zyme–analogue complex appeared unnecessary. Elimination of the triphosphate group of oATP bound to mitochondrial adenosinetriphosphatase (Lowe et al., 1979) was initially proposed to lead to the formation of a stable conjugated Schiff base with lysine. Subsequently, while this paper was in preparation, an alternate mechanism was suggested for the inactivation of this adenosinetriphosphatase by oATP which involved the formation of a morpholino derivative with lysine (Lowe & Beechey, 1982). Formation of a stable dihydroxymorpholino derivative of lysine was previously suggested for the reaction of oATP with phosphofructokinase (Gregory & Kaiser, 1979) and phosphorylase kinase (King & Carlson, 1981). The results obtained in the present study of the inactivation of isocitrate dehydrogenase by oADP also provided evidence for an elimination reaction, which led to a loss of the pyrophosphate group from the enzyme-bound oADP and suggested that the final product is a 4′,5′-didehydro-2′,3′-dihydroxymorpholino derivative.

Registry No. oADP, 64060-84-0; ADP, 58-64-0; ATP, 56-65-5; NADH, 58-68-4; isocitric acid, 320-77-4; L-lysine, 56-87-1; isocitrate dehydrogenase, 9001-58-5; Lys-oADP, 84560-16-7.

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Chemical Modification of L-Lactate 2-Monooxygenase with Fluorodinitrobenzene: Evidence for Two Essential Histidine Residues†

Christopher J. Murphy, Maxwell G. Shepherd, and Patrick A. Sullivan*

ABSTRACT: The modification of L-lactate 2-monooxygenase (lactate oxidase) with radioactively labeled fluorodinitrobenzene in 50 mM tris(hydroxymethyl)aminomethane (Tris)–acetate, pH 6.0, resulted in complete inactivation with the incorporation of 1 mol of dinitrophenyl per catalytic site. Analysis of acid hydrolysates using high-pressure liquid chromatography and an amino acid analyzer showed that N^{im}-(dinitrophenyl)histidine accounted for 95% of the incorporated label. Approximately 50% of the flavin in the modified enzyme was reduced by lactate, formed a sulfite complex, and underwent a photochemical reaction with oxalate. These results suggest that each active site in lactate oxidase contains two essential histidine residues that are modified in a mutually exclusive manner.

L-Lactate 2-monooxygenase (lactate oxidase) (EC 1.13.12.4) from mycobacteria catalyzes the oxidative decarboxylation of L-lactate and other L α-hydroxy acids according to the equation:

$$R\cdot CHOH\cdot COOH + O_2 \rightarrow R\cdot COOH + H_2O + CO_2$$

This FMN-containing flavoenzyme has been classified as a dehydrogenase/oxidase (Massey & Hemmerich, 1980). Ex-

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tensive studies on the dehydrogenation mechnism including rapid reaction studies (Lockridge et al., 1972), the elimination reaction with β-chlorolactate (Walsh et al., 1973), the suicide reaction with α-hydroxybutynoate (Ghisla et al., 1976), binding studies with oxalate (a putative transition-state analogue) (Ghisla & Massey, 1977), and the formation of a covalent adduct during glycollate oxidation (Ghisla & Massey, 1980) are consistent with a reaction mechanism that includes abstraction of the α-H as a H^+, electron transfer via the formation of a covalent flavin–substrate adduct and formation of a reduced flavin–pyruvate charge-transfer complex [for reviews, see Sullivan et al. (1981) and Ghisla (1982)]. Direct O_2 oxidation of the charge-transfer complex (Lockridge et al., 1972) results in a peroxidative decarboxylation of the enzyme-bound pyruvate with release of acetate as the last and rate-limiting step in catalysis.

In previous studies using diethyl pyrocarbonate (ethoxyformic anhydride) (Choong et al., 1977) and FDNB (Choong et al., 1978), we obtained evidence for essential histidine and lysine residues. The properties of the modified enzyme suggested that these residues participate in H^+ abstraction and substrate binding, respectively (Sullivan et al., 1981). In the present paper, we describe evidence for two essential histidine residues at each active site that react with FDNB[1] in a mutually exclusive manner.

Experimental Procedures

L-Lactate Oxidase. The enzyme was prepared from *Mycobacterium smegmatis* as described previously (Sullivan et al., 1977). The assay for lactate oxidase activity, protein determinations, units of activity, specific activity, and measurement of enzyme-bound FMN concentration were as described previously (Choong et al., 1975; Sullivan et al., 1977). The enzyme used in this study had a specific activity of 1200 units (mg of protein)$^{-1}$.

Chemicals. N^{α}-Acetylhistidine, FDNB, DNP, N^{ϵ}-DNP-lysine, *O*-DNP-tyrosine, and N^{α}-DNP-arginine were from the Sigma Chemical Co., St. Louis, MO.

Dinitrophenylation. All reactions were carried out in foil-wrapped vessels to exclude light. Stock solutions of FDNB, 200 mM, were prepared in absolute ethanol and assayed by the method of Murdock et al. (1966) with a molar extinction coefficient of 14.8×10^3 L mol^{-1} cm^{-1} for DNP at 360 nm. [U-^{14}C]FDNB (specific radioactivity 17 mCi/mmol) from Amersham Australia Pty Ltd., Sydney, Australia, was diluted with unlabeled compound to a specific activity of 3×10^5 to 3×10^6 cpm/μmol. Lactate oxidase (1–4 mg/mL) in 50 mM Tris–acetate, pH 6.0, was incubated with FDNB (2 mM) at 25 °C for 5–10 h. The ethanol concentration in the reaction system never exceeded 2% (v/v), and controls of the enzyme incubated alone with 2% v/v ethanol showed no measurable change in enzyme activity. When residual enzyme activity was 5% or less of the initial activity, the enzyme solution was dialyzed in the dark for 24 h against several changes of buffer.

Measurement of Radioactivity. Aqueous samples were measured in Bray's scintillant (Bray, 1960). Dried filters were counted in a Triton X-100 scintillant. ^{14}C incorporation during the inactivation was followed by precipitation with 10% w/v trichloroacetic acid; the precipitates were collected on Whatman GF/F filters, washed 3 times with trichloroacetic acid, twice with 90% w/v ethanol, and once with ether, dried, and counted.

Protein Hydrolysis. The ^{14}C-labeled enzyme was precipitated with 10% w/v trichloroacetic acid and washed 5 times each with trichloroacetic acid and ice-cold 90% v/v ethanol. The pellets were transferred to hydrolysis tubes, 0.5 mL of redistilled 5.7 M HCl was added, and the tubes were sealed under vacuum after repeated cycles of evacuation and flushing with O_2-free nitrogen. Hydrolysis at 110 °C was for 24 h, and the hydrolysates were evaporated to dryness.

Synthesis of N^{im}-DNP-histidine. N^{α}-Acetylhistidine was reacted with FDNB, and the acetyl group was hydrolyzed as described by Henkart (1971). Following ether extraction, the product was precipitated as the crystalline hydrochloride with petroleum ether. Recrystallization from methanol–ethyl acetate gave hygroscopic tan crystals. The product was characterized by thin-layer chromatography (20 cm × 5 cm × 0.5 mm silica gel plates; 1-butanol–glacial acetic acid–water either 4:1:1 or 4:1:5; developing time 4 h). The R_f of the N^{im}-DNP-histidine was 0.31 (4:1:1) or 0.26 (4:1:5).

Other standards run were N^{ϵ}-DNP-lysine and DNP. N^{im}-DNP-histidine on these plates darkened after overnight exposure to light (Margoliash, 1955). Hydrolysis of the product in 2.5 M NaOH (1 h, 30 °C) gave free histidine and DNP.

Amino Acid Analysis. Amino acid analysis of synthetic N^{im}-DNP-histidine, DNP-amino acids, and acid hydrolysates of [^{14}C]DNP-labeled lactate oxidase was carried out on a Jeol JLC-6AH amino acid analyzer, using a short column (4.5 cm × 1.2 cm) only and a manual program. Elution was with 0.35 M citrate buffer, pH 5.28, and the column temperature was 55 °C. Samples were dissolved in 0.067 M citrate buffer, pH 2.2, before loading. With radioactive hydrolysate, 1- or 5-min fractions, as appropriate, were collected from the detector outlet directly into scintillation vials and counted. Quenching was determined with an internal standard added to both blank and sample fractions.

Reverse-Phase HPLC. Reverse-phase high-pressure liquid chromatography (HPLC) of amino acid DNP derivatives and acid hydrolysates of DNP-labeled lactate oxidase was carried out on a Varian 5000 liquid chromatograph, equipped with a Waters U6K universal injector and a Varian UV50 detector. Samples were analyzed on a Waters μBondapak C_{18} column (0.4 cm i.d. × 30 cm) at 25 °C with a linear gradient from 25% CH_3CN in 0.1% H_3PO_4 to 50% CH_3CN in 0.1% H_3PO_4 over 20 min at a flow rate of 1.0 mL/min. DNP-amino acids were detected by their absorption at 340 nm. Radioactivity was located by collecting 30-s fractions directly from the detector flowcell through microbore tubing directly into scintillation vials. Instrument dead volume, detector lag, and recovery efficiencies were determined with [^{14}C]DNP (prepared by NaOH hydrolysis of [^{14}C]FDNB) as an internal standard.

Spectrophotometry. Absorption spectra were recorded with a Cary 118 spectrophotometer. Anaerobic studies were carried out as described previously (Choong et al., 1977). For the photoreaction with oxalate, the light source was a 500 W slide-projector lamp 20 cm from the cuvette.

Results

Inactivation of Lactate Oxidase with FDNB. In a previous study (Choong et al., 1978), it was shown that incubation of lactate oxidase with FDNB in Mes–NaOH buffer, pH 6.0, resulted in dinitrophenylation of 2 ± 0.2 mol of residues/mol of enzyme-bound FMN for 100% inactivation of the enzyme. This stoichiometry was confirmed during the present work.

[1] Abbreviations: FDNB, fluorodinitrobenzene; DNP, 2,4-dinitrophenol; N^{im}-DNP-histidine, N^{im}-(dinitrophenyl)histidine; N^{ϵ}-DNP-lysine, N^{ϵ}-(dinitrophenyl)lysine; *O*-DNP-tyrosine, *O*-(dinitrophenyl)tyrosine; N^{α}-DNP-arginine, N^{α}-(dinitrophenyl)arginine; Tris, tris(hydroxymethyl)aminomethane; Mes, 4-morpholineethanesulfonic acid.

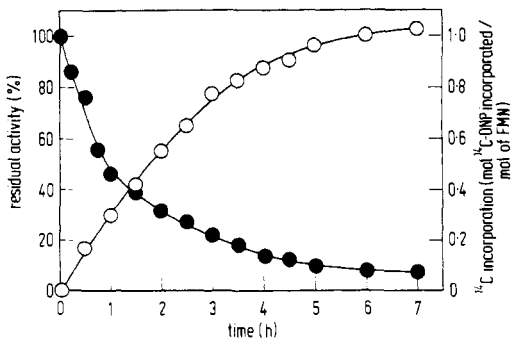

FIGURE 1: Modification of lactate oxidase with FDNB. Lactate oxidase (100 μM with respect to enzyme-bound FMN) in 50 mM Tris–acetate, pH 6.0, was incubated at 25 °C in the dark with 2 mM [^{14}C]FDNB (5×10^6 cpm/μmol), and the time course for inactivation (●) and DNP incorporation (○) was determined. The initial specific activity was 1200 units/(mg of protein).

In the present work, dinitrophenylation was carried out in 50 mM Tris–acetate, pH 6.0. Acetate, the product of the enzyme-catalyzed reaction, is a competitive inhibitor ($K_D = 1.1 \times 10^{-2}$ M) with respect to lactate (Ghisla & Massey, 1975). As shown in Figure 1, the reaction of lactate oxidase with FDNB in the Tris–acetate buffer resulted in complete inactivation with a concomitant incorporation of approximately 1.0 mol of DNP/mol of FMN. In seven subsequent experiments the incorporation for 100% inactivation was 1.04 ± 0.1 mol of DNP/mol of FMN. For routine preparation of DNP–enzyme, the enzyme was transferred to dialysis when the residual activity was 5% or less. After dialysis to remove excess FDNB (see Experimental Procedures), the modified enzyme did not possess measurable enzymic activity (<1% of the native enzyme), and the DNP incorporation remained at ~1 mol of DNP/mol of FMN.

Identification of Amino Acid DNP Derivative in Modified Enzyme. Modified enzyme was hydrolyzed as described under Experimental Procedures and analyzed for [^{14}C]DNP-amino acids. Analysis of the hydrolysate by reverse-phase HPLC is shown in Figure 2A. DNP-amino acid standards (N^{im}-DNP-histidine, N^{ϵ}-DNP-lysine, N^{α}-DNP-arginine, *O*-DNP-tyrosine, and DNP) were cochromatographed with the hydrolysate. Only one peak of radioactivity, coincident with the N^{im}-DNP-histidine, was detected in the effluent. The N^{im}-DNP-histidine peak had a retention time of 3.4 min and was well separated from the other standards: N^{α}-DNP-arginine (7.9 min), N^{ϵ}-DNP-lysine (9.1 min), *O*-DNP-tyrosine (11.2 min), and DNP (13.5 min). With [^{14}C]DNP chromatographed alone, the recovery of radioactivity in the peak was 85–90% of the original load. In analyses of the hydrolysate plus internal standards the recovery of counts was 70–85%. As described previously (Henkart, 1971; Fridkin & Shaltiel, 1971), N^{im}-DNP-histidine emerges before free lysine on the short column of the amino acid analyzer. We found that N^{ϵ}-DNP-lysine was eluted in a broad peak 12 min after free arginine. Both derivatives could be detected by the usual ninhydrin reaction. A mixture of labeled hydrolysate, N^{im}-DNP-histidine, and N^{ϵ}-DNP-lysine was chromatographed on the amino acid analyzer. As shown in Figure 2B, the eluate contained one peak of radioactivity that emerged with the N^{im}-DNP-histidine. Extensive quenching (>80%) was encountered in the measurement of the radioactivity of the ninhydrin-reacted effluent, but after correction with an internal standard, the recovery of counts with the N^{im}-DNP-histidine peak was 89% of the original load.

Properties of DNP–Enzyme. Native lactate oxidase has a typical flavoprotein spectrum with absorption maxima around

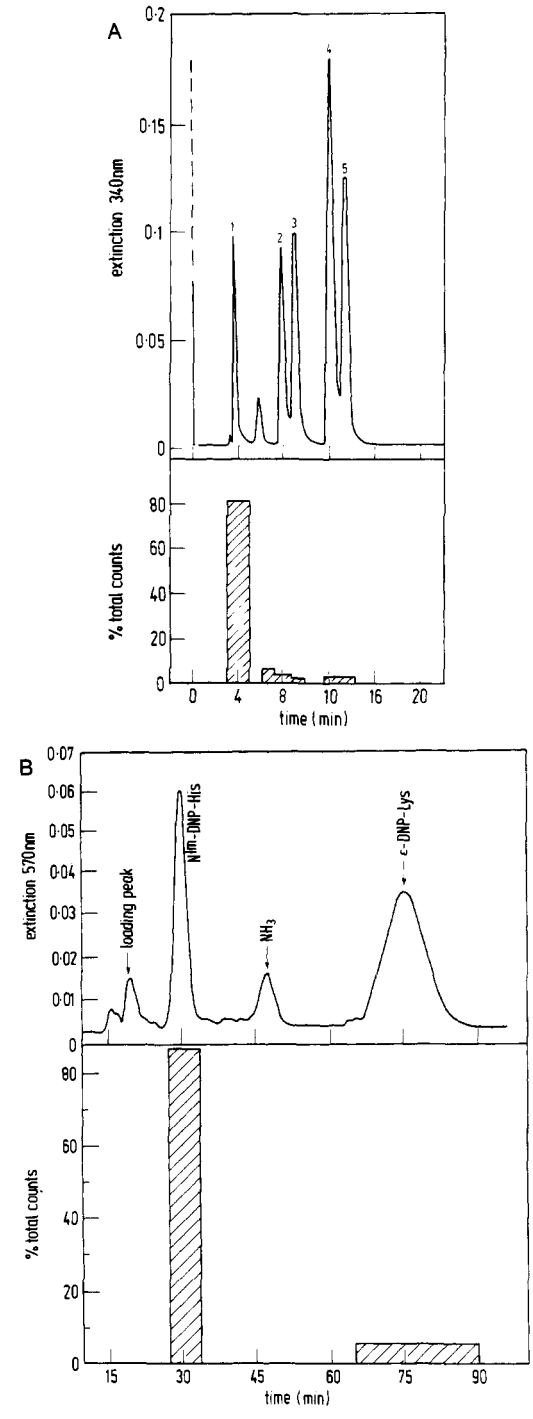

FIGURE 2: Analysis of acid hydrolysates for DNP-amino acids. (A) Analysis by reverse-phase HPLC. The sample applied to the column contained internal standards, (1) N^{im}-DNP-histidine (25 nmol), (2) N^{α}-DNP-arginine (10 nmol), (3) N^{ϵ}-DNP-lysine (10 nmol), (4) *O*-DNP-tyrosine (25 nmol), and (5) DNP (15 nmol), mixed with hydrolysate and dissolved in 0.1% v/v phosphoric acid. The sample contained 6.3 nmol of hydrolysate and 22 000 cpm. (B) Amino acid analysis. The sample analyzed contained 43 000 cpm, 12.3 nmol of hydrolysates (with respect to FMN), 20 nmol of N^{im}-DNP-histidine, and 40 nmol of N^{ϵ}-DNP-lysine. Conditions for the amino acid analyzer are described under Experimental Procedures.

375 and 450 nm (Lockridge et al., 1972) and the E_{450} varies from 9×10^3 to 12.8×10^3 L mol^{-1} cm^{-1}, depending on the solvent (Sullivan et al., 1977). Dinitrophenylation of lactate oxidase results in increased extinction below 400 nm, but the details of the spectrum from 400 to 500 nm were essentially unaffected (Figure 3). The flavin content of the modified enzyme was the same as that of the native enzyme.

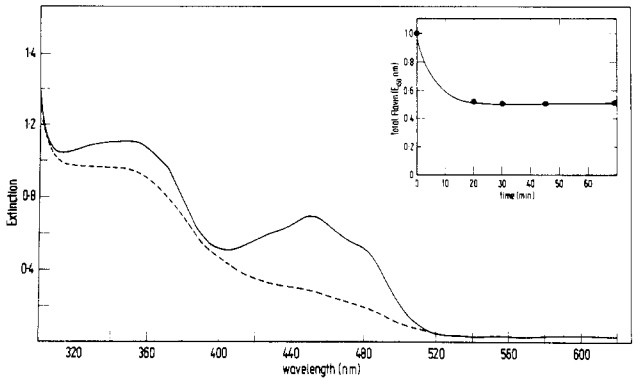

FIGURE 3: Modified enzyme (72 μM) in 50 mM Tris–acetate buffer, pH 6.0, in oxidized form (—) was incubated under anaerobic conditions for 2 h with 47.6 mM L-lactate (---). The inset shows the time course of reduction.

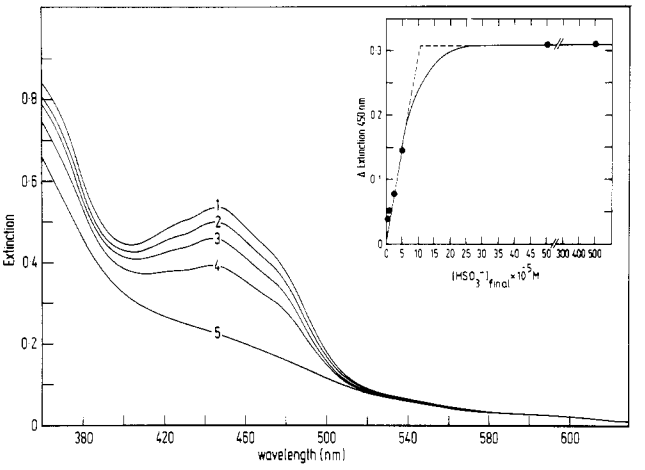

FIGURE 4: Titration of DNP–enzyme with sulfite. Modified enzyme (55 μM with respect to FMN) in 50 mM Tris–acetate, pH 6.0, was titrated with 5-μL aliquots of sodium sulfite in the same buffer to give the stated final concentrations (spectra were recorded 15 min after each addition): (1) no addition; (2) 0.5×10^{-5} M; (3) 2×10^{-5} M; (4) 5×10^{-5} M; (5) 5×10^{-4} M. There were no further changes over the range 5×10^{-4} to 1×10^{-2} M. The inset is a plot of E_{450} vs. sulfite concentration.

Although the modified enzyme exhibited no catalytic activity in the standard assay, it did however undergo a variety of reactions characteristic of native enzyme. Under anaerobic conditions only approximately 50% of the enzyme-bound FMN was reduced by excess L-lactate in a slow (0.14 min^{-1}) reaction (Figure 3). Native lactate oxidase readily forms a N(5) flavin complex with sulfite (Massey et al., 1969). Under the conditions used in the present work (50 mM Tris–acetate, pH 6.0), the flavin spectrum was completely quenched by a 2-fold molar excess of sulfite, and the titration yielded a K_D of 1×10^{-6} M. As shown in Figure 4 (and insert), titration of the DNP–enzyme with sulfite to a 100-fold molar excess resulted in a reaction with approximately 60% of the enzyme-bound flavin ($K_D = 4 \times 10^{-5}$ M). Ghisla & Massey (1977) have shown that the reaction of lactate oxidase with oxalate involves a two-step formation of a complex with the oxidized enzyme. Subsequent irradiation of the complex with visible light resulted in a facile photochemical decarboxylation of the oxalate and the formation of an $N(5)$-carboxylated flavin. Light irradiation (~10 s) of a solution containing native lactate oxidase and oxalate resulted in a rapid bleaching of the flavin spectrum (Figure 5, insert). The oxidized DNP–enzyme formed a complex with oxalate as judged by the changes in the flavin spectrum (curves 1 and 2 of Figure 5). Only 50%

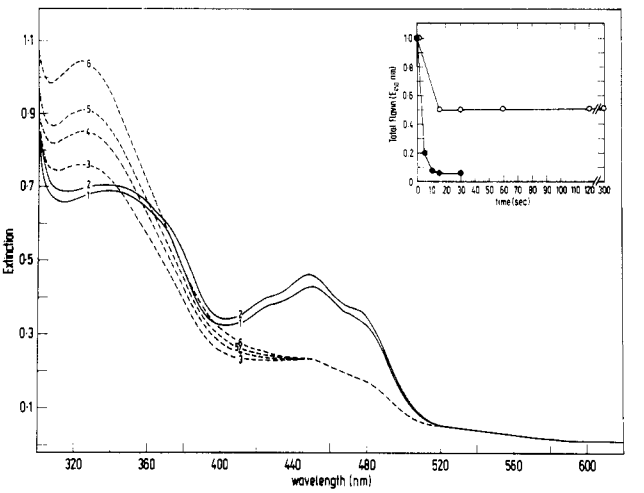

FIGURE 5: Photoreaction of the DNP–enzyme with oxalate. Modified enzyme (48 μM with respect to FMN) in 50 mM Tris–acetate, pH 6.0, containing 5×10^{-3} M sodium oxalate. The spectra recorded were as follows: (1) before the addition of oxalate; (2) plus oxalate and after 20-min incubation without light; (3) 15-s irradiation; (4) 60-s irradiation; (5) 120-s irradiation; (6) 300-s irradiation. The inset shows E_{450} vs. time for the reaction with native enzyme (●) and modified enzyme (○).

of the enzyme-bound flavin underwent the photochemical reaction even with irradiation for 300 s. Irradiation of the DNP–enzyme resulted in marked changes to the spectrum in the region 300–400 nm that were independent of the flavin photochemical reaction (Figure 5). This light-dependent reaction with DNP-modified lactate oxidase has been reported previously (Choong & Massey, 1980), and it was attributed to a reaction involving DNP. Consistent with previous studies on native enzyme (Ghisla & Massey, 1975), the N(5)-carboxyflavin complex of the DNP–enzyme decomposed slowly during incubation in darkness, yielding the oxidized flavin spectrum.

Discussion

The results presented in this paper have established conditions under which the dinitrophenylation of an average of one residue per catalytic subunit results in the complete inactivation of lactate oxidase. Analysis by two methods showed that N^{im}-DNP-histidine accounts for 90–95% of the ^{14}C label in the modified enzyme. It was therefore intriguing to find that the inactive enzyme contained two populations of active sites as judged by the ~50% reactivity with respect to lactate reduction, sulfite binding, and photochemical reaction with oxalate.

We therefore propose that each subunit contains two essential histidine residues designated His A and His B in this paper and that dinitrophenylation of either residue inactivates the enzyme. If either histidine residue were not at the active site, then at least 50% of the inactivation would result from secondary changes in structure. Native lactate oxidase could consist of subunits that have the same catalytic activity but in which different groups react with FDNB. This could arise from asymmetric subunit packing or because of two types of subunits. This model seems unlikely because previous studies (Sullivan et al., 1977) showed that lactate oxidase from *M. smegmatis* is composed of eight subunits of molecular weight 43 500 with the same N-terminal residue. Further, the native enzyme exhibits catalytically identical active sites in rapid reaction studies (Lockridge et al., 1972), titration with sulfite (Massey et al., 1969), flavin dissociation and reconstitution experiments (Choong et al., 1975), oxalate binding (Ghisla

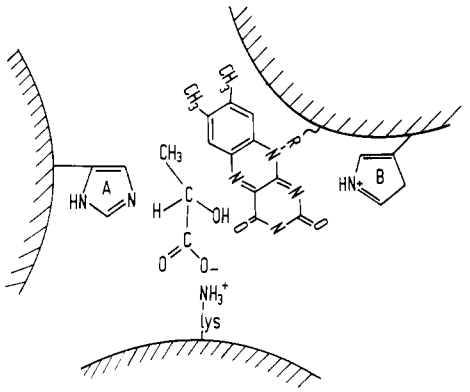

FIGURE 6: Proposed model for active site of lactate oxidase.

& Massey 1977), and photochemical reactions. Alternatively, each identical subunit could contain two essential histidine residues that react with FDNB in a mutually exclusive manner.

The mutual exclusion type model has previously been invoked by Swenson et al. (1982) to account for inactivation of D-amino acid oxidase by FDNB. In this work, it was found that the 90% decrease in enzyme activity could be accounted for by the incorporation of 0.89 mol of DNP/monomer. The label was distributed between two peptides, one containing 0.58 mol of *O*-DNP-tyrosine and the other containing 0.31 mol of N^{ϵ}-DNP-lysine.

Since dinitrophenylation of either histidine results in the inactivation of lactate oxidase, it is interesting to speculate on possible roles of these residues. Inactivation of lactate oxidase by diethyl pyrocarbonate occurs with the modification of one essential histidine residue (Choong et al., 1977). Reduction by lactate was completely abolished, but binding of competitive inhibitors and sulfite and the flavin reactivity were unaltered, and it was suggested that this histidine residue participates in the proton abstraction, generating a carbanion intermediate. This residue could be His A in the model for the active site shown in Figure 6.

When the reaction of lactate oxidase with FDNB was carried out in 50 mM Mes–NaOH, pH 6.0 (Choong et al., 1978), the inactive enzyme contained 1 mol/mol of FMN each of N^{ϵ}-DNP-lysine and N^{im}-DNP-histidine. In this modified enzyme, 50% of the flavin was reduced by L-lactate, and 50% of the flavin formed a complex with sulfite. In the present work, N^{ϵ}-DNP-lysine was present at less than 0.05 mol/mol of FMN. This indicates that acetate of the Tris–acetate buffer protected the lysine residue.

In 1969, Massey et al. reported that flavoprotein dehydrogenase/oxidases have high reactivity with sulfite, preferentially form stabilized red anionic semiquinone radicals, and form H_2O_2 as the first observed product when the dihydroflavin is oxidized with O_2. Evidence was also presented that suggested that these properties correlate with the presence of a positively charge amino acid residue at the active sites of oxidases. More recently, studies with flavoproteins in which the natural flavin nucleotides were replaced with flavin modified at various positions in the isoxalloxazine ring have strengthened the proposed role for a positively charged protein residue in the vicinity of the N(1)–C(2a) region of the flavin (Massey & Hemmerich, 1980, 1982). It is possible that the predicted protonated base that exerts an inductive effect on N(5) via N(1) of the flavin in lactate oxidase is His B (Figure 6). Thus, when His B is modified, the enzyme loses reactivity toward O_2 and sulfite. The modification of His A, however, would abolish the proton-abstraction step and oxalate reactivity but leave flavin reactivity intact. The active site model proposed from these DNP modification studies can be tested in further experiments.

Registry No. FDNB, 70-34-8; N^{im}-DNP-histidine, 25030-55-1; lactate 2-monooxygenase, 9028-72-2; histidine, 71-00-1; N^{α}-acetylhistidine, 2497-02-1.

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