enzyme-bound FMN) was incubated with 0.61 μmol of diethyl [^{14}C]pyrocarbonate in 20mM-Mes/NaOH buffer, pH6.0, at 25°C for 10min. The total volume was 2.5ml. Histidine modification was determined in the incubation by the change in A_{242}. A sample (2.0ml) was dialysed for 12h against the same buffer and samples were counted for ^{14}C radioactivity incorporated. Samples (0.5ml) of the dialysed modified enzyme were deacylated, with an appropriate control, as described in the Materials and Methods section, and radioactivity was determined in the apoprotein and supernatant fractions.

	Histidine modification (mol/mol of FMN)	^{14}C incorporation (mol/mol of FMN)	
Holoenzyme	1.37	—	
Apoenzyme	—	1.38	
		Apoprotein	Supernatant
After treatment with hydroxylamine		0.004	1.55
Control		1.31	0.005

reacts with an unprotonated histidine residue (pK_a' approx. 6.9) in lactate oxidase.

Specificity and stoicheiometry of enzyme modification

Incubation of enzyme with a 6-fold molar excess of reagent with respect to enzyme-bound FMN resulted in 92% inactivation in 13min. Histidine modification was proportional to the inactivation within the range 20–100% residual activity: extrapolation of this linear part of the plot intercepted the axis (0% residual activity) at 0.94mol of histidine modified/mol of enzyme-bound FMN, which indicates 1mol of histidine/mol of enzyme-bound FMN for enzyme inactivation. This stoicheiometry was further substantiated by titrating a fixed amount of enzyme with various amounts of reagent (Fig. 2). The linear region of the curve obtained at low concentrations of reagent extrapolated to 1mol of histidine/mol of FMN. Complete inactivation resulted in the modification of 1.7–2.0mol of histidine residues/mol of enzyme-bound FMN.

To show that diethyl pyrocarbonate reacts specifically with histidine residues in lactate oxidase, modification was carried out with ^{14}C-labelled reagent. Preliminary experiments showed that denaturation of the enzyme with 10% (w/v) trichloroacetic acid rather than ethanol precipitation or heat treatment after modification resulted in a 46% decrease of incorporation of label into the protein. In subsequent experiments enzyme denaturation and removal of the flavin group were achieved either by heating the inactivated enzyme at 100°C for 4min or by treatment with 90% (v/v) ethanol (final concentration). The supernatant obtained contained the FMN but negligible radioactivity. As shown in Table 1, the ethoxycarbonylhistidine and ^{14}C incorporation estimated as mol/mol of FMN were essentially the same. Further, treatment of the modified holoenzyme with neutral hydroxylamine, a nucleophile that has been shown to specifically deacylate ethoxycarbonylhistidine derivatives (Melchior & Fahrney, 1970), removed 99.3% of the ^{14}C label and restored enzyme activity.

Properties of ethoxycarbonylated lactate oxidase

Fig. 3(*a*) shows the u.v. difference spectrum that develops during the modification reaction. The absorption maximum after 15min at 242nm accounts for 1.56mol of histidine residues modified/mol of FMN. A decrease in A_{278} after protein modification with diethyl pyrocarbonate has been attributed to *O*-acetylation or *O*-alkylation of tyrosine residues (Simpson *et al.*, 1963). In view of the ^{14}C-incorporation stoicheiometry described above and direct determinations of amino acids described below, it seemed likely that the absorption trough in the region 260–290nm observed with ethoxycarbonylated lactate oxidase (Fig. 3*a*) resulted from a perturbation of the micro-environment of tryptophan, tyrosine or the flavin nucleotide. Treatment of the modified enzyme with hydroxylamine removed the 242nm peak, but increased the 270nm trough. After dialysis, however, the difference spectrum of the hydroxylamine-treated sample was similar to that of the original native enzyme (Fig. 3*b*).

When histidine, N^{α}-acetyl-L-histidine, tyrosine, tryptophan and cysteine in 20mM-Mes/NaOH buffer, pH6.0, were treated separately with diethyl pyrocarbonate only the imidazole derivatives reacted, as judged by u.v. spectra and amino acid analysis. L-Histidine treated with diethyl pyrocarbonate and applied to the amino acid analyser gave a ninhydrin-positive peak which emerged 15min earlier than L-histidine (at 32min). The derivative was not stable under conditions normally used for protein hydrolysis.

Direct determinations of tyrosine, tryptophan and cysteine in native and ethoxycarbonylated enzyme (4 histidine residues acylated/molecule of FMN) confirmed that these residues were not modified under the conditions used in this work (Table 2).

Several experiments were carried out to check that the gross structure and conformation of lactate oxidase were not altered by ethoxycarbonylation. Fluorescence emission is a particularly sensitive

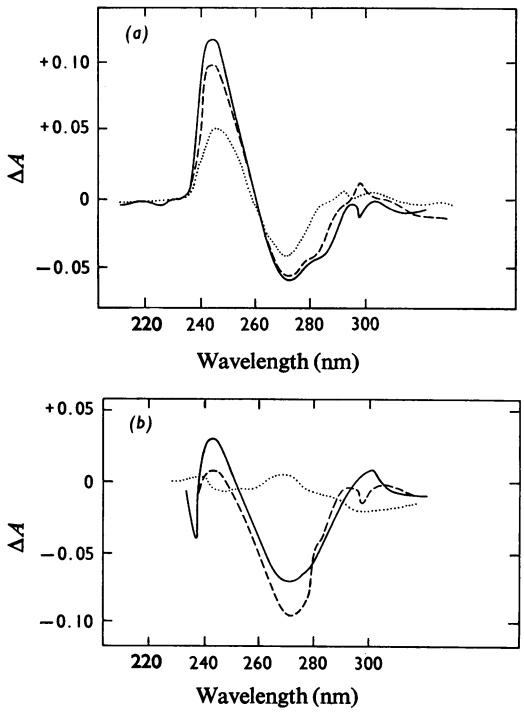

Fig. 3. *Difference spectra of diethyl pyrocarbonate-modified lactate oxidase*

(*a*) Lactate oxidase (19.5 μmol of enzyme-bound FMN) in 0.80 ml of 20 mM-Mes/NaOH buffer, pH 6.0, was added to two cuvettes. Diethyl pyrocarbonate (5 μl of a 15.7 mM solution) was added to the sample cuvette and 5 μl of ethanol was added to the reference. Spectra were recorded at 1 (····), 5 (----) and 15 min (——). (*b*) Deacylation was initiated by the addition of 0.5 ml of a solution containing 1 M-hydroxylamine and 2 mM-dithioerythritol in 20 mM-Mes/NaOH, pH 6.0, to both cuvettes. Spectra were recorded after 1 (——) and 5 (----) min, and after dialysis for 48 h against 20 mM-sodium phosphate buffer, pH 6.0 (····).

probe for conformational changes that affect the environments of tyrosine and tryptophan residues. The uncorrected fluorescence emission of ethoxycarbonylated lactate oxidase (excitation at 290 nm) showed an emission maximum at 330 nm and was the same as that of the native enzyme.

The rate of protein unfolding in urea has been used as an index of protein conformation in chymotrypsins (Hopkins & Spikes, 1968). As shown in Table 3 the rate of unfolding of ethoxycarbonylated lactate oxidase was not significantly different from that of the native enzyme.

The ethoxycarbonylated enzyme exhibited the same heat-stability at 55°C as the native enzyme. As

Table 2. *Tyrosine, tryptophan and cysteine analyses on native and modified enzyme*

Enzyme modification was carried out as described in Fig. 2. *N*-Ethoxycarbonylhistidine was determined from the change in A_{242}, and cysteine, tyrosine and tryptophan were determined as described in the Materials and Methods section. Enzyme activity is expressed as a percentage of that of the control (native) enzyme.

	Content (mol/mol of FMN)			
Enzyme activity (%)	Ethoxycarbonylhistidine	Cysteine	Tyrosine	Tryptophan
100	0	2.9	11.5	12.4
42	0.62	2.6	11.4	12.3
0	2.5	2.5	11.5	12.3

Table 3. *Unfolding of native and modified enzyme in urea*

Buffered solutions containing 2.04 mg of enzyme/ml were treated with diethyl pyrocarbonate for 15 min as detailed. The final volume was 2.5 ml. Samples were diluted, then assayed for enzyme activity and the change in A_{242}. The rate of enzyme unfolding was measured in 0.1 M-citric acid/sodium citrate buffer, pH 6.0, containing 4.5 M-urea (protein concentration 0.25 mg/ml), from the rate of decrease of A_{293}. The control contained 0.4% (v/v) ethanol.

Diethylpyrocarbonate (mM)	Enzyme (units)	Histidine residues modified (mol/mol of FMN)	Rate constant of unfolding (min^{-1})
0	5500	0	1.30
0.064	2700	1.3	1.07
0.16	380	2.0	1.21
0.32	0	3.0	1.36

shown in Fig. 4, the rate of decrease of enzyme activity followed pseudo-first-order kinetics with respect to time. The rate constant for native enzyme (specific activity 1450 units/mg of protein) was 0.14 min^{-1}. Ethoxycarbonylated samples of the enzyme containing 0.69 and 1.23 mol of ethoxycarbonylhistidine/mol of FMN, with specific activities of 460 and 88 units/mg of protein respectively, had the same rate constants (0.093 min^{-1}) for heat denaturation (Fig. 5). The slightly lower rate constant for the partially inactivated enzyme may be due to protection by the ethoxycarbonylated residues. Further, the modified enzyme probably undergoes deacylation and re-activation at the elevated temperature, since the ethoxycarbonylated enzyme underwent spontaneous re-activation at 4°C with a half-life of 14 days.

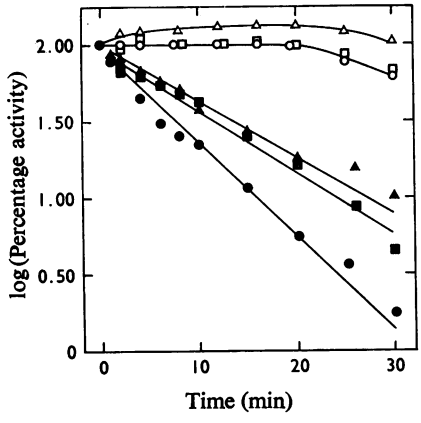

Fig. 4. *Heat-denaturation before and after modification with diethyl pyrocarbonate*

Solutions of lactate oxidase (2.4mg/ml) in 20mM-Mes/NaOH buffer, pH6.0, were treated with a 2-fold and 4-fold molar excess of diethyl pyrocarbonate respectively at 25°C. After 15min, imidazole/HCl buffer, pH7.0, was added to give a final concentration of 10mM and the samples were dialysed against 20mM-Mes/NaOH buffer, pH6.0, in the dark for 6h. A control was treated in the same way except that diethyl pyrocarbonate solution was replaced by an equal volume of ethanol. The enzyme solutions were incubated at 55°C in the dark with and without added L-lactate (final concn. 8.1mM). Samples (30μl) were diluted 5-fold in 20mM-Mes/NaOH, pH7.0, at 40°C and assayed for enzyme activity: the activity of each incubation was expressed as a percentage of the activity before incubation at 55°C. ●, Control; ○, control plus lactate; ■, 0.69mol of ethoxycarbonylhistidine/mol of FMN; □, 0.69mol of ethoxycarbonylhistidine/mol of FMN plus lactate; ▲, 1.23mol of ethoxycarbonylhistidine/mol of FMN; △, 1.23mol of ethoxycarbonylhistidine/mol of FMN plus lactate.

Several experiments were carried out to confirm that modification with diethyl pyrocarbonate had not affected the subunit structure or flavin-binding sites. The sedimentation coefficient and mobility in 7.5% polyacrylamide gels in the modified enzyme were the same as those reported previously for the native enzyme (Sullivan *et al.*, 1977; Choong *et al.*, 1975). It was also found that flavin dissociation and reconstitution of the modified enzyme were as described previously (Choong *et al.*, 1975) for the native enzyme, except that enzyme activity was not restored by the binding of FMN.

Effect of substrate and competitive inhibitors on the inactivation

A kinetic analysis was carried out with different concentrations of L-lactate (1.67–33.3mM) and air-saturated solutions in the assay system described previously (Choong *et al.*, 1975). Apparent V and apparent K_m values were determined from Lineweaver–Burk plots. The decrease in apparent V was proportional to the extent of modification, but the apparent K_m for L-lactate (5.0mM) was unaltered. Substrates and competitive inhibitors listed in Table 4 gave partial protection against diethyl pyrocarbonate. Although the first-order rate of histidine modification was unaltered, the maximum extent of histidine modification was decreased by ligands that bind at the active site to the extent of 0.8–1.2 histidine residues modified/molecule of enzyme-bound FMN.

Table 4. *Effect of substrate and competitive inhibitors on the rate of enzyme inactivation*

Lactate oxidase (21.7μM with respect to enzyme bound FMN) in 0.02M-Mes/NaOH, pH6.0, was preincubated with substrate or a competitive inhibitor for 5min before the addition of diethyl pyrocarbonate, final concentration 0.14mM. The volume was 1.0ml. Histidine modification was followed for 15min. Residual enzyme activity was measured and the rate constant of histidine modification was calculated from the slope of the plot of $\log[a/(a-x)]$ versus time, where a is the total mol of histidine modified/mol of FMN after 15min (the completion of the reaction) and x is mol of histidine modified/mol of FMN at time t.

Addition	Concn. (mM)	Residual activity (%)	Histidine modification (mol/mol of FMN)	Rate constant of histidine modification (min^{-1})
None	—	2	1.9–2.1	0.191
L-Lactate	24.2	46	1.18	0.170
	12.2	50	1.09	0.161
D-Lactate	24.4	50	1.1	0.209
Nitrate	24.2	46	0.75	0.165
	12.2	46	1.18	0.153
α-Hydroxymalonate	24.2	45	0.83	0.230

Studies on the enzyme-bound FMN in the modified enzyme

Thomé-Beau *et al.* (1971) reported that diethyl pyrocarbonate does not react with FAD. Similarly, we found that FMN was not affected by a 10-fold excess of the reagent, as judged by absorption spectrum and reduction with sodium dithionite. Inactivation of lactate oxidase with diethyl pyrocarbonate, however, modified the flavin absorption spectrum: the 457nm band decreased and the spectrum of the modified enzyme was isosbestic with that of the native enzyme at 500, 408, 385 and 310nm (Fig. 5).

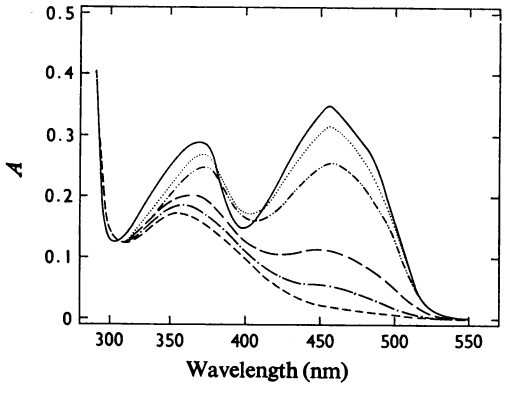

Fig. 5. *Substrate reduction of diethyl pyrocarbonate-modified lactate oxidase*

Solutions of lactate oxidase (0.8ml, containing 39μM enzyme-bound FMN) were treated with various amounts of diethyl pyrocarbonate listed below for 15min. Imidazole/HCl buffer, pH7.0 (0.1ml), was then added to each sample to a final concentration of 10mM. Enzyme reduction was carried out by the addition of a 3-fold molar excess of L-lactate, with respect to FMN, from the side arm of an anaerobic cuvette. Spectra corrected for dilutions are: ——, oxidized native enzyme; ····, oxidized modified enzyme (the spectra of solutions modified with different amounts of diethyl pyrocarbonate were the same); – – – –, reduced native enzyme. The spectra of partially reduced enzyme solutions —··—··) (——) (—·—·) were of enzyme treated with 1.43, 2.13 and 4.28μmol of diethyl pyrocarbonate/μmol of FMN respectively.

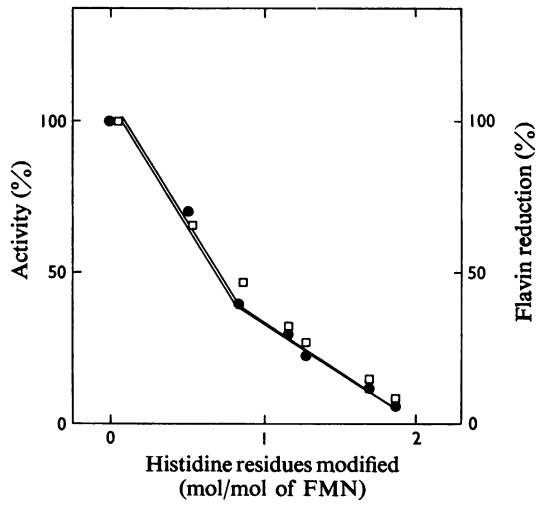

Fig. 6. *Relationship between substrate reduction and histidine modification of diethyl pyrocarbonate-treated enzyme*

Solutions of lactate oxidase (39μM enzyme-bound FMN) were treated at 25°C with various concentrations of diethyl pyrocarbonate (in a molar excess of 0–5.3) as described in Fig. 2. Histidine modification was followed at 242nm, and after 15min imidazole/HCl buffer, pH7.0, was added to give a final concentration of 10mM. Enzyme activity was then compared with a control solution, specific activity 1200 units/mg of protein. Treatment with diethyl pyrocarbonate was carried out in cuvettes fitted with ground-glass stoppers. These cuvettes were made anaerobic and L-lactate (0.2μmol) was added from a side arm. Flavin reduction was expressed as the ratio of change in A_{455} of the treated sample to that of the untreated control. The Figure shows percentage residual activity (●) and percentage flavin reduction (□).

Previous studies have shown that the flavin spectrum of lactate oxidase was perturbed in ligand binding to the active site (Lockridge *et al.*, 1972; Ghisla & Massey, 1975). Fully inactivated enzyme was reduced by sodium dithionite.

Anaerobic incubation of native lactate oxidase with a small molar excess of L-lactate results in the complete rapid reduction of the enzyme. Fig. 5 shows the reduction of the enzyme modified to different extents with diethyl pyrocarbonate. There was a direct correlation between the extent of the inactivation, histidine modification and the flavin reduction (Fig. 6).

Although the inactivated enzyme was not reduced by L-lactate, the modified enzyme still exhibited the characteristic binding of the competitive inhibitors phosphate, nitrate and α-hydroxymalonate, in addition to binding of bisulphite to the flavin group (Lockridge *et al.*, 1972; Massey *et al.*, 1969). Dissociation constants in 20mM-Mes/NaOH buffer, pH6.0, were determined by plots of changes in A_{450} versus ligand concentration by the method of Benesi & Hildebrand (1949). The values obtained for phosphate (2.3mM), α-hydroxymalonate (15μM) and bisulphite (24μM) all agreed with the values obtained for the native enzyme. It was also found that excess of L-lactate changed the flavin spectrum of the modified enzyme slightly from an unresolved to a resolved-type spectrum (Muller *et al.*, 1973), but the very small absorbance changes precluded an estimation of the dissociation constant. Inactivated enzyme was reduced with phenazine methosulphate plus NADH to the red anionic semiquinone derivative. The native enzyme undergoes the same reduction with this system (P. A. Sullivan, unpublished work).

Discussion

Although previous studies have established that diethyl pyrocarbonate acylates a number of amino acid side chains, including tyrosine, tryptophan, cysteine and histidine (Melchior & Fahrney, 1970), it has been clearly shown that this reagent often

reacts preferentially with histidine residues (for references see the introduction). In a number of studies it has been shown that diethyl pyrocarbonate distinguishes essential histidine residues [pig liver L-lactate dehydrogenase (Holbrook & Ingram, 1973); arginine mono-oxygenase (Thomé-Beau *et al.*, 1971)], but enzymes such as the amino acid oxidases contain a number of reactive histidine residues (Thomé-Beau *et al.*, 1971).

In the present study the close agreement between the overall protein modification as determined by the incorporation of ethoxy[^{14}C]carbonyl groups and the direct spectrophotometric estimation of histidine modification established clearly that diethyl pyrocarbonate reacts specifically with histidine residues in lactate oxidase (Table 1). Under the experimental conditions used throughout this work, direct experiments showed that low concentrations of the reagent did not acylate either free amino acids, other than imidazole derivatives, or amino acid residues in lactate oxidase other than histidine. Further, treatment of the inactive enzyme with neutral hydroxylamine removed all the ^{14}C label, completely abolished the difference-spectrum maximum at 242nm and restored enzyme activity. Melchior & Fahrney (1970) reported that this treatment removes the ethoxycarbonyl group from the imidazole ring, but does not affect modified amino groups. In addition, it was established that the rates of inactivation ($0.20\,min^{-1}$) and of ethoxycarbonylation of histidine residues ($0.18\,min^{-1}$) in lactate oxidase were the same within experimental error.

Amino acid analysis has established the histidine content of lactate oxidase as 11 residues/FMN (Sullivan *et al.*, 1977). Treatment of the enzyme with a 6-fold excess of the reagent resulted in the modification of 1.5–2 histidine residues/molecule of FMN and greater than 95% enzyme inactivation (Fig. 2). A kinetic analysis (Fig. 1) and the titration experiment (Fig. 2) indicate that modification of 1mol of histidine/mol of FMN was responsible for enzyme inactivation. Clearly lactate oxidase contains two hyper-reactive histidine residues. It is not possible, however, from the results presented to distinguish whether one or both reactive histidine residues are essential. Protection against enzyme inactivation by substrates and competitive inhibitors confirmed that one of the histidine residues was essential and suggested that the second may also be within the active-site domain (Table 1). With substrate and competitive inhibitors such as D-lactate, nitrate and α-hydroxymalonate present, the rate constant of histidine modification was unaltered, the extent of histidine modification was decreased from 2 to 0.75–1.2 residues/molecule of FMN, and the residual activity was 42–50% of the original value (Table 4). These results may be interpreted as follows: competitive inhibitors provided almost complete protection for the essential residue; the second residue was not protected and reacted at the same rate as in the native enzyme. Because this modified residue is in the active-site domain, modification has in some manner affected the active site so that the enzyme activity is decreased to 42–50%. This and other possible interpretations will require further work.

A number of experiments, including an analysis of protein fluorescence, a sedimentation-velocity analysis, gel electrophoresis and the rate of protein unfolding in urea indicated that modification with diethyl pyrocarbonate did not result in any significant changes in protein conformation. Further, the heat-stability experiments (Fig. 4) on native and partially inactivated enzyme showed that modification had not affected the parameters that determine the heat-stability of the remaining functional active sites in the enzyme. Lactate protected the functional active sites in the native and partially inactivated enzyme to the same extent. In this argument it is assumed that active-site modification is a random process with respect to the eight active sites rather than a concerted attack on all the active sites in the same molecule. In addition, the active sites in the modified enzyme were very similar to those of the native enzyme, as judged by the binding of bisulphite, formation of the red anionic semiquinone derivative, reduction by dithionite and binding of competitive inhibitors. The only evidence obtained for unaltered binding of the substrate to the inactive enzyme was a small perturbation of the flavin spectrum caused by lactate, but substrate analogues such as α-hydroxymalonate were still bound to the modified enzyme. The weak binding of L-lactate (K_D 22mM; Lockridge *et al.*, 1972) to the native enzyme and the very slight perturbation of the flavin spectrum preclude an accurate measurement of the binding constant of lactate with the modified enzyme. If the substrate binding is unaffected, then ethoxycarbonylation of a histidine residue blocks the next step in catalysis. Previous studies (Lockridge *et al.*, 1972) have established this step as the formation of a reduced flavin–pyruvate complex. This step may be initiated by the abstraction of the hydrogen from the α-carbon as a proton. It is suggested that the essential histidine residue modified by diethyl pyrocarbonate participates in this reaction as the initial proton acceptor.

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References

Averill, B. A., Schonbrunn, A., Abeles, R. H., Weinstock, L. T., Cheng, C. C., Fisher, J., Spencer, R. & Walsh, C. (1975) *J. Biol. Chem.* **250**, 1603–1605

Benesi, H. A. & Hildebrand, J. H. (1949) *J. Am. Chem. Soc.* **71**, 2703–2707
Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
Bredderman, P. J. (1974) *Anal. Biochem.* **61**, 298–301
Burstein, Y., Walsh, K. A. & Neurath, H. (1974) *Biochemistry* **13**, 205–210
Choong, Y. S., Shepherd, M. G. & Sullivan, P. A. (1975) *Biochem. J.* **145**, 37–45
Dann, L. G. & Britton, H. G. (1974) *Biochem. J.* **137**, 405–407
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Dawson, R. M. C. & Elliott, W. H. (1959) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), pp. 192–209, Oxford University Press
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
Fisher, J., Spencer, R. & Walsh, C. (1976) *Biochemistry* **15**, 1054–1064
Ghisla, S., & Massey, V. (1975) *J. Biol. Chem.* **250**, 577–584
Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H., & Walsh, C. (1976) *Biochemistry* **15**, 1791–1797
Hegyi, G., Premecz, G., Sain, B. & Mühlrad, A. (1974) *Eur. J. Biochem.* **44**, 7–12
Hellerman, L. & Coffey, D. S. (1967) *J. Biol. Chem.* **242**, 582–589
Hersh, L. B. & Schuman-Jorns, M. (1975) *J. Biol. Chem.* **250**, 8728–8734
Holbrook, J. J., & Ingram, V. A. (1973) *Biochem. J.* **131**, 729–738
Hopkins, T. R. & Spikes, J. D. (1968) *Biochem. Biophys. Res. Commun.* **30**, 540–545
Huc, C., Olomucki, A., Thi-Lan, L., Pho, D. B. & Thoai, N. V. (1971) *Eur. J. Biochem.* **21**, 161–169
Lockridge, O., Massey, V. & Sullivan, P. A. (1972) *J. Biol. Chem.* **247**, 8097–8106
Massey, V. & Ganther, H. (1965) *Biochemistry* **4**, 1161–1173
Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G. & Foust, G. P. (1969) *J. Biol. Chem.* **244**, 3999–4006
Melchior, W. B., Jr. & Fahrney, D. (1970) *Biochemistry* **9**, 251–258
Muller, F., Mayhew, S. G., Massey, V. (1973) *Biochemistry* **12**, 4654–4662
Schachman, H. K. (1957) *Method Enzymol.* **4**, 32–103
Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H. & Massey, V. (1976) *Biochemistry* **15**, 1798–1807
Simpson, R. T., Riordan, J. F. & Vallee, B. L. (1963) *Biochemistry* **2**, 616–622
Steenkamp, D. J., Schabort, J. C., Holzapfel, C. & Ferreira, N. P. (1974) *Biochim. Biophys. Acta* **358**, 126–143
Sullivan, P. A., Choong, Y. S., Schreurs, W. J., Cutfield, J. F. & Shepherd, M. G. (1977) *Biochem. J.* **165**, 375–383
Thomé-Beau, F., Thi-Lan, L., Olomucki, A. & Thoai, N. V. (1971) *Eur. J. Biochem.* **19**, 270–275
Walsh, C. T., Schonbrunn, A. & Abeles, R. H. (1971) *J. Biol. Chem.* **246**, 6855–6866
Walsh, C. T., Krodel, E., Massey, V. & Abeles, R. H. (1973*a*) *J. Biol. Chem.* **248**, 1946–1955
Walsh, C. T., Lockridge, O., Massey, V. & Abeles, R. H. (1973*b*) *J. Biol. Chem.* **248**, 7049–7054