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Chapter 8

L-LACTATE OXIDASE

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I. INTRODUCTION

The first recognition of an unusual enzyme responsible for oxidation of lactate in mycobacteria came from the work of Edson,[1] who found that crude extracts of *Mycobacterium phlei* oxidized only the L-isomer of lactate in a reaction requiring molecular oxygen. Pyruvate and acetate were not oxidized by these extracts, nor could pyruvate or H_2O_2 be detected to accumulate during the oxidation of L-lactate. Instead, acetate was found to accumulate, and as there was liberation of CO_2 stoichiometric with lactate and O_2 consumed, the overall reaction could be written:

$$\text{L-Lactate} + O_2 \longrightarrow \text{Acetate} + CO_2 + H_2O$$

Partial purification of the crude extract showed that activity was associated with a yellow color, and Edson concluded that the lactate oxidase was probably a flavoprotein. He detected flavine adenine dinucleotide (FAD) in the supernatant after acid denaturation by using the apo-D-amino acid oxidase test of Warburg and Christian,[2] a not surprising finding given the small degree of purification. As will be discussed later, the enzyme on extensive purification was indeed found to be a flavoprotein, but containing flavin mononucleotide (FMN) as prosthetic group. Edson also found that under anaerobic conditions, with methylene blue as acceptor, lactate was oxidized to pyruvate. This was later shown to be due to the presence of another enzyme,[3-5] but fortuitously led Edson to suggest what is in essence the sequence of reactions responsible for the oxidative decarboxylation reaction:

$$CH_3CHOHCOOH + Fl_{ox} \longrightarrow CH_3COCOOH + Fl_{red}H_2$$

$$Fl_{red}H_2 + O_2 \longrightarrow Fl_{ox} + H_2O_2$$

$$CH_3COCOOH + H_2O_2 \longrightarrow CH_3COOH + CO_2 + H_2O$$

Edson initially suggested that the catalytic reaction was completed by the known free chemical reaction of oxidative decarboxylation occurring nonenzymically; it was later shown by Lockridge et al.[6] that the latter reaction is indeed a crucial step in catalysis, but occurs while the two reactants, pyruvate and H_2O_2, are still enzyme bound.

Several years after Edson's studies, Yamamura et al.[3] partially purified two L-lactate oxidizing enzymes from *Mycobacterium tuberculosis avium*, one similar in catalytic properties to the Edson enzyme, the other oxidizing L-lactate to pyruvate with methylene blue as acceptor, aerobically or anaerobically. The oxidative decarboxylase activity correlated with the FAD content, so it was concluded, in agreement with Edson, that the enzyme was a FAD-containing flavoprotein. Uncertainty was soon injected, however, by a communication in *Nature*,[7] where it was stated that "our assumption identifying the lactic enzyme with a flavoprotein has proved to be false." While no details were given, this conclusion was reinforced by the first of a series of papers by Sutton[4] on the purification and properties of the enzymes from *M. phlei*. Although the enzyme purification was modest (~10×), no FAD could be detected, and Sutton concluded that the absorption spectrum also excluded "other riboflavin-containing nucleotides." He also concluded that since neither arsenite nor keto acid fixatives such as hydroxylamine or hydrazine influenced the reaction, pyruvate could not be an intermediate. Similarly, since catalase was without effect, he argued that H_2O_2 could not be involved. However, as pointed out by Edson and Cousins,[7] the possibility existed that "pyruvate and hydrogen peroxide (may) react spontaneously on the enzyme surface where they originate" and so could escape detection as free compounds. As will be discussed in more detail later, this prediction proved to be valid.

Further electrophoretic purification of the enzyme[8] resulted in the separation of an inactive brown fraction, with the result that the "preparation was bright yellow and gave a typical flavoprotein absorption spectrum." Unfortunately, no spectrum was reported, nor any details given which would permit assessment of the purity. Analysis of the flavin released by acetone-HCl treatment or by acid-$(NH_4)_2SO_4$ showed a single flavin with properties closely similar to those of FMN. Finally, the enzyme was obtained in pure crystalline form, sedimenting in the ultracentrifuge with a single Schlieren pattern clearly associated with the yellow color of the enzyme.[9] The molecular weight was calculated from sedimentation and diffusion, assuming a partial specific volume of 0.73, to be approximately 260,000. Based on spectroscopic data, which unfortunately were not reported, the minimum molecular weight per flavin was estimated to be ~ 126,000. These values would indicate the enzyme to be a dimer, a conclusion at variance with all later studies, and concluded to be in error, because of an unreliable protein determination.[10] In his 1957 paper, Sutton also demonstrated the formation of pyruvate with substrate quantities of enzyme and lactate under anaerobic conditions, using 2,4-dinitrophenylhydrazine as keto-acid trap, and concluded, in agreement with Edson's original suggestion, that pyruvate was a catalytic intermediate.

When the crystalline enzyme from *M. phlei* was incubated with L-lactate in an atmosphere of $^{18}O_2$ and $H_2{}^{16}O$, the acetate product isolated was found to have incorporated approximately one atom of ^{18}O.[11] Thus lactate oxidase is formally a flavoprotein monooxygenase:

$$CH_3CHOHCOOH + {}^{18}O_2 \longrightarrow CH_3CO^{18}OH + CO_2 + H_2{}^{18}O$$

Accordingly, it is also frequently called L-lactate 2-monooxygenase, as recommended by the Enzyme Nomenclature Commission (EC 1.13.12.4).

At the same time that Sutton was working on the enzyme from *M. phlei,* Cousins was working on the enzyme from *M. phlei, M. stercoris,* and *M. smegmatis*. He chose the latter organism for large-scale preparation because of its higher content of the enzyme.[5] Cousins achieved considerable purification and showed by electrophoretic separation that the major component in his preparation (approximately 50% of the total protein) was associated with

activity and yellow color. The published absorption spectrum has some of the characteristics of a flavoprotein, but was clearly obscured by the presence of an impurity with absorbance maximum near 400 nm. Cousins failed to find a correlation between catalytic activity and FAD content, and unfortunately did not assay for FMN; hence in this work the nature of the prosthetic group was left unclear.

In 1968, improved purification procedures resulting in crystalline preparations from both *M. phlei*[10] and *M. smegmatis*[12] were reported, as well as more refined analyses of the physical properties. In both cases, it was concluded that the prosthetic group is FMN, that the molecular weight is large (340,000 to 400,000 for the *M. phlei* enzyme and 300,000 to 400,000 for the *M. smegmatis* enzyme). The minimum molecular weight per flavin was estimated as 53,300 to 56,200 for the *M. phlei* enzyme and 48,000 to 51,000 for the *M. smegmatis* enzyme, indicating a hexameric-octameric subunit structure. Further analysis of the *M. phlei* enzyme led to the conclusion that it had a molecular weight of approximately 350,000 and was composed of six subunits of molecular weight 56,000.[13,14] This was based on the estimation of flavin content from the absorption spectrum of the isolated enzyme in 0.1 *M* phosphate, pH 7.0, and by the finding of one N-terminal serine residue and one C-terminal arginine residue per 56,000 mol wt. On the other hand, the *M. smegmatis* enzyme, on careful analysis, was shown to be an octamer of subunit molecular weight 43,500.[15] The molecular weight was determined by sedimentation analysis to be in the range 345,000 to 350,000, and the subunit molecular weight was based on the FMN content and quantitative amino acid analysis. The octameric structure has recently been confirmed by determination of the amino acid sequence of 393 residues, yielding a molecular weight of 42,616 for the apoprotein, or 43,072 for the holoenzyme.[16] This corresponds to exactly 8.0 subunits per molecular weight of 345,000. It seems likely that the *M. phlei* enzyme should have the same structure, since the *M. smegmatis* enzyme has been shown to have the same N-terminal serine residue and the same C-terminal arginine. As pointed out by Lockridge et al.[6] and Sullivan et al.,[15] the extinction coefficient of the enzyme-bound flavin is very dependent on the nature of the buffer, and small errors in estimation of flavin content and protein concentration could account for the differences in interpretation of structure. In this respect it is interesting to note that the related enzyme, spinach glycollate oxidase, has recently been shown by X-ray crystallographic analysis to have an octameric structure.[17] Similarly, glycollate oxidase from pumpkin cotyledons was also shown to be an octamer by FMN content and molecular weight determinations.[18]

II. SPECTROSCOPIC PROPERTIES OF THE OXIDIZED AND REDUCED ENZYME

The absorption spectrum of the crystalline enzyme isolated from *M. smegmatis* is quite dependent on the presence or absence of low molecular weight ligands. Most inorganic anions bind to lactate oxidase as competitive inhibitors, with K_d values in the range 0.4 to 50 m*M*.[6,12,15] The effect of phosphate on the flavin absorption spectrum is shown in Figure 1. In general, such perturbation of flavoprotein spectra is common on binding of competitive inhibitors and is useful for determination of dissociation constants of enzyme-ligand complexes, as illustrated in the inset of Figure 1. Thus, both the absorption maxima and extinction coefficients of the enzyme-bound flavin depend strongly on the medium. In 10 m*M* imidazole chloride, pH 7.0, 25°C, the enzyme has λ_{max} at 278, 373, and 458 nm with extinction coefficients (ϵ) of 113, 8.94, and 11.06 $mM^{-1}cm^{-1}$, respectively. The oxidized enzyme is unusual among flavoproteins in having its flavin fluorescence almost completely quenched; this may be due to two conserved tyrosine residues close to the enzyme flavin (see later section on active site structure). On reduction with substrate (after dissociation of the intermediate E~$FMNH_2$·keto acid complex), there is almost complete loss of A_{458}; the reduced

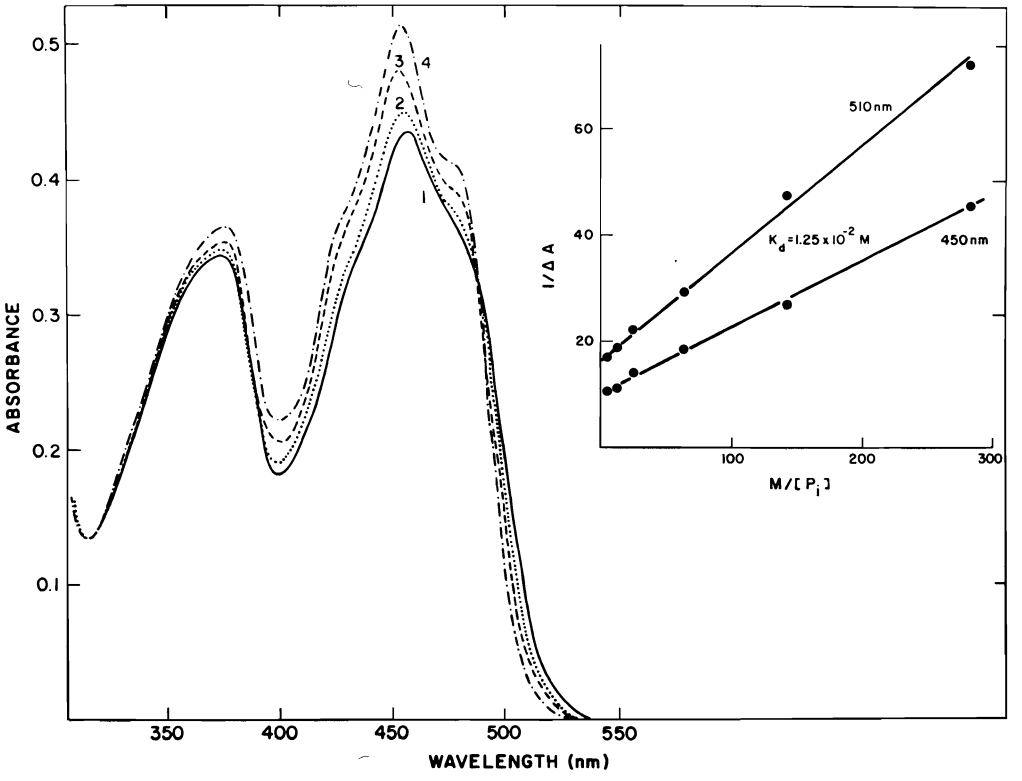


FIGURE 1. Effect of phosphate on the absorption spectrum of lactate oxidase. Curve 1 shows the spectrum of the enzyme, $4 \cdot 10^{-5}$ *M* in 0.01 *M* imidazole, pH 7.0 at 25°C. Curves 2, 3, and 4 are of the same sample after addition of 7, 41, and 197 m*M* phosphate, respectively. The inset shows the dependence of the absorbance at 450 and at 510 nm on phosphate concentration. (From Lockridge, O., Massey, V., and Sullivan, P. A., *J. Biol. Chem.*, 247, 8097, 1972. With permission.)

enzyme flavin has a λ_{max} of 360 nm, $\epsilon = 4.8$ mM^{-1} (see Figure 2). Curiously, the reduced enzyme is quite fluorescent, with an emission maximum at 507 nm and excitation spectrum closely similar to that of the absorption spectrum.[19] This fluorescence is quenched on complex formation with pyruvate and other keto acids, a property which proved very useful in elucidation of the mechanism of substrate dehydrogenation (see later section).

III. THE REACTION WITH L-LACTATE: STEADY-STATE AND STOPPED-FLOW KINETICS

The overall reaction mechanism of lactate oxidase was elucidated with the enzyme from *M. smegmatis,* employing a combination of steady-state and stopped-flow kinetics.[6] The ready interpretation of results was made possible by the recognition that lactate oxidase binds most common anions, including phosphate, with K_d values in the range 10 to 50 m*M*, and that these serve as competitive inhibitors.[12] It was found that the complex stopped-flow results reported by Takemori et al.[20,21] were due to their experiments being conducted in 50 m*M* phosphate, pH 7.0, conditions under which the enzyme exists predominantly as the enzyme phosphate complex, which has to dissociate for reaction with substrate to proceed.[6] Accordingly, all subsequent experiments were carried out in 10 m*M* imidazole·HCl buffer pH 7.0, where minimal inhibition was observed.

The reduction of the enzyme by L-lactate under anaerobic conditions was found to

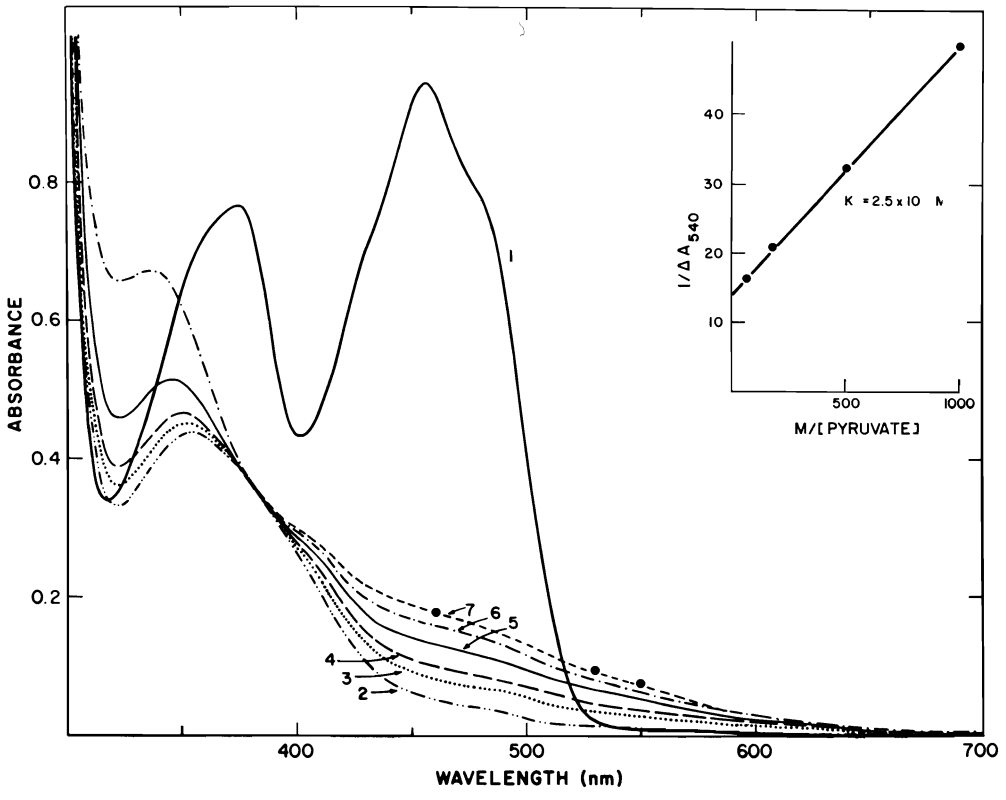


FIGURE 2. Spectrophotometric course of the reaction of lactate oxidase with L-lactate. Curve 1 represents the absorption spectrum of oxidized enzyme $8.6 \cdot 10^{-5}$ *M* under anaerobic conditions in 0.01 *M* imidazole buffer, pH 7.0. Curve 2: same sample 5 min after addition of $8 \cdot 10^{-4}$ *M* L-lactate. Curves 3, 4, 5, and 6: after the addition of $1 \cdot 10^{-3}$ *M*, $2 \cdot 10^{-3}$ *M*, $5.33 \cdot 10^{-3}$ *M*, and $15.35 \cdot 10^{-3}$ *M* pyruvate. The inset shows the extrapolation of the absorbance changes at 540 nm to ∞[pyruvate], Curve 7 (----) shows the spectrum of the fully formed complex calculated in this way. The points coinciding with curve 7 show the absorbance obtained at the end of the rapid phase of reduction of enzyme by L-lactate, determined in separate stopped-flow experiments. (From Lockridge, O., Massey, V., and Sullivan, P. A., *J. Biol. Chem.*, 247, 8097, 1972. With permission.)

proceed via an intermediate species with long wavelength absorbance, whose spectrum is shown in Figure 2, curve 7. The rate of formation of this intermediate was very fast and strongly dependent on L-lactate concentration. The rate of disappearance of the intermediate to yield the spectrum of the free reduced flavoenzyme was independent of substrate concentration, 2.5 min^{-1} at 25°C. At all concentrations of substrate employed, the rate of formation of the intermediate was orders of magnitude faster than the decay, allowing a clean separation of the kinetics. The rate of formation of the intermediate was clearly saturating at high concentrations of substrate, a double reciprocal plot of $1/k_{obs}$ vs. 1/[L-lactate] was linear with a limiting velocity at infinite lactate concentration of 14,000 min^{-1}, consistent with the formation of a Michaelis complex prior to formation of the intermediate:

$$\text{E}\sim\text{FMN} + \text{L-Lactate} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{E}\sim\text{FMN}\cdot\text{Lactate} \xrightarrow{k_2} \text{Intermediate}$$

The K_d (k_{-1}/k_1) for the enzyme-lactate complex can be obtained from the slope/intercept of the double reciprocal plot and is $5 \cdot 10^{-2}$ *M*, indicating rather weak binding of substrate to

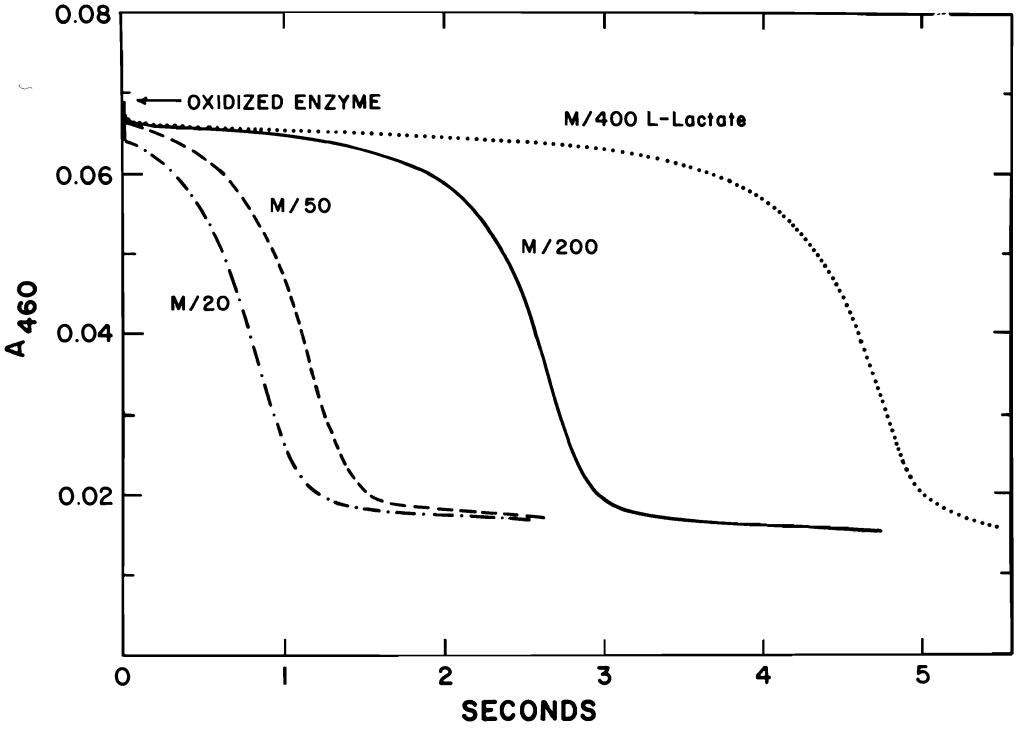


FIGURE 3. Stopped-flow turnover studies with lactate oxidase reacting with L-lactate and oxygen. The enzyme in 0.01 *M* imidazole, pH 7.0, and at 25°C was at a final concentration of $6.35 \cdot 10^{-6}$ *M* and was reacted with the concentrations of lactate shown at an initial oxygen concentration of $2.6 \cdot 10^{-4}$ *M*. The absorbance changes at 460 nm were followed with time. (From Lockridge, O., Massey, V., and Sullivan, P. A., *J. Biol. Chem.*, 247, 8097, 1972. With permission.)

the enzyme, followed by rapid conversion (k_2 = 14,000 min^{-1}) to the intermediate. The latter was shown to be a complex of reduced enzyme and pyruvate, since the spectrum of the intermediate could be developed by equilibrium titration of reduced enzyme with pyruvate, as shown in Figure 2, to yield a K_d for the complex of $2.5 \cdot 10^{-3}$ *M*. Essentially the same value was obtained by measuring the kinetics of formation of the complex from reduced enzyme at different pyruvate concentrations. Hence the overall anaerobic reduction of the enzyme by lactate could be defined:

$$\text{E}\sim\text{FMN} + \text{Lactate} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{E}\sim\text{FMN}\cdot\text{Lactate} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{E}\sim\text{FMNH}_2\cdot\text{Pyruvate} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{E}\sim\text{FMNH}_2 + \text{Pyruvate}$$

with the value of k_3 as 2.5 min^{-1} and k_{-3} as 10^3 $M^{-1}min^{-1}$.

The stopped-flow technique was also used to measure steady-state turnover, using the enzyme absorbance itself to follow the course of catalysis. Typical turnover traces are shown in Figure 3, and Figure 4 shows the results of analysis of these traces as described by Gibson et al.[22] to generate a set of parallel Lineweaver-Burk plots, which define V_{max} as 6250 min^{-1}, K_m(lactate) as $2.2 \cdot 10^{-2}$ *M*, and K_m (O_2) as $7.1 \cdot 10^{-5}$ *M*. The catalytic turnover number is clearly very much larger than the rate of dissociation (k_3) of the E~$FMNH_2$-pyruvate com-

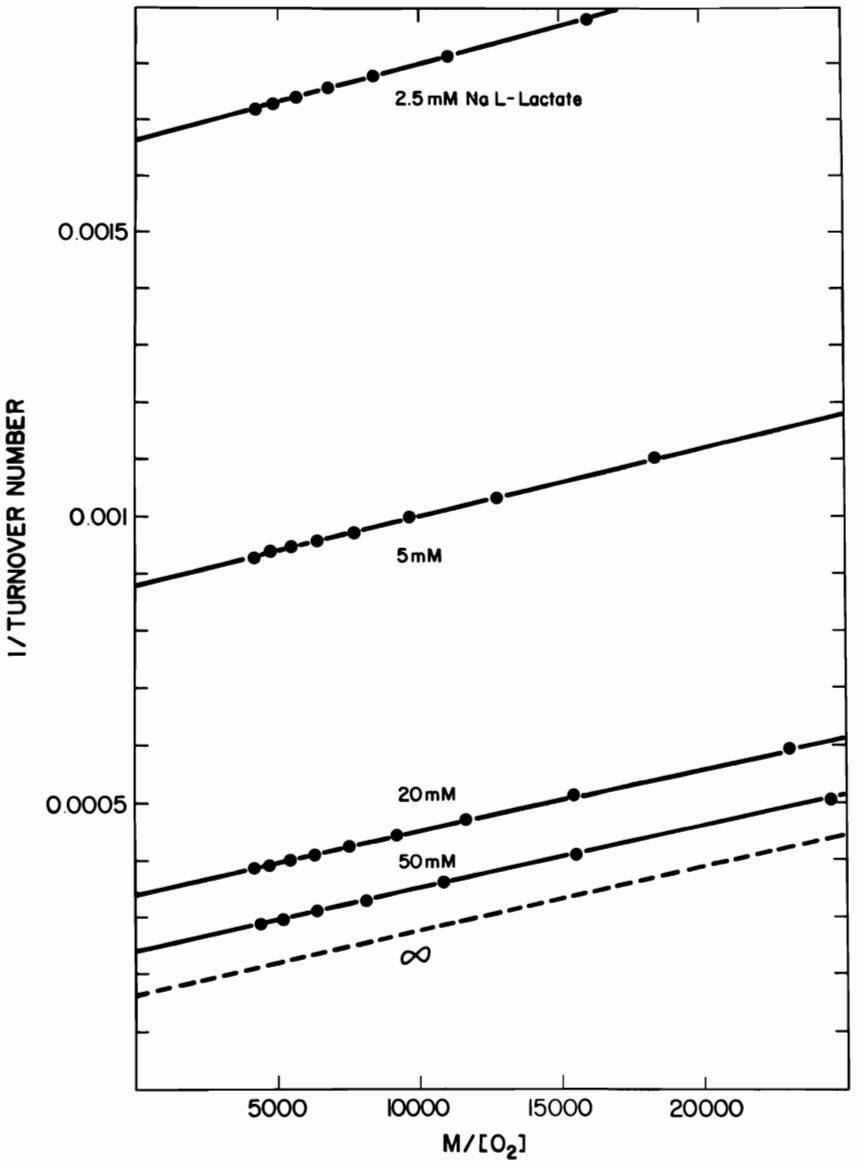


FIGURE 4. Secondary plot of the data obtained from Figure 3 plotted according to Lineweaver-Burk. (From Lockridge, O., Massey, V., and Sullivan, P. A., *J. Biol. Chem.*, 247, 8097, 1972. With permission.)

plex; hence free E~$FMNH_2$ could be ruled out as a catalytically viable species. On the other hand, the rate of formation of the E~$FMNH_2$-pyruvate complex (k_2 = 14,000 min^{-1}) clearly placed it as a likely catalytic intermediate. The turnover traces of Figure 3 reveal that during the steady state of turnover, the enzyme is maintained largely in the oxidized state, implying that the reduced enzyme species which reacts with O_2 must be reoxidized even faster than the oxidized enzyme is reduced by L-lactate.

Further stopped-flow experiments with reduced enzyme, either free or in complex with pyruvate, showed that both species reacted with O_2 in a second-order fashion, but at very different rates: $5.4 \cdot 10^5$ $M^{-1}min^{-1}$ for E~$FMNH_2$ and $1.1 \cdot 10^8$ $M^{-1}min^{-1}$ for E~$FMNH_2$-pyruvate. Parallel Lineweaver-Burk plots such as those of Figure 4 are diagnostic of binary complex (ping pong) mechanisms or of ternary complex mechanisms with certain rate

constants sufficiently small that the usual pattern of converging Lineweaver-Burk plots disappears.

For a binary complex mechanism to operate, O_2 would have to be reacting in catalysis with free E~$FMNH_2$, but as already discussed, this can be ruled out since the formation of free E~$FMNH_2$ is too slow to be of catalytic importance. Thus it is apparent that O_2 reacts catalytically with the E~$FMNH_2$-pyruvate complex, i.e., that a ternary complex mechanism is operational. In this reaction the spectrum of oxidized enzyme is regenerated in a monophasic fashion, i.e., there is no evidence for a flavin C(4a)-hydroperoxide intermediate as found with true flavoprotein monooxygenases (see References 23 and 24 for reviews). The logical chemical reaction to occur in this step is thus the conversion of the E~$FMNH_2$-pyruvate complex to an E~FMN-pyruvate-H_2O_2 complex. If the release of products is slow, then the oxidative decarboxylation reaction between pyruvate and H_2O_2, which occurs slowly in free solution, can be envisaged as occurring rapidly in the complex of oxidized enzyme and primary products. In this way the observations of earlier workers that neither catalase nor carbonyl trapping reagents affected catalysis was also explained. Further evidence for this mechanism was provided by the finding that when reduced enzyme equilibrated with $CH_3{}^{14}COCOOH$ was reacted with O_2, quantities of ^{14}C-labeled acetate stoichiometric with the enzyme were formed.[6]

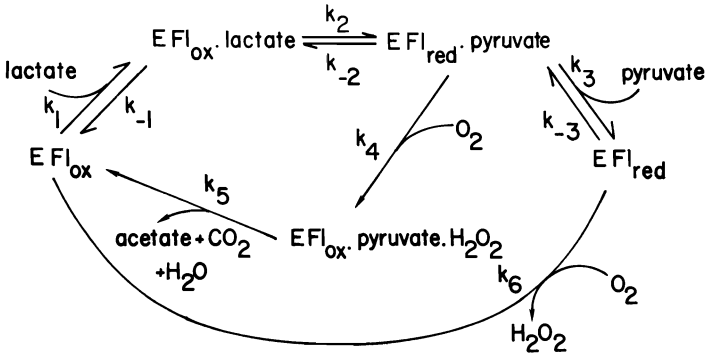
The conclusions concerning the catalytic cycle which could be derived from this work are summarized in Scheme 1. The inner cycle, composed of reactions 1,2,4,5, constitute normal catalysis, while the outer cycle of reactions 1,2,3,6 would constitute a conventional oxidase-type reaction, with free keto acid and H_2O_2 as products. This would represent an "uncoupling" of the normal oxidative decarboxylation sequence and has been found experimentally to occur to a limited extent with glycollate as substrate (see Section XII.C). In normal catalysis, a further rate-limiting step, k_5, is required for a fit of the steady-state and stopped-flow kinetic data. In the original paper,[6] this was proposed to be liberation of products; the actual chemical rate-limiting step could be the oxidative decarboxylation of pyruvate by H_2O_2 at the active site followed by rapid dissociation of products. The initial rate equation for the catalytic cycle of Scheme 1 is

$$V = \frac{V_{max}}{1 + \frac{K_{lactate}}{[lactate]} + \frac{K_{O_2}}{[O_2]} + \frac{K}{[lactate]\cdot[O_2]}}$$

$$V_{max} = \frac{k_2 \cdot k_5}{k_2 + k_5}$$

$$K_{lactate} = \frac{k_5(k_{-1} + k_2)}{k_1(k_2 + k_5)} \qquad K_{O_2} = \frac{k_5(k_2 + k_{-2})}{k_4(k_2 + k_5)} \qquad K = \frac{k_{-1}\cdot k_2 \cdot k_5}{k_1 \cdot k_4 \cdot (k_2 + k_5)}$$

Rate constants for reaction of the enzyme with L-lactate and a number of other L-α-hydroxy acid substrates which show the same type of kinetic behavior (derived from similar stopped-flow data) are listed in Table 1. From the observed values of V_{max} and k_2, the value of k_5 can be calculated. The value of k_4 has been determined directly in the case of L-lactate and L-α-hydroxy-β-methyl valerate as substrates, allowing a comparison of K_{O_2} calculated from the kinetic constants above with the experimentally observed values. The agreement is impressive: $K_{O_2} = 5.5 \cdot 10^{-5}$ *M* (calculated) versus $7.1 \cdot 10^{-5}$ *M* (observed) with lactate as substrate and $8.1 \cdot 10^{-5}$ *M* (calculated) versus $8.0 \cdot 10^{-5}$ *M* (observed) with L-α-hydroxy-β-methyl valerate as substrate. These calculations assumed that k_{-2} is negligible, an assumption justified by the anaerobic titration data of Figure 2, where addition of large concentrations of pyruvate failed to result in any detectable accumulation of oxidized enzyme species. This



SCHEME 1. Kinetic sequence depicting the steps involved in turnover of lactate. The inner loop represents normal catalysis, while the outer loop shows the steps involved in the so called uncoupling. (Adapted from Lockridge, O., Massey, V., and Sullivan, P. A., *J. Biol. Chem.*, 247, 8097, 1972. With permission.)

TABLE 1
Kinetic Constants of Lactate Oxidase with Various Substrates

The values were obtained as described in Reference 6, chiefly from stopped-flow studies. All values were obtained in 0.01 *M* imidazole, pH 7.0, 25°C

Constant	L-lactate	β-Phenyl-L-lactate	L-α-OH-iso-valerate	L-α-OH-β-Me-valerate
$K_d = k_{-1}/k_1$	$5 \cdot 10^{-2}$ *M*	0.34 *M*	$9 \cdot 10^{-2}$ *M*	$3 \cdot 10^{-2}$ *M*
k_2	14,000 min^{-1}	6,700 min^{-1}	3,700 min^{-1}	590 min^{-1}
k_3	2.5 min^{-1}	~2 min^{-1}	~0.2 min^{-1}	
k_{-3}	$1 \cdot 10^3$ M^{-1} min^{-1}			
k_4	$1.1 \cdot 10^8$ M^{-1} min^{-1} (observed)	$5.7 \cdot 10^6$ M^{-1} min^{-1} (calculated)	$3.3 \cdot 10^6$ M^{-1} min^{-1} (calculated)	$2.7 \cdot 10^6$ M^{-1} min^{-1} (observed)
k_5	11,300 min^{-1}	1,060 min^{-1}	1,370 min^{-1}	350 min^{-1}
	$V_{max,catalytic}$ 6,250 min^{-1}	910 min^{-1}	1,000 min^{-1}	220 min^{-1}
	$K_{RCHOH\text{-}COOH}$ $2.23 \cdot 10^{-2}$ *M*	$5 \cdot 10^{-2}$ *M*	$2.5 \cdot 10^{-2}$ *M*	$1.13 \cdot 10^{-2}$ *M*
K_{O_2}	$7.1 \cdot 10^{-5}$ *M*	$1.6 \cdot 10^{-4}$ *M*	$3 \cdot 10^{-4}$ *M*	$8 \cdot 10^{-5}$ *M*

k_6 (free enzyme) = $5.4 \cdot 10^5$ M^{-1} min^{-1}

same factor would also account satisfactorily for the observation with all the substrates of Table 1 of parallel Lineweaver-Burk plots, since then the last term in the denominator of the initial rate equation, K, would become negligible.

From the data of Table 1, it is evident that the active site is able to accommodate α-hydroxy acids much larger than lactate, although in all cases, including L-lactate, binding is weak. Interestingly, the D-isomers of these substrates are all competitive inhibitors and bind more tightly than the L-isomers. For example, under the conditions of Table 1, stopped-flow spectroscopic studies of the perturbation of the visible absorbance spectrum show that D-lactate binds to the enzyme with a K_d of $\sim 1.7 \cdot 10^{-3}$ *M*, with $k_{on} = 4.5 \cdot 10^3$ $M^{-1}s^{-1}$ and $k_{off} = 7.5$ s^{-1}.[103] With the substrates of Table 1, it should also be noted that the keto acid products also dissociate slowly from the reduced enzyme, α-keto isovalerate dissociating ~10 times more slowly than pyruvate and α-keto-β-methyl valerate dissociating so slowly that it was not possible to determine accurately its rate constant. With all these reduced enzyme keto acid complexes, the reaction with O_2 is appreciably slower than with E~$FMNH_2$-pyruvate, presumably reflecting more limited access of O_2 to the reduced flavin when the bulky products are bound.

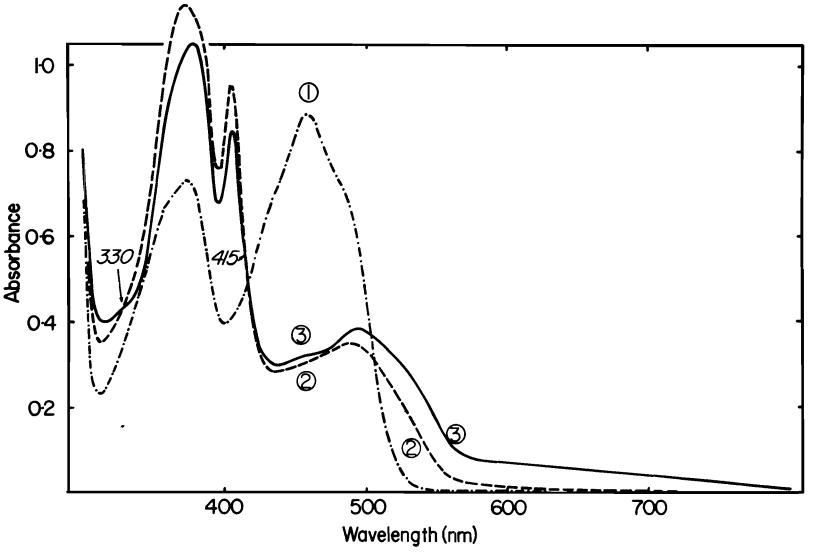


FIGURE 5. Effect of pyruvate on the absorption spectrum of the semiquinone of FMN-lactate oxidase. Curve 1 is that of the oxidized enzyme, $8.3 \cdot 10^{-5}$ *M* in $1.3 \cdot 10^{-2}$ *M* imidazole, pH 7, 25°C, under anaerobic conditions. Curve 2: enzyme semiquinone obtained by photoirradiation with catalytic amounts of deazaflavin. Curve 3: after the addition of $1.3 \cdot 10^{-2}$ *M* pyruvate.

IV. REDOX PROPERTIES OF LACTATE OXIDASE

In common with most flavoprotein oxidases, lactate oxidase has been found on enforced one-electron reduction, e.g., by photochemical reduction,[25,26] to stabilize the red-colored flavin anion radical over the whole pH range of stability of the enzyme. As will be discussed in a later section, this observation provided one of the first pieces of evidence for the presence of a positively charged residue of the protein positioned close to the flavin N(1)-position. Despite its thermodynamic stabilization by the protein, the anion radical is found to react reasonably rapidly with O_2, with a second-order rate constant of $4 \cdot 10^5$ $M^{-1}\text{min}^{-1}$ at pH 7.0, 25°C,[27] only slightly smaller than that for E~$FMNH_2$ under the same conditions, $5.4 \cdot 10^5$ $M^{-1}\text{min}^{-1}$.[6] Remarkably, the enzyme radical form binds pyruvate and many other keto acids much more tightly than either the oxidized or fully reduced enzyme. For example, at pH 7.0, 25°C, pyruvate binds to E~$FMNH_2$ with a K_d of $2.5 \cdot 10^{-3}$ *M*,[6] to E~FMN with a K_d of $3.3 \cdot 10^{-3}$ *M*,[104] and to E~$FMN^{\cdot -}$ with a K_d of $1.36 \cdot 10^{-5}$ *M*. It should be noted that a similar tight binding of pyruvate to the anion semiquinone form of yeast flavocytochrome has been reported.[28] The complex between keto acid and the radical form of the enzyme is characterized by appreciable changes in absorbance spectrum (see Figure 5) which permit the determination of k_{on} and k_{off} in the equilibrium:

$$\text{E}\sim\text{FMN}^{\cdot -} + \text{Pyruvate} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \text{E}\sim\text{FMN}^{\cdot -}\ \text{Pyruvate}$$

From stopped-flow experiments, the value of k_{on} was determined as $1.9 \cdot 10^3$ $M^{-1}\text{min}^{-1}$, while k_{off} was remarkably slow, 0.021 min^{-1}. Thus K_d determined kinetically from the ratio k_{off}/k_{on} was $1.17 \cdot 10^{-5}$ *M*, in good agreement with the value of $1.36 \cdot 10^{-5}$ *M* determined from static titration data.[27] It should be noted that this value of k_{on} is similar to that found for binding of pyruvate to E~$FMNH_2$; the remarkable difference is with k_{off}, which is two

orders of magnitude slower than that for the E~$FMNH_2$-pyruvate complex. The possible reason for this effect will be considered in a later section, taking into account recent information about the structure of the active site.

The most remarkable feature of the enzyme radical-keto acid complex is that the ability to be reoxidized rapidly by O_2 is almost completely lost. The enzyme radical-keto acid complex can be formed anaerobically at 4°C by mixing the O_2-sensitive radical with an excess of keto acid, then opened to air, and excess keto acid removed by gel filtration with Sephadex G-25 at 4°C, to yield essentially 100% E~$FMN^{\cdot -}$-keto acid complex containing equimolar amounts of flavin and keto acid. Such complexes can then be warmed to 25°C and the kinetics of reoxidation followed. With the pyruvate complex, the rate of reoxidation was shown to be the same as that of k_{off} determined independently under anaerobic conditions. Thus, while the binding of pyruvate to E~$FMNH_2$ increases the O_2-reactivity by approximately 200-fold,[6] binding of pyruvate to E~$FMN^{\cdot -}$ essentially abolishes reaction with O_2!

The thermodynamic stabilization of the flavin anion radical by lactate oxidase was confirmed by Stankovich and Fox[29] by redox potential determinations. At pH 7.0, 25°C, in 0.01 *M* imidazole buffer, the E_m value for the E~FMN/E~$FMN^{\cdot -}$ couple was found to be -67 ± 6 mV and that for the E~$FMN^{\cdot -}$/E~$FMNH^-$ couple to be -231 ± 4 mV. From these data the potential of the overall $2e^-$ reduction can be calculated as E_mE~FMN/E~$FMNH^-$ $= -149 \pm 5$ mV. This value is quite consistent with the pH 7 value of the pyruvate/lactate couple of -189 mV[30] and approximately stoichiometric reduction of the enzyme by lactate under anaerobic conditions. The much tighter binding of pyruvate to the semiquinoid state than to either the oxidized or reduced enzyme would predict on thermodynamic grounds an even wider separation of the two half potentials in the presence of pyruvate, with the E_m value of the E~FMN/E~$FMN^{\cdot -}$ couple being raised to approximately $+80$ mV and that of the E~$FMN^{\cdot -}$/E~$FMNH^-$ couple lowered to approximately -370 mV. This could be in part the explanation of the greatly lowered O_2-reactivity of the radical-keto acid complexes, since the high potential of the E~FMN/E~$FMN^{\cdot -}$ couple would make the one-electron reduction of O_2 thermodynamically difficult (E_m, pH 7 for O_2/O_2^- $= -160$ mV[31]).

V. INHIBITION BY SULFITE

Sulfite is a very potent inhibitor of lactate oxidase due to its reversible equilibrium reaction with the enzyme flavin to form a flavin N(5)-sulfite adduct.[32,33] This is a reaction which is common to most flavoprotein oxidases, but binding to lactate oxidase and the related glycollate oxidase is particularly strong.[32] Sulfite also binds tightly to the flavin of yeast flavocytochrome b_2, which also oxidizes L-α-hydroxy acids, but is not an oxidase.[34] It has long been proposed that the ability of flavoproteins to form the flavin N(5)-sulfite adduct and to stabilize a variety of other negatively charged flavin species is linked to the inductive effect of a positively charged residue of the protein located near the flavin N(1)-position.[32,35] The X-ray crystal structure of cytochrome b_2 indeed reveals that Lys-349 is located in just the right position to stabilize flavins bearing a negative charge in the N(1)C(2) = 0 region, and thus provides a striking verification of the earlier hypothesis.[36] An almost identical situation has been shown in the crystal structure of spinach glycollate oxidase, with Lys-230 adjacent to the flavin N(1)-position.[37] As will be discussed in detail in a later section, in lactate oxidase, Lys-266 occupies the homologous position in the highly conserved amino acid sequence.[16] The crystal structure of the cytochrome b_2-sulfite adduct has recently been reported, and shows very beautifully the linkage of sulfite to the flavin N(5)-position.[38]

In the initial paper describing the tight binding of sulfite to lactate oxidase,[32] a K_d of 3.7 μ*M* at pH 7.0, 25°C was reported. However, this determination was made in the presence of 0.1 *M* phosphate, which was subsequently found to be a competitive inhibitor.[6] It has

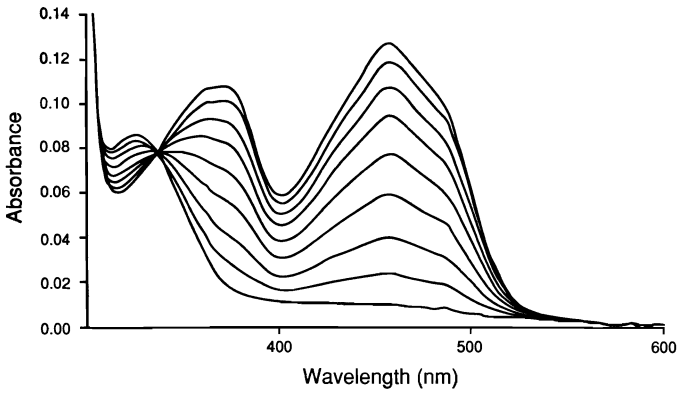


FIGURE 6. Absorption spectrum of the lactate oxidase-sulfite N(5) covalent adduct and decay in the presence of MMTS. The lowest curve is that of the enzyme $1.2 \cdot 10^{-5}$ *M* in 0.01 *M* imidazole, pH 7, 25°C upon addition of $2 \cdot 10^{-4}$ *M* sulfite. The subsequent curves were recorded with a Hewlett Packard Diode Array spectrophotometer at 3, 7, 13, 20.5, 30, 42, 62, and 128 min after addition of 10^{-3} *M* MMTS. From this sequence the rate of decay (k_{off}) of the sulfite complex was estimated. See text for further details.

therefore been obvious for a long time that the true K_d must be considerably lower, of the order of 10^{-7} *M* or less, but this has been difficult to determine experimentally. Recently we have done this by kinetic means. The formation of the sulfite adduct was followed by the stopped-flow technique, and k_{on} was determined as $1.29 \cdot 10^4$ $M^{-1}s^{-1}$ at pH 7.0, 24°C.[105] The value of k_{off} was determined by making use of the reactivity of sulfite with methylmethane thiolsulfonate (MMTS), which thus removes sulfite from the equilibrium as it is released from the enzyme flavin:

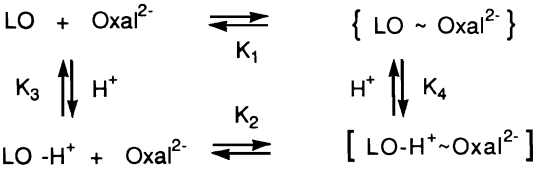
$$\text{E}\sim\text{FMN}\text{–N(5)–}\text{SO}_3^- \underset{k_{on}}{\overset{k_{off}}{\rightleftarrows}} \text{E}\sim\text{FMN} + \text{SO}_3 =$$

$$\text{CH}_3\text{–SO}_2\text{–S–CH}_3 + \text{SO}_3 = \xrightarrow[\text{I}]{\text{fast}} \text{CH}_3\text{–S–SO}_3^- + \text{CH}_3\text{SO}_2^-$$

A typical experiment is shown in Figure 6, from which the value of k_{off} can be calculated as $7.2 \cdot 10^{-4}$ s^{-1}. As expected from these equations, this value was independent of the concentration of MMTS used. Control experiments showed that MMTS affected neither the spectrum nor the activity of the free enzyme. The K_d, calculated from the ratio k_{off}/k_{on}, is $5.6 \cdot 10^{-8}$ *M*.

VI. BINDING OF DICARBOXYLIC ACIDS AND INHIBITION

The simplest dicarboxylic acid, oxalate, has long been recognized as an inhibitor of enzymes oxidizing lactate[39] and as such has been studied extensively. It is a potent competitive inhibitor and it binds very tightly to lactate oxidase and with strong perturbations of the flavin spectrum.[39] What sets aside oxalate, and to a lesser extent also higher dicarboxylic acids, is the mode of binding to the enzyme.[39,40] While most monocarboxylic acids bind to lactate oxidase in a simple equilibrium with K_d's in the range 10^{-2} to 10^{-3} *M*,[6] dicarboxylic acids bind in a two-step equilibrium process. The first step, K_1, has K_d values around 10^{-2} *M* and is fast; it leads to the {LO~$Oxal^{2-}$} complex shown in Scheme 2. The second step, K_4, is established slowly and leads to the marked spectral changes mentioned earlier (complex



SCHEME 2. Steps involved in the formation of the lactate oxidase-oxalate complex. Free oxidized enzyme (LO) binds oxalate rapidly and reversibly (K_1). Subsequently the complex becomes protonated in a slow and pH-dependent step (K_4) to form the stable and photoreactive complex (lower right). Steps K_3 and K_2 would lead to the same complex via the reversed sequence of events. The equilibria of steps K_3 and K_4 represent the ionizations of uncomplexed and complexed enzyme. See text for details.

[LO-H+~$Oxal^{2-}$]). Most importantly, it goes along with uptake/dissociation of one H^+ per molecule of oxalate bound. Thus, the overall constant for the binding of oxalate to lactate oxidase at pH 7 and in imidazole·HCl buffer is $1.6 \cdot 10^{-5}$ *M*.[40] The overall binding can thus be formulated as shown in Scheme 2.

The second complex is also the one which is photochemically reactive,[39] as discussed later. The rates of proton uptake and release are linearly dependent on [H^+] in the neutral range and show breaks which correspond to the protein ionization constants K_3 and K_4. K_3, the dissociation of uncomplexed enzyme, corresponds to a pK of 4.7; K_4 is that of lactate oxidase complexed to oxalate and its pK value of 9.8 indicates a microscopic pK shift corresponding to ~7 kcal/mole. A similar behavior was found with malonate with the difference that in this case the second ionization of malonate (pK_{a2} = 5.7) is higher than pK_3, resulting in a monophasic binding in the pH range below pK_{a2} (where malonate is monoanionic). From these results it has been proposed that oxalate and, to a lesser extent, malonate are analogs of the carbanionic transition state proposed to occur during dehydrogenation of α-hydroxycarboxylic acids.[40] Based on the observed pK value of 4.7, it was also proposed that the amino acid function is a histidine, and that this base is involved in α-proton abstraction. This has turned out to be correct, as nicely demonstrated by the three-dimensional structures of cytochrome b_2 and glycollate oxidase. In order to rationalize the findings in structural terms, we have fitted oxalate into the frame of the active center analogous to that of flavocytochrome b_2 (see Chapter 7) by simply replacing oxalate for pyruvate, the ligand cocrystallizing in the case of cytochrome b_2. The result of this exercise is shown in Figure 7.

One of the carboxylates of oxalate binds to Arg-293 and interacts with Tyr-44 exactly the same way as normal substrate does. His-290 stabilizes the second negative charge of oxalate, and this would thus be the function having the pK of 4.7. Additionally, one oxalate oxygen could also interact with Tyr-152, while the carbonyl function takes the place of the lactate methyl group. The molecular interactions shown in Figure 7 provide a rationale for the pK shift of ~5 units occurring upon binding of oxalate. The slowness of the steps of proton uptake ($k_4 = 4 \cdot 10^8\ M^{-1}\ min^{-1}$) and release ($k_{-4} = 0.06\ min^{-1}$) can be rationalized if one assumes a well-shielded active center. Three-dimensional displays of the protein portion around the active center are compatible with restricted access of solvent in the presence of small ligands. This assumption goes along with the findings of Walsh et al.,[41] which require restricted exchange of the H^+ abstracted from the substrate α-position. It is also interesting to note that the relatively small change in size between oxalate and malonate increases the rates of proton uptake and release by His-290 by approximately two orders of magnitude. This indicates a much looser fitting, which would induce a better accessibility or mobility at the active center.

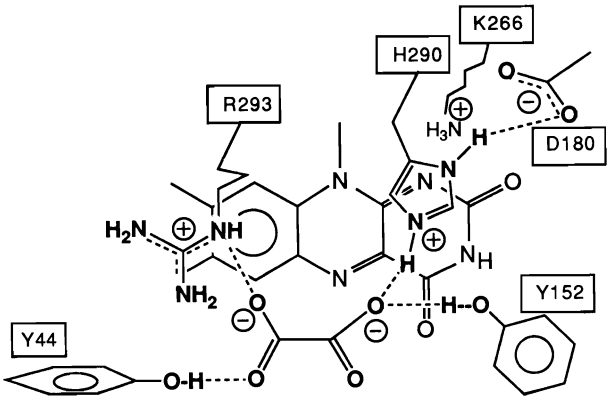


FIGURE 7. Representation of the active site of lactate oxidase binding oxalate. The orientation of the functional groups was adapted from the drawings of the active centers of cytochrome b_2[36] and of glycollate oxidase[37] (see Chapter 7). Oxalate was fitted into the active center simply by replacing pyruvate in the representation of the cytochrome b_2 active center. Note that this complex corresponds to the [LO-H$^+$~Oxal^{2-}] complex of Scheme 2.

VII. PHOTOCHEMICAL REACTIONS AND FORMATION OF COVALENT ADDUCTS

L-Lactate oxidase is probably the flavoenzyme for which photochemical reactions have been most studied and which yields a wide range of products. In general, both the oxidized and the reduced form of the flavin and of flavoenzymes could be expected to be photoreactive. Free reduced flavins and reduced flavoenzymes, on the other hand, are generally assumed not to be photoactive. We will discuss the two types of photoreaction separately.

A. PHOTOCHEMICAL REACTIONS OF OXIDIZED LACTATE OXIDASE

The 5-deazaflavin-mediated photoreduction of oxidized lactate oxidase is facile and yields the anionic semiquinone;[26] this is, strictly speaking, a photoreaction of free deazaflavin and will not be addressed here. The common pattern of photoreactions of free oxidized flavin,[42] and also of oxidized lactate oxidase, involves decarboxylation of a substrate and the addition of the resulting residue to the flavin:

$$Fl_{ox} + R\text{–}COOH + h\nu \rightarrow Fl_{red}H\text{–}R + CO_2$$

This stoichiometry has been demonstrated for the reaction of oxalate using the ^{14}C-labeled compound.[39] Since one carboxylate group is fixed by the interaction with Arg-293, as shown in Figure 7, the α-position of oxalate comes to lie above N(5). Indeed all photoadducts formed starting from oxidized lactate oxidase are N(5) derivatives. The nature of the residue R in the adduct depends on that of the starting carboxylic acid; it can be either a substituted alkyl or a functional group.[43,44] Interestingly, however, "unsubstituted" carboxylic acids such as acetate are not photoreactive.

The absorption spectra of lactate oxidase flavin covalent adducts are similar to those of the corresponding free species, although somewhat red shifted. Most remarkable is their fluorescence, which contrasts with the nonfluorescence of most analogs free in solution. The stability of lactate oxidase photoadducts depends entirely on the nature of the N(5) substituent. Thus, N(5)acetyl is stable, while carbonates, carbamates, or enolamines are labile.[43,44] Worth noting is the good photoreactivity of 2-thio- and 4-thio-FMN-lactate oxidase, in contrast to the free molecule.[43] No specific deductions can be made with respect to the mechanism of photoreaction. A likely possibility is a reaction in which the excited

flavin (a strong oxidant) oxidizes the carboxylate by abstraction of an electron, thus initiating decarboxylation, as has been suggested for the photoreaction of the free system.[45]

B. PHOTOCHEMICAL REACTIONS OF REDUCED LACTATE OXIDASE

The discovery of this reaction[43] is instructive, since it occurred during attempts to alkylate reduced lactate oxidase with bromoacetate, a reaction which was expected to lead to "normal" alkylation of the flavin positions N(5) or C(4a) or to alkylation of a cysteine residue, which is supposed to be located in the vicinity of the active center (see further). As a matter of fact, neither of these processes occur in the dark, but attempts to follow the course of the reaction by monitoring fluorescence changes of reduced lactate oxidase led to the discovery that changes, i.e., alkylation, did occur, but that the process was induced by the excitation light. These findings suggest that the orientation of the reactants is not correct for reaction in the ground state and that irradiation might affect the mobility to an extent sufficient for reaction. The three-dimensional structure of the active center of b_2 and LO suggests that the α-halide of a carboxylic acid might take the place normally occupied by the lactate $-CH_3$ group (see Figure 7 or Figure 10); this would not be a position favorable for a "simple" SN_2 substitution. Thus the alkylation might be induced by the vibrations arising from the relaxation of the excited state and, strictly speaking, the reaction in question is probably not a classical photochemical one, in a general sense.

The photochemistry of reduced lactate oxidase, while of limited interest as such, has allowed the synthesis of several well-defined flavin adducts not readily obtainable by other methods, which have been of great value as model compounds.[43] The comparison of the velocity of reaction and the type of product obtained using ω-halogenated carboxylic acids of varying chain length has led to the proposal of a mode of their binding with respect to the flavin,[43] which has later been proven to be correct in its essence by X-ray crystallography.

VIII. INFORMATION ON THE FLAVIN BINDING SITE FROM INCORPORATION OF ARTIFICIAL FLAVINS

In recent years much valuable information about the nature of the flavin binding site in flavoproteins has come from studies where the native flavin has been replaced by artificial flavins with selective properties specific to the individual flavin (for reviews, see References 46 and 47). Thus, even though the crystal structure of lactate oxidase is not yet available, a lot of information about the protein in the immediate vicinity of the flavin is known from flavin replacement studies, much of which has been corroborated in a striking fashion from the recent X-ray crystal structures of the related enzymes, yeast flavocytochrome b_2, and glycollate oxidase. For example, the series of studies on the stereospecificity of flavin-substrate interactions using 5-deaza-8-hydroxyflavins showed that in both D-lactate dehydrogenase and L-lactate oxidase, substrate reacts with the enzyme flavin only on the Si-face, the opposite face than that employed by glutathione reductase and a series of other NAD(P)H-interacting enzymes.[48,49] The same Si-face of the flavin has been found to interact with the product, pyruvate, in the crystal structure of flavocytochrome b_2[50] and the inhibitor, thioglycollate, in the crystal structure of spinach glycollate oxidase.[17,37]

Studies with 8-Cl-FMN- and 8-mercapto-FMN-substituted lactate oxidase revealed that the chemically reactive 8-substituent of these flavins was not accessible to solvent-borne reagents.[51] The same conclusion could be drawn from the crystal structures of cytochrome b_2 and glycollate oxidase (previous references).

Lactate oxidase containing 2-thio-FMN was found to be unreactive with methylmethane thiolsulfonate[52] as well as with H_2O_2 and *m*-chloroperbenzoate,[53] indicating that this portion of the flavin is also shielded from solvent by the protein. Reconstitution of the apoenzyme with FMN-2-S-oxide gave a stable enzyme with spectral properties similar to those of the

TABLE 2
Kinetic Constants of Native Lactate Oxidase and of Enzyme Reconstituted with 2-Thio-FMN and with Iso-FMN

	Native enzyme[a]	2-Thio-FMN[b]	Iso-FMN[c]
V_{max}	6200 min^{-1}	4500 min^{-1}	Large but indeterminate
$K_{lactate}$	22 m*M*	8 m*M*	Large but indeterminate
K_{O_2}	0.071 m*M*	0.22 m*M*	Large but indeterminate
K	~0	~0	~0
k_{-1}/k_1	50 m*M*	28 m*M*	80 *M*
k_2	$1.4 \cdot 10^4$ min^{-1}	$1.0 \cdot 10^4$ min^{-1}	$2.0 \cdot 10^4$ min^{-1}
k_{-2}	~0	~0	~0
k_3	2.5 min^{-1}	105 min^{-1}	2.6 min^{-1}
k_{-3}	10^3 $M^{-1}min^{-1}$	$8.4 \cdot 10^3$ $M^{-1}min^{-1}$	not determined
k_4	$1.1 \cdot 10^8$ $M^{-1}min^{-1}$	$5.4 \cdot 10^7$ $M^{-1}min^{-1}$	$1.4 \cdot 10^5$ $M^{-1}min^{-1}$
k_5	$1.13 \cdot 10^4$ min^{-1}	$8.2 \cdot 10^3$ $M^{-1}min^{-1}$	large but indeterminate
k_6	$5.4 \cdot 10^5$ $M^{-1}min^{-1}$	$2.9 \cdot 10^5$ $M^{-1}min^{-1}$	$8.4 \cdot 10^4$ $M^{-1}min^{-1}$

All values were obtained in 10 m*M* imidazole·HCl buffer, pH 7.0 at 25°C.

[a] Reference 6.
[b] Reference 54.
[c] Reference 58.

free flavin-2-S-oxide in apolar solvents, indicating that this portion of the flavin might be experiencing a hydrophobic environment in the protein.[53] However, this conclusion does not appear to be compatible with results from other studies, to be described later, which strongly suggest the presence of a protein positively charged residue interacting with the flavin N(1)-C(2 = 0) locus. The 2-thio-FMN enzyme has proved useful in other ways. It has been subjected to a detailed kinetic analysis and found to be very similar to native enzyme in practically all its catalytic properties, including values of individual rate constants, as shown in Table 2.[54] It also reacts with the suicide substrate α-hydroxybutynoate to give a covalent adduct with the flavin,[43] presumably a C(4a)-N(5)-cyclic adduct as with the native flavin.[55,56] This adduct of the 2-thio-FMN, and a similar C(4a)-adduct produced photochemically, have very different spectral properties than N(5)-adducts of 2-thioflavins and in this way have proved useful in differentiating between N(5)- and C(4a)-catalytic intermediates in this[44] and other[57] enzymes. 4-Thio-FMN also binds to lactate oxidase to give a holoenzyme which is reduced rapidly by L-lactate, resulting in a reduced flavin anion species with intense fluorescence.[59] However, the holoenzyme is not very stable, and over a period of days at 0°C gradually undergoes desulfuration of the flavin to yield normal FMN and what appears to be a protein persulfide. The latter imparts a long wavelength absorption band to the spectrum, typical of a charge transfer complex, which is discharged on reaction with cyanide.

These properties indicate that there may be a protein thiol residue near the flavin which is able to induce the desulfuration of the 4-thio-FMN with the concomitant formation of a persulfide. The slowness of the conversion leaves open the possibility that the initial reaction occurs not in the flavin binding site, but with a thiol on the protein surface reacting with dissociated 4-thio-FMN, followed by rebinding of the nascently formed FMN. However, the concomitant appearance of the long wavelength band would imply that the persulfide residue must be close to the flavin. The protein-bound 4-thio-FMN reacts rapidly with H_2O_2, again with desulfuration, showing that the flavin 4-position is accessible to small molecules. Addition of sulfite to 4-thio-FMN enzyme results in rapid formation of a 4-thio-FMN-N(5)-sulfite adduct, analogous to that of native enzyme.[60] This reaction, of course, requires ac-

cessibility from the solvent to the flavin 5-position. Similar conclusions were reached by Jorns et al.,[61] who showed the formation of a stable flavin 4a,5-epoxide on treatment of 5-deaza-FMN lactate oxidase with H_2O_2 or *m*-chloroperbenzoate. It is interesting to note that pig liver glycollate oxidase, which also binds sulfite strongly,[32] when substituted with 5-deaza-FMN and reacted with H_2O_2, also forms the flavin 4a,5-epoxide, but then undergoes further reactions involving disruption of the flavin pyrimidine ring.[62] These results imply some significant differences in the flavin binding site of lactate oxidase and glycollate oxidase.

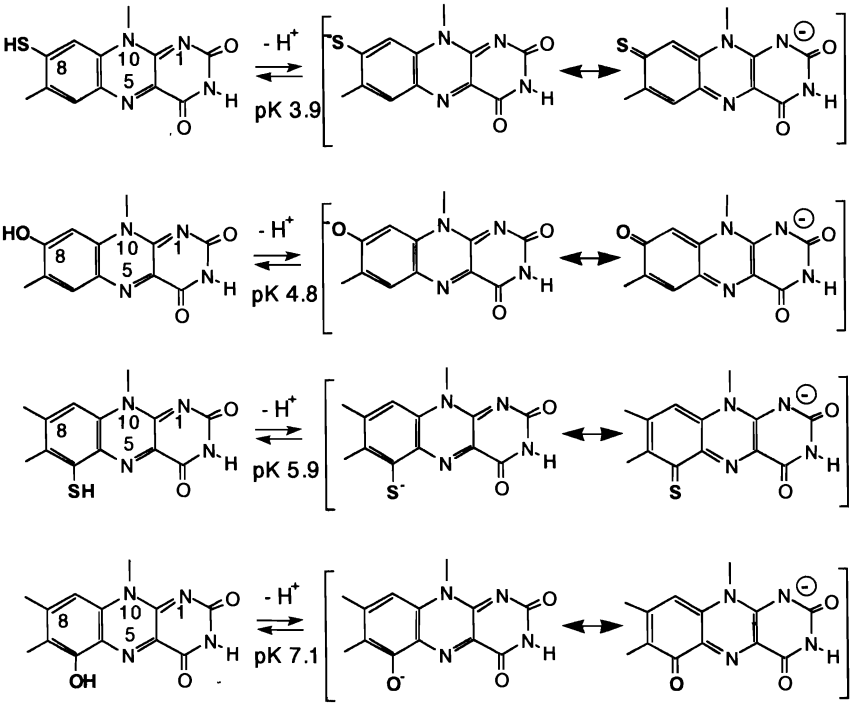
6-Thiocyanato-FMN also reacts spontaneously with the apoprotein of lactate oxidase to yield a stable 6-mercapto-FMN enzyme, with the elimination of cyanide.[63] Unfortunately it has not yet been possible to determine whether this process occurs in the active site, or whether the conversion occurs with surface thiols followed by incorporation of the resultant 6-mercapto-FMN in the active site. The 6-mercapto-FMN enzyme reacts only slowly with iodoacetamide, *N*-ethylmaleimide, or methyl methane thiolsulfonate, all of which react rapidly with free 6-mercaptoflavins, thereby implying limited access of these reagents to the flavin 6-position. However, reaction with H_2O_2 or *m*-chloroperbenzoate to yield the flavin 6-S-oxide is quite fast. These results would imply reasonable accessibility, but unfortunately this apparent dilemma cannot be properly evaluated, as it was not possible to follow the rate of the same process with free 6-mercaptoflavins, where the product of oxidation is the 6-mercaptoflavin dimer. Flavins containing OH- or SH-substituents at the 6- or 8-positions constitute a group of artificial flavins which are very sensitive to the protein environment, especially to positively or negatively charged residues interacting with the flavin pyrimidine ring (see Reference 47 for a review). In their anionic state, they can exist in various resonance forms, predominant ones of which are listed in Scheme 3.

With all four flavins, binding to apo-lactate oxidase results in stabilization of the anionic flavin, and in all cases it appears to be the benzoquinoid form shown on the right which is stabilized (bound) by the protein. This is particularly obvious in the case of 8-mercapto-FMN, where the resonance form with the negative charge on the 8-sulfur atom is the one predominant in the free flavin, with a λ_{max} of 530 mm. This is very little perturbed on binding to apo-flavodoxin, but is dramatically shifted on binding to apo-lactate oxidase, as shown in Figure 8.

With the other flavins of this group, the anionic state of the free flavin appears to have the major resonance contribution from the benzoquinoid forms, even without stabilization by the protein. In these cases, the effect of the protein is best evaluated by its effect on the pK of the bound flavin. With lactate oxidase, the pK of 8-OH-FMN is lowered from 4.8 to <4.0, that of 6-mercapto FMN from 5.9 to <4.5, and that of 6-OH-FMN from 7.1 to <5.0. Thus, in all cases, there is a distinct preference for the enzyme to bind the anionic flavin, with its negative charge localized in the N(1)C(2)=O locus. Together with the stabilization of the anionic flavin radical and the N(5)-sulfite adduct already discussed, these properties provided compelling evidence for a positively charged protein residue in the vicinity of the flavin N(1)-position, a prediction for this group of enzymes which is being nicely borne out by the recent X-ray structural information discussed in another section.

Further valuable information about the active site was obtained from a detailed study of the properties of the enzyme in which the native flavin was replaced with iso-FMN (where the two methyl groups of the benzene ring are at positions 6 and 7 instead of 7 and 8). This enzyme displays most of the properties possessed by native enzyme, as described in other sections.[58] It forms covalent derivatives at the flavin N(5)- and C(4a)-positions in facile photochemical reactions analogous to those of native enzyme. It also forms a red anionic semiquinone on one-electron reduction, and this radical is remarkably stabilized toward O_2 when it is in complex with pyruvate, again similar to native enzyme.

The iso-FMN enzyme also reacts rapidly and stoichiometrically with α-hydroxybutynoate



SCHEME 3. Representation of structures of modified flavins substituted for normal FMN in lactate oxidase. The formulae on the left row are those of the protonated species, which ionize with the pK values shown. In brackets the canonical structures of the mesomeric anions are represented. The right row depicts the quinoid mesomeric forms thought to be stabilized at the active center of lactate oxidase by the interaction with Lys-266.

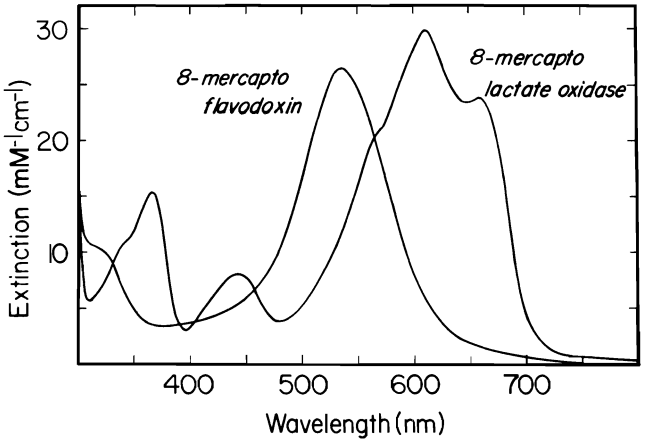


FIGURE 8. Absorption spectra of 8-mercapto-FMN bound to lactate oxidase and flavodoxin. The two spectra are representative of the two possible extreme mesomeric forms of 8-mercapto flavin. With lactate oxidase the structure with the negative charge at N(1) is stabilized (see right row of Scheme 3), while with flavodoxin the charge is located on the 8-S substituent (see Scheme 3, middle row). (Adapted from Massey, V., Ghisla, S., and Moore, E. G., *J. Biol. Chem.*, 25, 9640, 1979. With permission.)

to yield a highly fluorescent C(4a)-N(5)-cyclic adduct. Rapid reaction studies showed that the iso-FMN enzyme reacts even more rapidly with L-lactate than does native enzyme to form a reduced enzyme-pyruvate complex of stability very similar to that of native enzyme. Kinetic constants are summarized in Table 2. The only striking difference between the iso-FMN enzyme and native enzyme is the reactivity of the reduced enzyme-pyruvate complex with O_2. With native enzyme, this reaction is some 200 times faster than with free reduced enzyme; with the iso-FMN enzyme, there is only a marginal increase in O_2 reactivity. It would appear that while the bulky methyl group at the flavin 6-position does not interfere with substrate or product binding, access of O_2 to the reduced flavin-pyruvate complex is considerably hindered. It is because of the slow reaction of the reduced enzyme-pyruvate complex with O_2 that the steady-state kinetics show very high K_m values for both lactate and O_2, making the determination of the true catalytic maximum velocity experimentally very difficult.

IX. INFORMATION ABOUT THE ACTIVE SITE FROM PROTEIN CHEMICAL MODIFICATIONS

In the absence of real knowledge of the three-dimensional structure of a particular protein, one has to turn to information gained by indirect means, such as that described in the previous section. Another approach which has long been used is that of reaction of the protein with reagents known to modify specific amino acid residues and testing the effect on catalytic activity. If activity is lost, and the presence of substrate or competitive inhibitors is shown to protect against loss of activity, it is not unreasonable to infer that the amino acid residue modified specifically is either involved directly in catalysis or that the modification results in blockage of access of substrate to its binding site. In either case the modified residue would be expected to be close to the active site, and hence such studies could be expected to give information about the protein environment of the active site, particularly if the location of the modified residue in the amino acid sequence can be identified. Now that the amino acid sequence of lactate oxidase has been determined,[16] we can attempt to relate the results of such modifications to the recently determined crystal structures of flavocytochrome b_2 and glycollate oxidase.

Evidence for the catalytic role of a histidyl residue came from modification of the enzyme by diethyl pyrocarbonate.[64] Two histidine residues were found to react in enzyme inactivated to 10% of its initial activity. However, incubation with limited amounts of reagent and extrapolation of the residual activity vs. moles histidine modified led to the conclusion that only one histidyl residue was essential for activity. The modification is sufficiently labile to make identification of the specific residue impossible, but it could well have been His-290, since the homologous His-373 in cytochrome b_2 and His-254 in glycollate oxidase are positioned in such a way as to make them likely to be the enzyme base responsible for abstraction of the substrate α-proton as an early step in catalysis.[36,37]

Lactate oxidase is also inactivated by reaction with fluorodinitrobenzene, and the presence of competitive inhibitors such as nitrate or α-hydroxymalonate (tartronate) provided strong protection.[65] Unfortunately it is difficult to draw firm conclusions from this modification as the results appear to be influenced by the nature of the buffer used. In the initial paper,[65] the modification was carried out in 50 m*M* 2-(N-morpholino) ethane sulfonate (Mes) buffer, pH 6.0, and inactivation was claimed to be accompanied by incorporation of two DNP residues, one resulting in modification of an imidazole residue and the other modification of a lysyl side chain. In the presence of nitrate or α-hydroxymalonate, where almost complete protection was obtained, only 0.5 mol imidazole-label was incorporated, suggesting that it was the modification of a lysine residue which caused inactivation. In a later paper from the same laboratory,[66] the inactivation was carried out in 50 m*M* Tris-acetate buffer,

pH 6.0, and resulted in complete inactivation with the incorporation of one DNP residue per mole enzyme-bound FMN. Analysis of protein hydrolysates indicated that only histidine was modified. The inactivated enzyme was found to react only partially with sulfite and only partially in a photochemical reaction with oxalate. It was also found to be only partially reduced by L-lactate under anaerobic conditions. It was concluded that acetate, a known competitive inhibitor,[6] protected the essential lysyl residue from reaction, and that the approximately 50% reaction with sulfite, oxalate, and substrate was due to modification of two active site histidines in a mutually exclusive fashion, with each histidyl residue having a specific role in controlling the reactions with the earlier mentioned ligands and with both being required for catalysis.

We have recently reinvestigated the reaction with FDNB under conditions similar to those employed by Choong et al.[65] except that 20 m*M* Mes buffer, pH 6.0, was employed.[67] Peptide mapping by high performance liquid chromatography (HPLC) of radioactively labeled enzyme digested with trypsin showed three peaks of radioactivity, and sequencing of the peptides showed that two different cysteine residues and a histidine residue had been modified. These correspond to Cys-104, Cys-203, and His-240 in the amino acid sequence.[16] Both cysteine residues were protected from modification in the presence of sulfite (which forms a tight N-5 adduct with the flavin; see earlier section) or by 2-methyl lactate, a competitive inhibitor; the histidine modification was, however, only partially protected. The extent of cysteine modification was 1 mol per mole FMN, suggesting that the two different residues modified must be labeled in a mutually exclusive fashion, and since they are both protected by inhibitors, must both be in or near the substrate-binding site of the enzyme. Lactate oxidase is also inactivated by reaction with 5,5′-dithiobis(2-nitrobenzoic acid)(DTNB) with the incorporation of one-DNP residue per mole enzyme flavin, with inactivation proceeding from an enzyme-DTNB complex with a K_d of ~1 m*M*.[68] Taken together with the spontaneous reactions of 4-thio-FMN enzyme and 6-SCN-FMN enzyme described in the previous section, these results seemed to provide strong suggestive evidence for at least one (and more probably two) cysteine residues in or close to the flavin binding site of the enzyme. However, in neither the glycollate oxidase nor the flavocytochrome b_2 sequence is there any homologous cysteine residue. Unless both types of evidence discussed earlier are artifactual, it would appear that this is a feature of the lactate oxidase structure distinguishing it from those of flavocytochrome b_2 and glycollate oxidase.

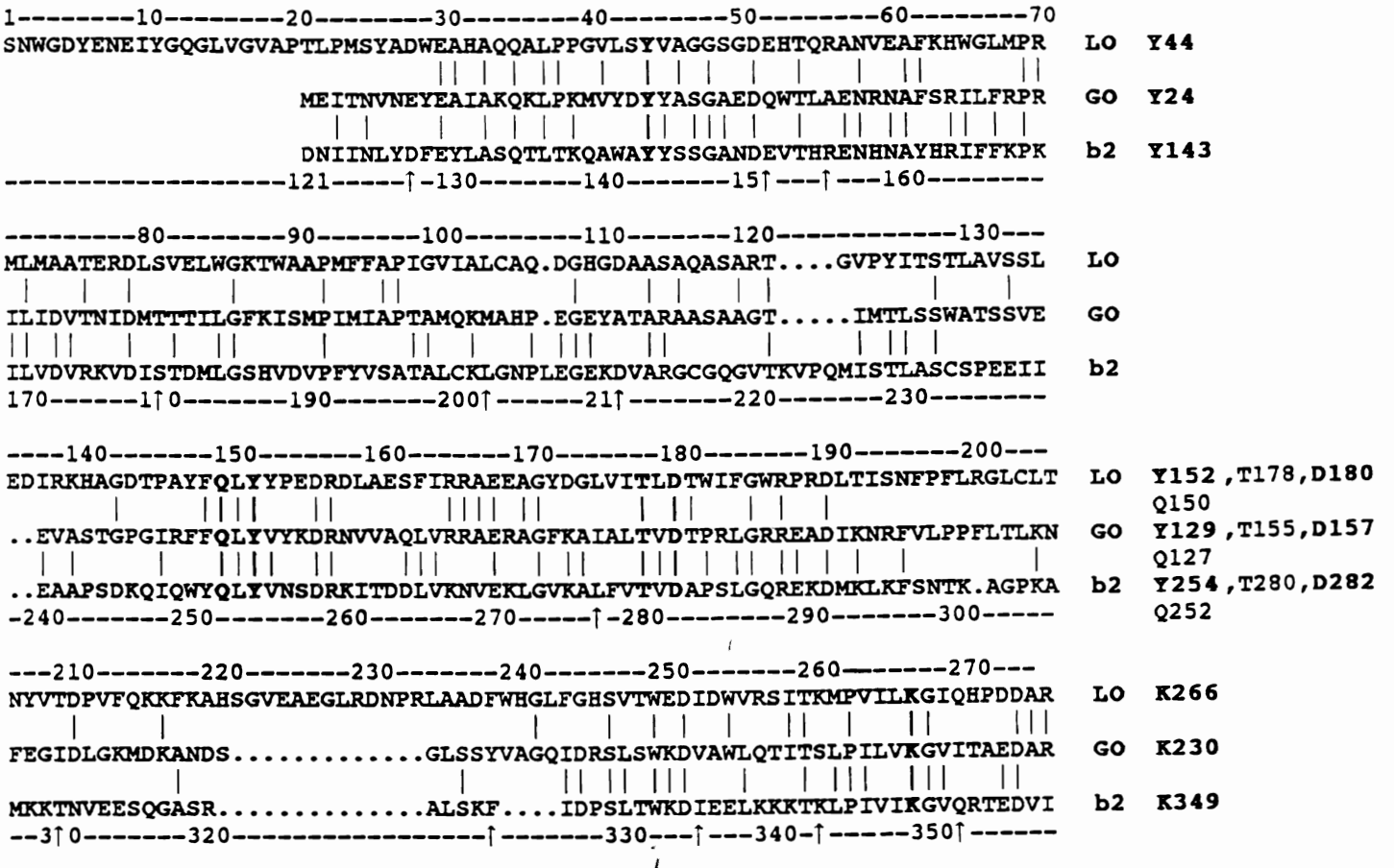
Lactate oxidase from *M. phlei* was found to be inactivated irreversibly in lutidine buffer by reaction with 2,3-butanedione or phenylglyoxal.[69] Analysis showed that a single arginine residue was modified, and it was concluded that this residue was probably involved in binding the carboxylate of the substrate. This conclusion was based on the protective effect of D-lactate, and from the observation that phosphate failed to perturb the spectrum of the inactivated enzyme. The modified enzyme also failed to form a flavin N(5)-sulfite adduct. In the crystal structures of flavocytochrome b_2 and glycollate oxidase, an arginine residue has been found on the Si side of the flavin and is believed to bind the substrate carboxylate (Arg-376 in flavocytochrome b_2 and Arg-257 in glycollate oxidase) (see Chapter 7). In lactate oxidase, Arg-293 occupies the homologous position and is therefore likely to be the residue modified by Peters et al.[69] The important role of this arginine residue in flavocytochrome b_2 has been shown by site-directed mutagenesis; replacement by a lysyl residue resulted in complete loss of catalytic activity.[70,71]

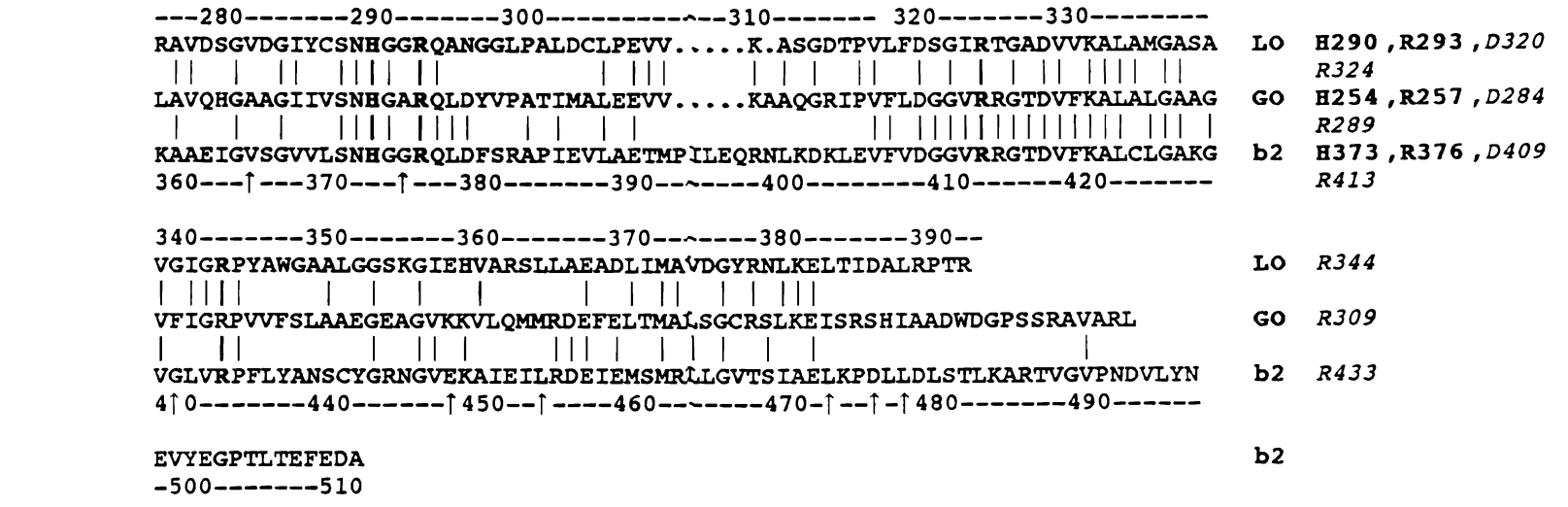
X. STRUCTURAL COMPARISONS WITH SPINACH GLYCOLLATE OXIDASE AND YEAST FLAVOCYTOCHROME b_2

The amino acid sequence of lactate oxidase is now available via the cDNA sequence of the cloned gene.[16] The subunit sequence of 393 amino acids is shown in Table 3 and is

TABLE 3

Sequence Comparison of L-Lactate Oxidase (LO), Glycollate Oxidase (GO), and Flavocytochrome b_2 (b_2)





(|) Denotes matches between corresponding lines, (↑) matches between b_2 and LO in addition to matches common to all three proteins.

(|) Denote residues implicated in FMN binding or catalysis.
Residues identified in italic script are involved in FMN(N10)side chain-protein interactions, those in light print in isoalloxazine-protein interactions, while those in bold type are involved in substrate binding and catalysis.

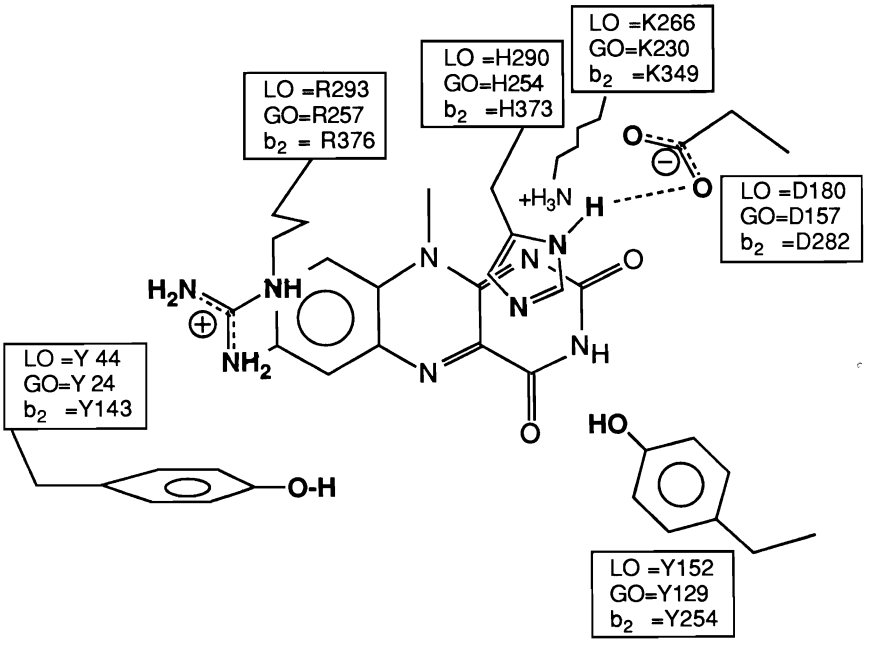


FIGURE 9. Comparison of active center functional groups for lactate oxidase (LO), glycollate oxidase (GO), and flavocytochrome b_2 (b_2).

aligned for maximal sequence identity with spinach glycollate oxidase[72,73] and yeast cytochrome b_2.[74] It is clear that there is considerable similarity between the three enzymes, which might be expected to be reflected also in their three-dimensional structures. This assumption is shown to be largely correct in the case of glycollate oxidase and cytochrome b_2, which have very similar structures around the FMN prosthetic group[17,50] (see Chapter 7). In the sequence comparison of Table 3, there are 69 residues showing sequence identity between all three enzymes. Included among these are residues which are involved in binding to the FMN side chain or isoalloxazine ring, or residues implicated in substrate binding and catalysis. Thus, as discussed in detail elsewhere in this volume for cytochrome b_2 (Chapter 7), Arg-413 and Arg-433 form salt linkages to the phosphate of FMN, Asp-409 is H-bonded with the FMN ribityl-3′-OH, and Lys-349 is H-bonded with the ribityl-2′-OH. Exactly the same functions in the glycollate oxidase crystal structure are ascribed to the homologous residues Arg-289, Arg-309, Asp-284, and Lys-230.[37] While the crystal structure of lactate oxidase has not yet been determined, the existence of the same homologous residues, Arg-324, Arg-344, Asp-320, and Lys-266, suggests strongly a very similar binding of FMN also in this enzyme. Similar interactions are also seen between the protein and isoalloxazine ring, with O(2) making a hydrogen bond with Thr-155 in glycollate oxidase, Thr-280 in cytochrome b_2, and presumably Thr-178 in lactate oxidase. Interaction of O(4) with Gln-127 in glycollate oxidase is paralleled by the same interaction with Gln-252 in cytochrome b_2 and presumably by the equivalent Gln-150 in lactate oxidase. Among the amino acid functions, which are assumed to be part of the catalytic machinery, the conservation is impressive, as shown in Figure 9.

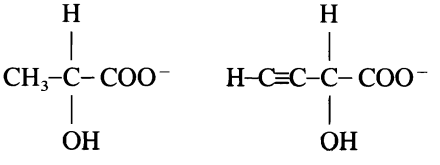
The importance of a positively charged residue close to the flavin N(1)-position has long been recognized in the case of lactate oxidase and other flavoproteins with similar properties, as being involved in the stabilization of anionic flavins with the negative charge in the N(1)-O(2) locus[34,76] (see also later sections). The mechanistic significance of this residue will be considered in detail in another section, but it is gratifying to see in the crystal

structures of glycollate oxidase and cytochrome b_2 that the same role is ascribed to Lys-230 and Lys-349, respectively.[36,37] In the lactate oxidase sequence, the homologous residue is Lys-266.[16]

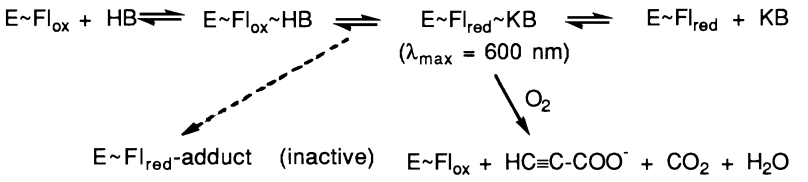
Binding of the substrate in the active site is also similar in all three enzymes. From the crystal structures it is known that substrates or competitive inhibitors are bound on the Si-side of the flavin;[17,36] the same conclusion was reached independently with lactate oxidase from enzyme substituted with stereospecifically labeled 8-OH-5-deaza-$FMNH_2$.[48] With cytochrome b_2, the carboxylate of the substrate is held in a salt bridge with Arg-376 and by a hydrogen bond with the hydroxyl of Tyr-143 (see Chapter 7). The same interactions with glycollate oxidase are fulfilled by Arg-257 and Tyr-24[37] and presumably with lactate oxidase by the strictly homologous residues Arg-293 and Tyr-44.[16] All three proteins contain a strictly conserved histidine residue which is believed to be the active site base responsible for abstraction of a proton from the substrate α-position in one of the primary steps in catalysis. This is His-373 in cytochrome b_2, His-254 in glycollate oxidase, and presumably His-290 in lactate oxidase. The N(1)-position of this histidine residue is also close to the carboxylate of Asp-282 in cytochrome b_2, Asp-157 in glycollate oxidase, and presumably Asp-180 of lactate oxidase. Finally, a tyrosine residue located on the substrate binding side of the flavin appears to be strictly conserved, and as considered in detail in a later section, probably plays an important role in catalysis. This is Tyr-254 in cytochrome b_2, Tyr-129 in glycollate oxidase, and Tyr-152 in lactate oxidase. Some of these common features are also illustrated in Table 3, based on the interpretation of the crystal structure of cytochrome b_2 by Lederer and Mathews.[36] While the exact position of bound substrate relative to flavin in the case of lactate oxidase is not yet proved, this description of the active site is fully consistent with the deductions made previously from covalent adducts formed photochemically from complexes of α-bromoacetate and β-bromopropionate and reduced enzyme.[43]

XI. REACTION WITH SUICIDE (MECHANISM-BASED) INACTIVATORS

Following Bloch's original report[77] of irreversible inactivation of a dehydrase by 3-decynoyl thiolesters, substrate analogs, L-lactate oxidase was among the first enzymes for which the mechanism of a "suicide" or "mechanism-based" inactivation was worked out in detail. 2-Hydroxy-3-butynoic acid (right) is an analog of lactate (left):



and its use was conceived also as a mechanistic probe since, as in Bloch's case,[77] the formation of a negative charge adjacent to an alkyne function can lead to an allene and subsequently to covalent reaction with a function at the active center. In fact 2-hydroxy-3-butynoic acid was found by Walsh et al.[78] in 1972 to irreversibly inactivate lactate oxidase by covalent modification of the FMN cofactor. Later studies[55] showed that it behaves in most respects as a reasonably good substrate, the catalytic parameters being consistent with the steps of Scheme 4, and its turnover number (~220 min^{-1}) being approximately one tenth the value observed with lactate under the same conditions (3200 min^{-1}). The products to be expected in the presence of oxygen are those also shown by the sequence of Scheme 4, i.e., 2-propynoic acid and CO_2 (compare to Scheme 1 for lactate).

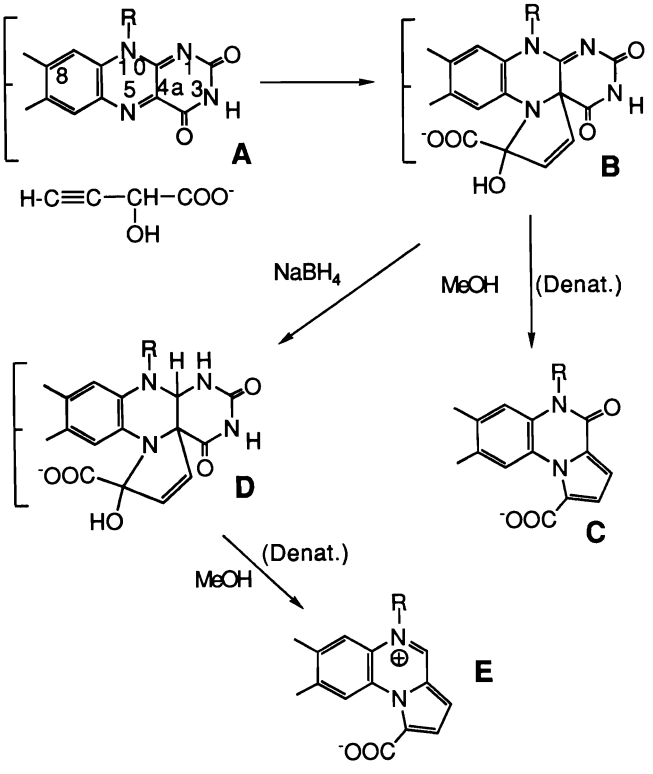


SCHEME 4. Kinetic scheme for the catalytic turnover of 2-hydroxy-3-butynoate (HB) and for the irreversible inactivation. Note that inactivation can occur starting either from oxidized enzyme and HB or from reduced enzyme and 2-ketobutynoate (KB).

Under the same conditions, the rate of irreversible enzyme inactivation is ~2 min^{-1}, and thus one of every 110 turnovers results in modification of the enzyme. The independence of this ratio on the concentration of inactivator is consistent with a partitioning of some common precursor between oxidation and inactivation.[55] Also in line with this assumption, the rate of enzyme inactivation is much higher in the absence of oxygen, and inactivation also occurs when the reduced enzyme is incubated anaerobically with 2-ketobutynoate. Stopped-flow studies of the inactivation process demonstrated the formation of an intermediate preceding inactivation, which is characterized by a long wavelength absorption typical of complexes of reduced enzyme and α-ketoacids.[55] These studies[55,56] could not, unfortunately, differentiate between the inactivation event originating at the level of the complex of oxidized enzyme and a putative α-deprotonated inhibitor, or the complex of reduced enzyme and 2-ketobutynoate. This follows from the observation that the species shown on the top line of Scheme 4 are in a sufficiently rapid equilibrium that a discrimination of pathways was not possible.

One main issue in the context of this inactivation process was the structure of the adduct formed by addition of the inhibitor moiety to the flavin, since this would provide important mechanistic insights. This task proved to be a difficult one since the adduct is labile while bound to the protein, and even more so upon release from the protein by denaturation. These decay reactions were found not to yield a single product, but several species depending on the conditions. The absorption spectrum of the species present prior to decay was typical for a 4a,5-dihydro-isoalloxazine chromophore suggesting, at the time, a new type of biochemical flavin reaction. A stabilization of this primary adduct, to which structure B, Scheme 5, was assigned, was achieved by reduction with sodium borohydride,[56] a reaction in line with the assumed structure, which leads to the colorless phenylenediamine (D) (Scheme 5). This is also unstable; it decays, however, to a single main product which was identified as the pyrroloquinoxaline (E).[56] This information led to the identification of (C) as a major product from the decay of the primary adduct (B) in the absence of $NaBH_4$ treatment.[56] The sum of the results was thus compatible with structure (B) as the primary product of the inactivation event and involves a cyclic addition of the inactivator to the isoalloxazine.

Unfortunately, these studies did not provide specific clues on the mechanism of formation of (B). Formation of this species could be initiated either by attack of a 2-OH-butynoate α-carbanion on the oxidized flavin or by Michaelis addition of the reduced flavin to the product 2-ketobutynoate. On the other hand, these studies provided important information on the mode of binding and orientation of substrate with respect to the flavin in a complex preceding inactivation. In conjunction with similar experiments obtained with D-lactate dehydrogenase,[79,80] they showed that the substrate α-position must be located in close proximity to the flavin N(5) in such a complex, a proposal again fully supported by the deduced three-dimensional structure discussed in the previous section.

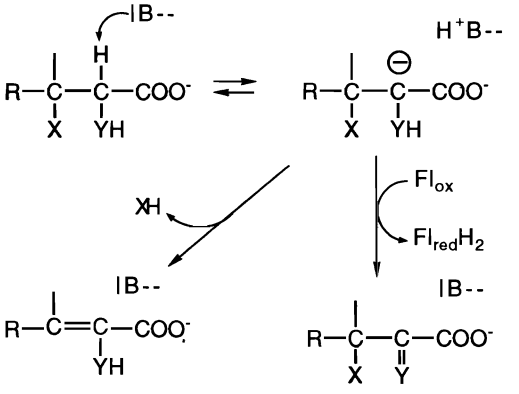


SCHEME 5. Formation of covalent adducts between the flavin of lactate oxidase and the inactivator 2-hydroxybutynoate and decay. B is the adduct thought to be formed directly from addition of the inactivator to the oxidized flavin A. C is the structure of one of the major products arising from the decay of B during and subsequent to denaturation of the protein with methanol. Treatment of B with sodium borohydride yields D, which in turn decays to E upon denaturation. E is the product which was identified. (From Schonbrunn et al., *Biochemistry*, 15, 1798, 1976. With permission.)

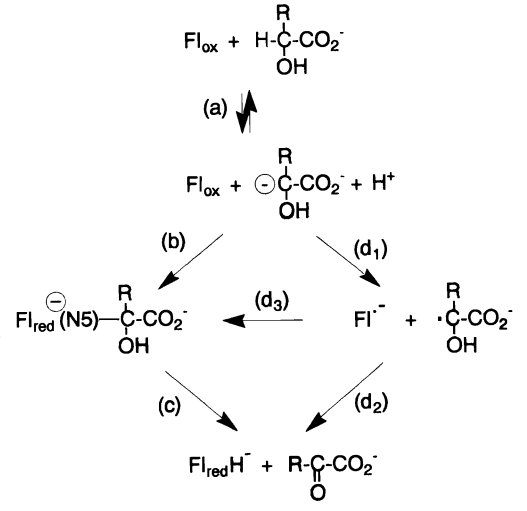
XII. THE REDUCTIVE HALF-REACTION, MECHANISM OF DEHYDROGENATION

A. EVIDENCE FOR CARBANION MECHANISM

The catalytic cycle of lactate oxidase is initiated by substrate dehydrogenation in the complex with oxidized enzyme. As discussed elsewhere in detail,[81] the cleavage of the α-C-H bond of an α-hydroxy (or α-amino) acid can be formulated by different chemical mechanisms, i.e., homolytically, by expulsion of a hydride, or by formation of an α-carbanion. In the case of lactate oxidase and of the related enzymes D-amino acid oxidase and cytochrome b_2, evidence compatible with an α-carbanion initiated process (Scheme 6) has accumulated in the past two decades and this mechanism is now accepted by general consensus (see Chapter 7). This assumption is based on the use of substrate or product analogs, in which initial formation of a carbanion induces a partitioning between the normal dehydrogenation process and a reaction typical of the particular substituent. A classical example is β-elimination induced by α-carbanion-forming substrates as shown in Scheme 6. Such a behavior was first observed by Miyake et al.[82] while studying the interaction of β-Cl-alanine and D-amino acid oxidase, and was subsequently interpreted correctly by Walsh et al.[83] in their classical work, which first suggested the carbanion theme in the present context. The same partitioning was found soon thereafter for lactate oxidase using β-Cl-lactate,[41,85] and the reaction type was studied in greater detail later in the cytochrome b_2



SCHEME 6. Mechanism of elimination of halide from the β-position of an α-OH-carboxylic acid and competition with catalysis. −B| is an enzyme active center base and X a leaving group such as Cl^- or Br^- and Y is O or NH.



SCHEME 7. Modes of transfer of redox equivalents from a substrate carbanion to the oxidized flavin. The carbanion is formed in the first step (a). Step (b) represents addition of the carbanion to the flavin N(5) position to yield the covalent adduct shown. Step (c) represents fragmentation of the intermediate to the reduced enzyme flavin-keto acid complex. The right-hand side of the scheme depicts steps involving transfer of single electrons. In (d_1) this forms a (transient?) complex to flavin radical anion and the corresponding substrate radical. Further transfer of $1e^-$ leads to the products (steps d_2 or d_3 + c).

system (see Chapter 7). Present knowledge on the mode of inactivation of lactate oxidase and D-lactate dehydrogenase by suicide substrates such as α-hydroxybutynoic acid (see earlier) can similarly be taken as supporting initial carbanion formation. For a detailed discussion of the arguments in favor of carbanion mechanism in the case of cytochrome b_2, we refer to Chapter 7.

B. MODES OF TRANSFER OF "REDOX EQUIVALENTS" FROM A CARBANION INTERMEDIATE TO THE OXIDIZED FLAVIN ACCEPTOR

Subsequent to carbanion formation, for the transfer of negative charge to the oxidized flavin of lactate oxidase, three alternative pathways can be formulated as depicted in Scheme 7. As pointed out by Bruice,[86,87] in particular for nitroalkane anions, the oxidation of carbanions proceeds via radical mechanisms, which might be initiated by a step such as d_1,

in Scheme 7, and results in formation of a pair of radicals. Collapse of the unpaired electrons in the radical pair will result in formation of a covalent bond (step d_3). Step (d_2), i.e., the transfer of $1e^-$ and $1H^+$ to the flavin also must obey certain rules. First, there cannot be transfer of $H^{\cdot}$, e.g., from the α-O-H, since the hydrogen ending up at N(5) of the flavin originates from the substrate α-position, and will rest on the abstracting base (His-290, His-254, and His-373 in lactate oxidase, glycollate oxidase, and cytochrome b_2) at the stage of the radical pair or of the covalent intermediate. Thus, formation of products via a putative step (d_2) must be either concerted with protonation of N(5) by histidine or will involve a negative charge at the flavin N(5) as an intermediate (Scheme 7). This sequence contrasts with the direct attack of the carbanion on the flavin as shown by step (b) to form the covalent intermediate, a formal sequence which has to be followed by fragmentation, step (c), to yield the same products.

As pointed out elsewhere,[48,57] a differentiation between two sequences such as (b + c) or (d_1 + d_3 + c) (covalent adduct mechanism) and (d_1 + d_2) (radical transfer mechanism) might be very difficult if not impossible if steps (c) and (d_2) are faster than the preceding ones, as they are likely to be. A discussion of these alternatives follows in a later section.

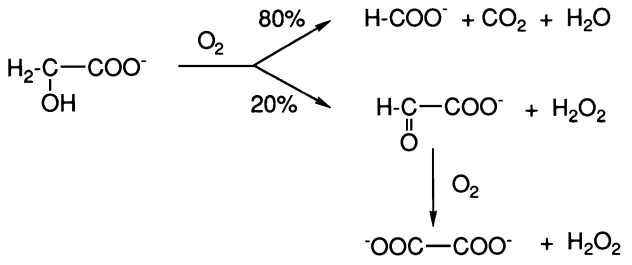
In this context it is important to point out that reduction of the lactate oxidase flavin by L-lactate (R = CH_3 in the substrate structure shown in Scheme 7) proceeds monophasically to yield the complex of reduced enzyme and pyruvate.[6] Thus neither the biradical nor the covalent species shown in Scheme 7 are observable reaction intermediates. Their lifetimes must be either extremely short, or they might occur as transient species. While this is true for the normal substrate, lactate, the reaction of lactate oxidase with glycollate, the lower homologue, yields a drastically different picture, and indeed intermediates have been observed and identified.

C. REACTIONS OF LACTATE OXIDASE WITH GLYCOLLATE

Glycollate is the simplest α-OH-carboxylic acid and the only one having two (equivalent, but prochiral) α-hydrogens. Lactate oxidase recognizes glycollate as a substrate and it catalyzes its oxidation in a fashion which is superficially similar, but in important details significantly different from that observed with lactate. In view of the importance of this reaction, which yields mechanistic information not obtainable with normal substrate, it will be discussed in some detail.

1. Turnover of Glycollate by Lactate Oxidase

Glycollate undergoes a dehydrogenation and an oxidative decarboxylation in the presence of oxygen, the main products being formate, CO_2, and water. In this respect its reaction is analogous to that found with lactate and depicted in Scheme 1. However, and in contrast to the reaction with lactate, there is some 20% uncoupling of the decarboxylative degradation which yields glyoxylate and H_2O_2 as shown in Scheme 8. The turnover number for glycollate is 66 to 70 min^{-1} [88] and is lowered to ~20 with α-dideuteroglycollate, yielding an isotope effect of ~3.6, which is essentially the same as found for lactate/deuterolactate (Table 4). The product of the uncoupled reaction, glyoxylate, probably in its hydrated form, is also a good substrate, the rate of reduction of oxidized lactate oxidase being ~13 s^{-1}, and the K_d for formation of the Michaelis complex 0.05 *M*, i.e., closely similar to that found for lactate (see earlier). The product of this reaction is oxalate, and lactate oxidase thus behaves in this case as a true oxidase in contrast to the normal monooxygenase catalysis. Note that oxalate is a potent inhibitor (see Section VI), and that this reaction thus progressively decreases in velocity as oxalate accumulates. For this reason the determination of a classical turnover number for glyoxylate is not possible.



SCHEME 8. Modes of turnover of glycollate and formation of different products. Note the partial "uncoupling" of the reaction, which leads to formation of glyoxylate, which, in turn, can react further, probably in its hydrated form, to yield the final product oxalate. (Adapted from Massey, V., Ghisla, S., and Kieschke, K., *J. Biol. Chem.*, 255, 2796, 1980. With permission.)

TABLE 4
Kinetic Constants Estimated for the Steps Involved in the Reaction of Lactate Oxidase with Glycollate

glycollate/([2-$^2H_2$]glycollate)

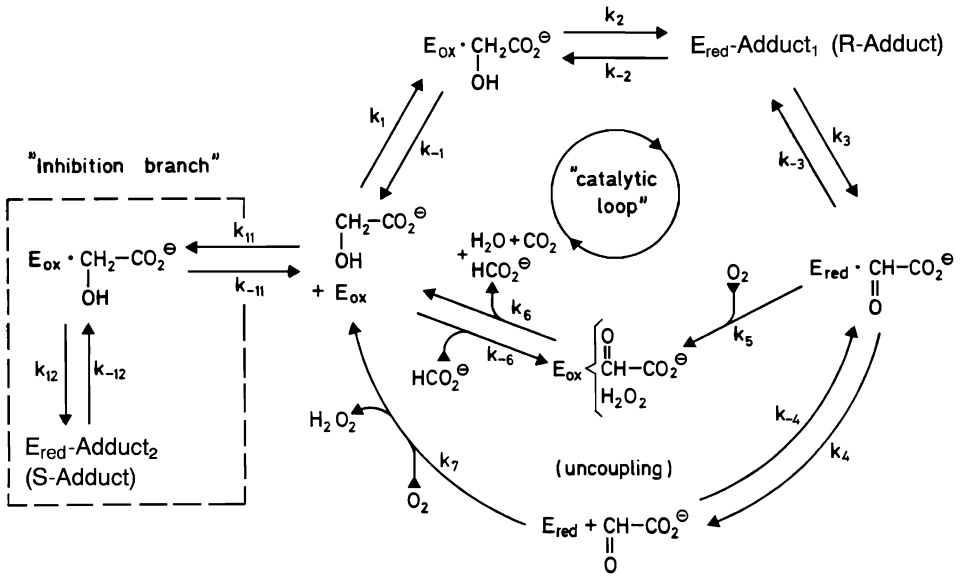
Rate constant	Temperature 25°C	0°C
k_{-1}/k_1	$6.5 \cdot 10^{-3}$	$1.8 \cdot 10^{-3}$
k_2	76 min^{-1}/(17 min^{-1})	6 min^{-1}
k_{-2}	13 min^{-1}/(13 min^{-1})	1 min^{-1}
k_{-3}/k_3	~1	
$k_4 + k_{12}$	≤0.6 min^{-1}	
k_{-4}	fast	
k_5	$2 \cdot 10^5$ M^{-1} min^{-1}	
k_6	900 min^{-1}	
k_7	$5.4 \cdot 10^5$ M^{-1} min^{-1}	
k_{-12}	0.035 min^{-1}	0.001 min^{-1}

The reactions were measured in 0.01 *M* imidazole/HCl buffer, pH 7.0.

From Massey, V., Ghisla, S., and Kieschke, K., *J. Biol. Chem.*, 255, 2796, 1980. With permission.

2. Progressive Inactivation of Lactate Oxidase During Turnover with Glycollate

One striking peculiarity of the enzymatic turnover with glycollate is a progressive decline of the enzymatic activity. It should be stressed that this is independent of oxalate formation from glyoxylate via uncoupling, as deduced from the observation that the inactivation also occurs in the presence of hydroxylamine, which effectively traps α-keto acids. The enzyme is, however, not completely inactivated, thus after a prolonged period (20 to 30 min) of incubation under turnover conditions, inactivation levels off. At this point in the incubation there is ~96% inactive and ~4% active enzyme, and this was found to be a steady-state level in which the two forms are interconverted. From the rate of inactivation, as compared to turnover, it was estimated that for 300 catalytic cycles, there is one event of inactivation.[88]



SCHEME 9. Reaction sequences catalyzed using glycollate as substrate. This scheme is essentially an amplification of that described in Scheme 1 for L-lactate. The "catalytic loop" describes the sequence leading to substrate monooxygenation. The lower part represents the so-called "uncoupling" in which the enzyme behaves as an oxidase. The reactions described in these two cycles involve abstraction of the substrate "Re-hydrogen", which corresponds stereochemically to the α-hydrogen of L-lactate. The "inhibition branch" is derived from reaction of the glycollate "Si-hydrogen". See text for details and Table 4 for the estimated values of the single rate constants. (Adapted from Massey, V., Ghisla, S., and Kieschke, K., *J. Biol. Chem.*, 255, 2796, 1980. With permission.)

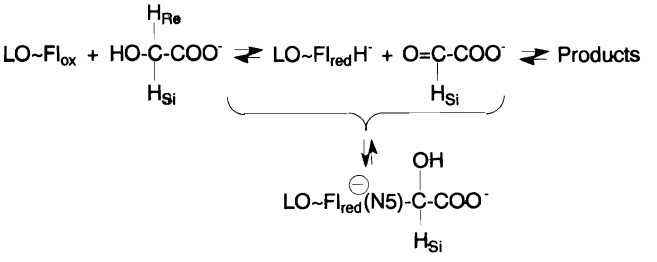
3. Formation of Intermediates During Turnover of Glycollate

A main difference between the lactate and glycollate reductive half-reactions is that in the former case a long wavelength absorbing species is formed immediately upon mixing of the reactants, which is absent with glycollate (see Figure 2). Instead two highly fluorescent species are formed, which can be differentiated spectrally from uncomplexed reduced lactate oxidase. These fluorescent species eventually decay to form reduced uncomplexed lactate oxidase with a very long half-life of ~50 min at 25°C under anaerobic conditions.[88] One of the fluorescent species is labile and does form oxidized lactate oxidase very rapidly upon admission of air. The second, in contrast, is comparatively very stable and can be isolated free of substrate or products by gel filtration at 0 to 4°C. It is identical with a metastable species formed upon irradiation of the lactate oxidase-tartronate complex and also with a species which can be obtained from reduced lactate oxidase and glyoxylate.[44,88]

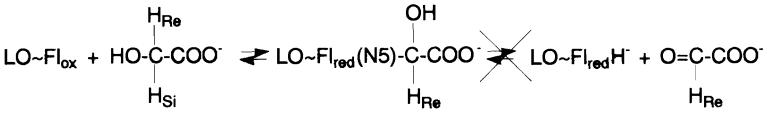
Furthermore, conditions were devised which allow the accumulation of the stable species as a major component and its isolation and study. Formation and accumulation of this species, which is not catalytically active, is the cause of progressive inactivation of the enzyme referred to earlier. The kinetic properties of the labile species, on the other hand, are such that it can be competent in catalysis. The kinetic behavior of the lactate oxidase glycollate couple has been summarized in Scheme 9, and the single rate constants are listed in Table 4. In the so-called catalytic loop, glycollate is turned over very much the same way as lactate, the difference being the occurrence of the labile fluorescent species on the pathway, an intermediate which cannot be observed with lactate.

4. Reaction of Either the H_{Re} or the H_{Si} of Glycollate, Formation of Isomeric Adducts

As already mentioned earlier, with glycollate, in contrast to lactate, there are two α-C-



SCHEME 10. Possible formation of the covalent adduct between glycollate and the flavin occurring in a side reaction.



SCHEME 11. Pathway of formation and decay of the stable "S-Adduct" from lactate oxidase and glycollate.

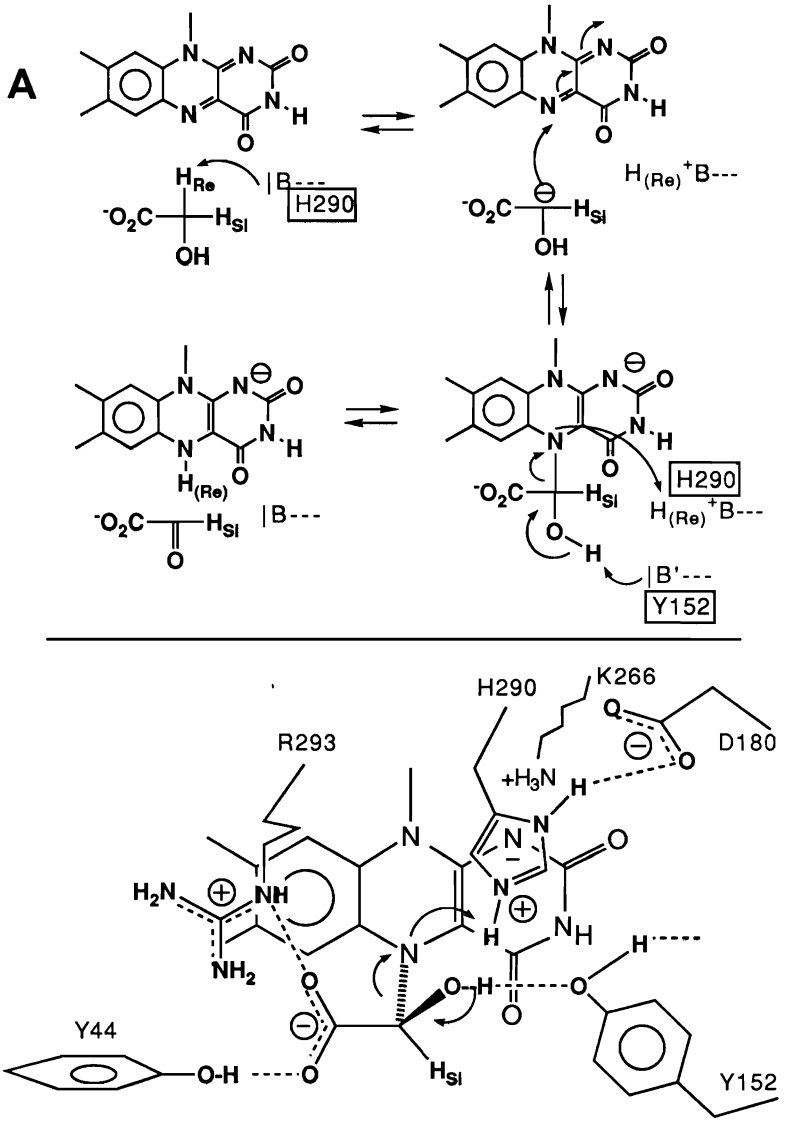
H bonds which can be oxidized. Lactate oxidase is selective for the Re-hydrogen of glycollate, the one which corresponds to that in L-lactate. The Si-hydrogen is also acted upon, albeit at a rate approximately 1% that observed with the Re-hydrogen. These deductions were arrived at using selectively R- or S-, tritium- and deuterium-labeled glycollates.[88] The intermediate fluorescent species referred to in the previous sections were found to be N(5) covalent adducts of glycollate to reduced 1,5-dihydroflavin. They are derived from reaction of glycollate involving breaking of either the α-C-H_{Re}, or the α-C-H_{Si} bonds (see Figure 10).[44] The two adducts are markedly different in their properties.

The "labile" adduct is identical with $Adduct_1$ of Scheme 9 and with the so-called "R-Adduct" discussed in References 44 and 88. It is in a sufficiently rapid equilibrium with both glycollate and glyoxylate to be a kinetically viable intermediate, and thus the suggestion that dehydrogenation of glycollate proceeds via a covalent flavin N(5)-adduct was considered. It should be pointed out, however, that the kinetic parameters determined for formation and decay of the R-Adduct are not sufficient for answering the question whether it is on the reaction pathway as shown in Figure 10A below or in a side equilibrium as shown in Scheme 10.

The second intermediate has been proposed to result from abstraction of the substrate α-H_{Si} and subsequent addition of the glycollyl carbanion to the enzyme flavin position N(5) (cf. Figure 10B). It should be noted that the stereochemistry of α-H_{Si} corresponds to that of the methyl group of D-lactate, and that the latter is not a substrate but an inhibitor of lactate oxidase to which it binds with a $K_d = 10^{-3}M$, which is approximately one order of magnitude lower than that for L-lactate (cf. Table 1). This adduct has been termed "$Adduct_2$" (cf. Figure 7) or "S-Adduct".[89]

It is sufficiently stable to be isolated and characterized in its salient physicochemical and kinetic features. Its most important property is its being in a slow equilibrium with glycollate, but *not* with glyoxylate. That means it does not decay to the reduced enzyme-flavin-glyoxylate complex, as does the "R-Adduct", nor is it formed from it, as one might have expected from chemical considerations (Scheme 11).

Therefore this stable "S-Adduct" must be formed *directly* from glycollate and oxidized lactate oxidase as shown in Figure 10B. This is a most important deduction since it essentially implies that reaction of glycollate must be initiated by abstraction of the H_{Si}-hydrogen. During formation and decay of this adduct, the hydrogen involved (H_{Si}) apparently does not



FIGURES 10A and 10B. Mechanisms proposed for the formation of the two covalent adducts from glycollate. The top half of both figures outlines the chemical events thought to lead to formation of the adducts. In the first step glycollate binds to the active center primarily via the interaction of its carboxylate to Arg-293 and Tyr-44. However two stereomeric Michaelis complexes are formed, which are different with respect to the orientation of the three α-substituents (compare top left structures in Figures 10A and 10B). In these complexes His-290 attacks either H_{Re} (Figure 10A) or H_{Si} (Figure 10B) to form the (transient) carbanions shown, which in turn add to the flavin N(5) position to yield the diastereomeric adducts. The bottom half of the figures depicts the postulated interaction of the two adducts with the active center functions. In the "R-Adduct" (Figure 10A), an interaction is formulated between Tyr-152 and the α-OH, which promotes the fragmentation of the adduct to yield the complex of reduced enzyme and glyoxylate. Such an interaction might not be possible in the case of the "S-Adduct" (Figure 10B, lower panel), thus rendering impossible its decay to form the products. Oxygen reaction or dissociation of the reduced enzyme glyoxylate complex (Figure 10A, center left) leads to the final products (not shown).

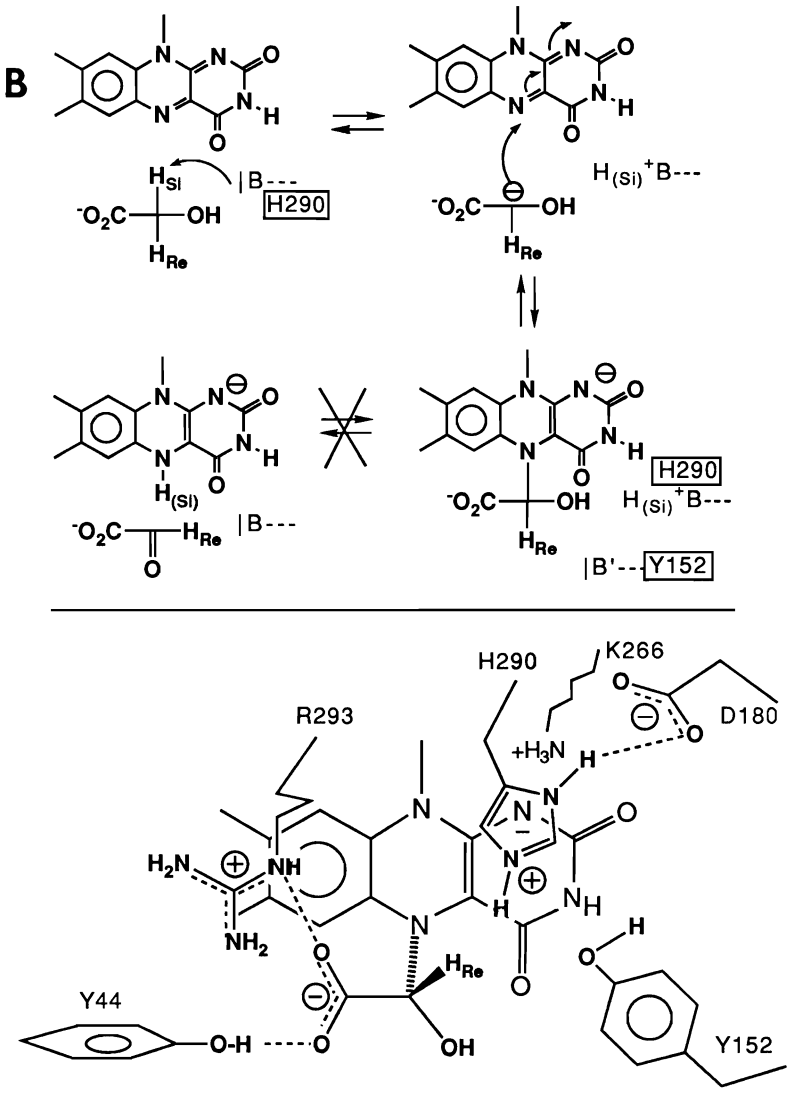


FIGURE 10B.

exchange with solvent, in line with the assumed shielding of the active site from solvent. This follows from the observation that decay of the S-Adduct exhibits an isotope effect, i.e., that the abstracted H_{Si} from glycollate remains bound to an active center base prior to retransfer to the glycollate skeleton.

Interestingly, the photoreaction of the oxidized enzyme-tartronate complex also leads to formation of the stable S-Adduct, and during this reaction, the α-hydrogen of tartronate is retained in the S-Adduct at the Re-position.[44]

A molecular interpretation of the reactions of glycollate and their differences can be attempted in view of the information available from the deduced three-dimensional structure. This is done with the sequences of Figures 10A and 10B. Figure 10A corresponds to the reduction half-reactions of the "catalytic loop" and Figure 10B to the "inhibition branch" of Scheme 9.

The general outline of the two figures is identical, with the difference that glycollate first forms two diastereomeric Michaelis complexes in full accordance with the rapid reaction studies detailed elsewhere.[88] By analogy with the kinetic behavior of D,L-lactate, it is tempting to speculate that the "Si-Michaelis complex" (Figure 10B) is thermodynamically more stable, as compared to the "Re-one", although its rate of formation might be slower. In the two Michaelis complexes, either the H_{Re} or the H_{Si} hydrogens of glycollate face the same active center base, which is proposed to be His-290, and which induces their abstraction as protons. The carbanionic species formed from this step relaxes in both cases by nucleophilic addition to the oxidized flavin N(5) position, this reaction being facilitated by the presence of Lys-266, which stabilizes the incoming negative charge on N(1). The crucial point in this context is the rationalization of the different stability or reactivity of the two covalent adducts. For this, at the time the original work was published, the hypothesis was put forward that the α-OH functions of the two adducts would be located in "different chemical environments", which would affect the subsequent fragmentation to yield reduced enzyme flavin and glyoxylate. The deductions, which can be made from the three-dimensional structure, provide a nice confirmation of the original assumption. In Figures 10A and 10B, which have been derived from the active center topography of cytochrome b_2[36,90] and glycollate oxidase,[37] the postulated reactions and interactions of the two covalent adducts are compared.

This mechanism assumes that for the decay of the adduct, Tyr-152 plays a role in acidifying the α-OH and is part of a charge relay system, which transfers the α-hydroxyl hydrogen as H^+ to a still unidentified function or to the solvent. In the case of the S-Adduct (Figure 10B), this hydrogen bridge involving Tyr-152 and the α-OH most likely is absent. The α-OH function of an S-Adduct should be located at the position which normally would be occupied by the lactate methyl group. These pictures and comparison of the reactivities suggest that removal of the proton from α-OH is not a marginal step in catalysis and that Tyr-152 plays an active role.

Finally, we have put forward the hypothesis[44] that the R-Adduct lies on the catalytic pathway for the dehydrogenation of glycollate as shown in Figure 10A and in contrast to the alternate mechanism of Scheme 10, which would relegate it to a fast side equilibrium. This is based on the reasonable assumption that the chemistry of formation of the R-Adduct is the same as for the S-Adduct.

D. COMMENTS ON THE STABILITY OF A COVALENT ADDUCT IN THE CASE OF GLYCOLLATE DEHYDROGENATION AND ITS APPARENT ABSENCE DURING LACTATE DEHYDROGENATION BY LACTATE OXIDASE AND THE ABSENCE OF COVALENT SPECIES DURING CATALYSIS BY GLYCOLLATE OXIDASE AND FLAVOCYTOCHROME b_2

The first hypothetical rationalization coming to mind for this difference in mechanistic behavior is the assumption that a covalent adduct occurs in all cases, but that its stability varies drastically and depends on the magnitude of the rates of decay as compared to those of formation. A second hypothesis would assume the occurrence of different mechanisms depending on the enzyme and on the substrate (i.e., lactate oxidase/glycollate vs. lactate oxidase, glycollate oxidase, and flavocytochrome b_2 with all other substrates). As pointed out earlier, the close similarity of the active sites of lactate oxidase, glycollate oxidase, and flavocytochrome b_2 provide a big temptation with respect to the drawing of mechanistic analogies. The finding of substantially different reactivities with oxygen, however, should caution us that apparent similarities might hide important differences. Nevertheless, as also discussed by others (see Chapter 7), it appears clear that at least with several substrates and analogs like lactate, α-hydroxybutynoic acid, and β-halogenated-α-OH-acids, the same basic type of catalysis occurs. We thus think it is unlikely that the interchange of a nonreactive

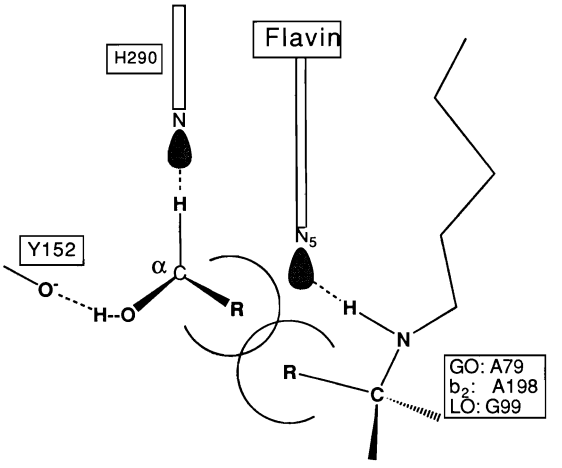


FIGURE 11. Schematic "side-view" of the active center of lactate oxidase and steric interactions of the substrate α-substituent (R) with the amino acid closest to position N(5) of the flavin. This figure was obtained from rotation of an active center diagram such as that of Figure 9 and simplification by omission of protein chain fragments and functional groups.

substituent such as $-CH_3$/H will completely alter the mechanism of dehydrogenation. Such a substitution might, however, have profound effects on single rates and on the thermodynamics of intermediates and transition states.

Along this line, there are two main differences between glycollate and lactate: (1) the difference in redox potential (−90 vs. −185 mV), which, *a priori,* cannot be excluded to be sufficient to induce a switch in mechanisms; and (2) differences of steric requirements. The catalytic cavity of lactate oxidase can accommodate a large variety of α-substituents (see Table 1). On the other hand, very subtle effects have been observed, e.g., in the case of cytochrome b_2,[91] the apoenzyme of which does not bind the analog 1-deaza-FMN. This suggests that the difference between the N(1) sp^2 free electron pair of normal flavin as compared to the C(1)-H function of the analog, which, at least sterically, should be indeed a minor one, is sufficient to induce such a large effect.

It is thus conceivable that a similar (steric) effect is at the origin of the different properties of putative intermediates in flavocytochrome b_2. The groups closest to the α-CH_3 of lactate in a complex preceding carbanion formation are the flavin N(5) and the $-CH_3$ of Ala-198 (3.3 to 3.5 Å).[90] In the case of lactate oxidase, however, instead of alanine we find Gly-99, which clearly is less sterically demanding (see Table 3). While this might also have to do with the oxygen reactivity of the reduced enzyme-pyruvate complex as discussed further, it could well affect the stability of an N(5) covalent adduct. This is illustrated in Figure 11.

From the steric interactions to be deduced from Figure 11, we think it is legitimate to speculate that in the case of lactate oxidase, there is a greater steric tolerance around N(5) than in flavocytochrome b_2. This, combined with the smaller steric requirements of −H as the α-substituent R vs. $-CH_3$, might be the crucial factor increasing the stability of the covalent intermediate. The higher electrophilicity of glyoxylate might be the second factor of importance. In fact, glyoxylate exists mainly in its hydrated form as opposed to pyruvate. Earlier model work with free flavins confirms the differences in stability of reduced flavin N(5) adducts depending on the substituents of a carbonyl function. Thus, with formaldehyde, N(5) adducts are indeed formed, while an additional methyl group, as in acetaldehyde, prevents directly observable formation.[92-94]

Thus, steric effects in lactate oxidase, combined with the considerably higher electrophilicity of glyoxylate as compared to pyruvate, might be sufficient to account for the

difference in stability of a glycollyl adduct as compared to a putative lactoyl one. However, it should be pointed out that although glycollate oxidase, like flavocytochrome b_2, has an alanine residue at the analogous position occupied by glycine in lactate oxidase, the crystal structure shows that its peptide N atom is 5.2 Å from the flavin N(5)-position and that a water molecule is positioned close to this part of the flavin.[37] While it is conceivable that this geometry is altered in a complex with substrate and/or in the case of a covalent adduct, this should serve as a warning against too much extrapolation from protein sequence homology, which while impressive, can obviously still permit significantly different peptide chain folding.

E. ON THE FEASIBILITY OF SINGLE E^- TRANSFER STEPS AND COMPARISON WITH COVALENT CATALYSIS

A basic discussion of this problem has been presented by Bruice[86] and also by ourselves;[57] it is essentially an extension of the discussion introduced earlier in the context of the mode of transfer of redox equivalents as depicted in Scheme 7 and revolves about the differentiation between a "radical mechanism", essentially steps $(d_1) + (d_3)$ (Scheme 7) and "covalent catalysis" (steps [b] + [c] or $[d_1] + [d_3]$ + [c]). There appears to be agreement that in a complex of an α-carbanion with oxidized flavin (see Scheme 7), the fastest event might be the reversible transfer of $1e^-$ (step d_1). Steps (b) and (d_3) are similar. The starting species, oxidized flavin and the flavin radical anion, are planar and also the structure of substrate α-radical and α-carbanion are likely to be similar, since in the latter, charge delocalization will occur (see further). Thus, both pathways will be restricted by the same steric factors inherent to the structure of the adduct (see Figure 11). This would mean that the pathways (b) and $(d_1) + (d_3)$ might indeed have very similar energetic profiles and that they can be viewed as being equivalent at first approximation, the $[Fl^{\cdot -} \sim {}^{\cdot}C^-]$ complex being a transient on the way to the covalent adduct.

In a "radical mechanism", the step (d_2), which can follow (d_1), has some underlying restrictions of a different nature: transfer of $1e^-$ will form a flavin N(5) anion. Recognition of this was probably what induced Urban and Lederer[98] to propose this species as an (observable) intermediate. As will be discussed later, we consider this intermediate to be most unlikely. Avoiding this intermediate in a radical mechanism requires the concomitant transfer of $1e^-$ and one of H^+ to the flavin N(5). This must be the His-290H^+ proton, which originates from the substrate α-C. Such a step cannot be excluded, but has apparently not been considered to be an obvious one by the same authors.[98] Furthermore, either simultaneously or subsequently, transfer of the α-OH proton of the lactoyl radical to (presumably) solvent or to an appropriate base, possibly via Tyr-152 (cytochrome b_2 Y-254) (see Figure 10), also must take place. In any event, one experimental observation, which is very clear, should be stressed again in this context: both the covalent adduct and the radical pair complex (Scheme 7) can be only transient species when lactate is a substrate since neither has ever been observed.

The difference between formation of a covalent bond and e^- transfer steps $(d_1 + d_2)$ is likely to depend on the mode of approach and the distance, as well as the orientation of the two radicals in the complex $[Fl^{\cdot -} \sim {}^{\cdot}C^-]$ (right-hand side, Scheme 7). To form a covalent bond (e.g., in step d_3), their half occupied orbitals must have the correct orientation and a sufficient overlap. For the transfer of the second single electron (step $[d_2]$), electronic restrictions might be comparatively less stringent; however, there is the requirement of the simultaneous H^+ transfer from His-290 to the flavin N(5), which also requires proper alignment of orbitals. The distance between the flavin N(5) and α-C of (planar) pyruvate is ~3.7 Å in cytochrome b_2 (complex of flavin radical anion and pyruvate; Chapter 7); this is favorable for single electron transfer. On the other hand, conversion of planar pyruvate to the pyramidal glycollyl skeleton will bring the α-C closer to N(5) by about 0.5 Å, i.e.,

towards distances (~3 Å) where orbital overlap (i.e., bond formation) will start to become important. This covalent bond formation might not reach completion if the subsequent step, which also involves protonation of the flavin N(5) by His-290H^+, ensues rapidly.

In our opinion, in the case of glycollate, which is an alternate substrate, the covalent intermediate observed is a "true" catalytic intermediate. With lactate, the normal substrate, its relative energy might be much higher for the reasons discussed earlier, or it might be viewed as a transition state in which the covalent bond is formed only partially. One should keep in mind that optimization of catalysis would not favor the occurrence of "very stable intermediates". Along this speculative line, one could hypothesize that nature has favored the Gly-99->Ala-79 exchange in order to *destabilize* the putative covalent adduct which might be formed with normal substrates such as lactate with lactate oxidase and glycollate with glycollate oxidase.

Reiterating the earlier discussion, formation of a covalent link between N(5) and substrate α-C might go to completion only when the steric constraints can be overcome, as in the case of lactate oxidase and glycollate. In other cases bond formation might be only partial and occur in a step concerted with the subsequent breakage; i.e., the N(5)-C(α) bond is a true transient state.

F. ON THE QUESTION OF INVOLVEMENT OF ANIONIC FLAVIN N(5) AS AN INTERMEDIATE DURING CATALYSIS

Lederer[98] (see also Chapter 7) favors formation of a reduced dianionic flavin (charges at N[1] and N[5]) coupled with release of the α-OH proton, e.g., by Tyr-152 as a mechanism for the transfer of electrons from the carbanion to the oxidized flavin. This is equivalent to sequences (d_1 + d_2) of Scheme 7, with the omission of a concomitant H^+ transfer such as discussed earlier. There are, in our opinion, several lines of evidence against N(5) carrying a negative charge, as formulated by Lederer. In view of the basic importance of the argument, in the following we compare the thermodynamics of the ionization states of charged groups at the enzyme active center and of functions involved in catalysis.

Since the N(5) function in free reduced flavin is neutral (>N[5]-H), the formulation of an anionic form implicitly raises the question about its pK_a in the free state and in the complex with the amino acid functions at the active centers of α-hydroxy acid oxidases/dehydrogenases. This important aspect has not been addressed by Urban and Lederer.[98] The pK_a value of the enzyme bound form should be in a range compatible with enzymatic functional groups, i.e., it should not be "too far" from neutrality. While direct measurements do not appear to be feasible, there is, however, information allowing a reasonable estimate of the molecule in solution. In Figure 12, we have plotted the nuclear magnetic resonance (NMR) chemical shifts of ^{15}N of a series of aromatic amines[99] against their estimated pK_a values,[100] an accepted method for the estimation of ionization constants outside the experimentally accessible range. The correlation found[101] is quite satisfactory, the largest deviation from the interpolating line being ±2 pK units. The chemical shift of $^{15}N(5)$-H in reduced flavin is ~323 ppm (using nitromethane as reference)[95,96] and this places it in close neighborhood of aniline, the estimated pK_a of which is ~28.[100] Thus, even if the error of such an estimation would be large, the pK of N(5)-H can safely be put around 25 or above, i.e., some 18 units above neutrality. An independent estimation of this pK_a value relying on the rate of exchange of N(5)-H with solvent is >21.[101] In this context it is intriguing to note that the chemical shift of N(5)-H is essentially unaltered by the ionization at N(1) of reduced flavin,[101] and that also the chemical shift of $^{13}C(4a)$ is little affected by the same ionization,[97] in contrast to what would be suggested by chemical intuition.

The pK of His-290 in lactate oxidase is most likely ~4.7 in the case of a freely accessible active site (see studies on binding of oxalate, earlier, and Reference 40), and it is increased by some ≥5 units if a negative charge is placed in its vicinity such as upon binding of

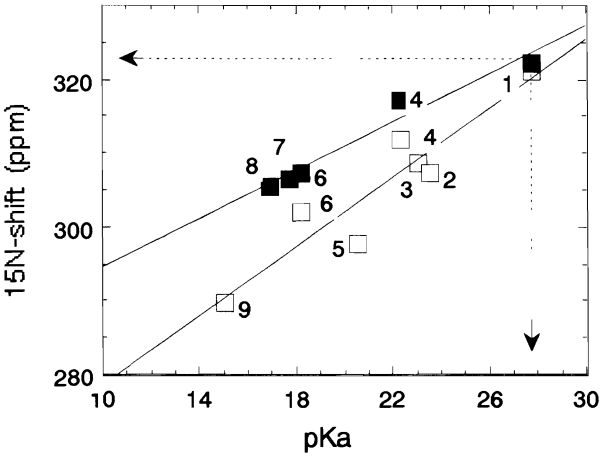


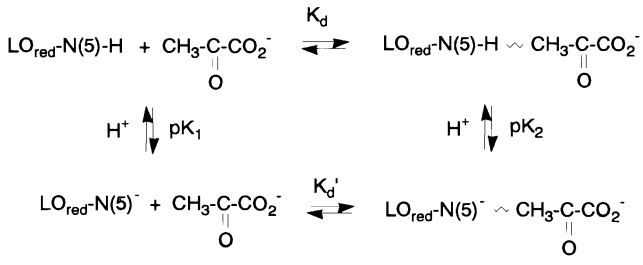
FIGURE 12. Correlation of ^{15}N-chemical shift with the pK_a of a series of aromatic amines. The values shown were taken from References 99 and 100 and are for data obtained in acetone (■) and in dimethylsulfoxide (□) as solvents, and referred to neat nitromethane as reference. The compounds are 1: aniline; 2: 2-azaaniline; 3: 4-cyanoaniline; 4: 4-azaaniline; 5: 2,6-diazaaniline; 6: 4-nitroaniline; 7: 2-nitroaniline; 8: 4-chloro-2-nitroaniline; and 9: 2,3-dinitroaniline. The horizontal arrow shows the chemical shift found for $^{15}N(5)$-H in reduced flavin,[95,96] and the vertical arrow extrapolates to a value ~28 for the pK_a of this function. (Adapted from Macheroux, P., Sanner, Ch., Rüterjans, H., Ghisla, S., and Müller, F., *Eur. J. Biochem.*, manuscript in preparation. With permission.)

dicarboxylic acids. The pKs of Tyr-44 and Tyr-152 are likely to be around 10 at first approximation. Thus in a (putative) transient imidazolium-α-carbanion pair, the pK of the latter might be lowered by at least 5 pK units, and thus possibly below 15. In Lederer and Mathews' scheme,[36] the step in catalysis subsequent to α-carbanion formation is proposed to generate two negative charges on the reduced flavin. The first one, at N(1), will be stabilized by (protonated) lysine (Lys-266 in lactate oxidase, Lys-349 in cytochrome b_2, see Figure 9). The second one, if on N(5), and regardless of its pK, would not be close to any positively charged species, except maybe for His-290.

It is thus clear that a presumed flavin N(5) anion will have the tendency to neutralize its charge at the expense of any one of its (protonated) neighboring functions having a lower pK. There is a further strong argument against the occurrence of an N(5) anionic species as an intermediate, which can be deduced from simple thermodynamic and kinetic considerations of the "reverse reaction", i.e., that of reduced enzyme with pyruvate. Reduction of lactate oxidase with L-lactate yields as a first observable product a species which is identical to that obtained upon reaction of pyruvate with reduced enzyme (see Figure 2 and earlier discussion). This leads to the conclusion that the long wavelength species assumed to be the complex of reduced enzyme (flavin N[5]-H) and pyruvate (Figure 2) would have to be a corresponding one in which N(5) is anionic. This $N(5)^-$ complex would have to be formed in two steps, namely binding of pyruvate and subsequent dissociation of N(5)-H as shown in Scheme 12. The (overall) binding constant, as expressed by the experimentally determined value $K_{d(exp)}$, should be as presented in Scheme 12.

At pH 7.0, the $K_{d(exp)}$ for pyruvate binding[6] is $2.5 \cdot 10^{-3}$ *M*; thus, assuming $K_1 \leq 10^{-25}$ *M*, it follows that K_d', the binding of pyruvate to N(5) anion flavin enzyme, would have an unreasonably low dissociation constant (i.e., $K_d' \sim 2.5 \cdot 10^{-21}$ *M*). If one assumes that the pK of the reduced flavin N(5) is lowered in the complex with pyruvate to, e.g., ~15, the value of K_d would still be unreasonably low ($K_d \sim 2.5 \cdot 10^{-11}$ *M*). (Based on the context of the discussion of Lederer in Chapter 7 and in Reference 98, N(5)-H is one of the functions to which an experimentally determined pK of ~15 might be attributed.) Assuming a still

$$K_{d(exp)} = \frac{K_d \cdot [H^+]}{K_2} = \frac{K_d' \cdot [H^+]}{K_1}$$



SCHEME 12. Steps which would be involved in the formation of an enzyme anionic reduced flavin complex with pyruvate. The scheme is purely hypothetical and represents the steps and equilibria, which are required for the formation of the reduced enzyme pyruvate complex in the case the flavin N(5) would exist in its anionic form. K_d and K_d' represent the binding of pyruvate to not ionized and to ionized reduced enzyme. K_1 and K_2 are the ionization constants of uncomplexed and of pyruvate complexed enzyme.

lower pK_a for this function might ease the problem of pyruvate binding, but will increase that of the pK shift of N(5)-H which must be induced by the protein. In this context it should be recalled that N(5) is accessible to solvent both in cytochrome b_2 (Chapter 7) and in glycollate oxidase,[17] rendering a large shift unlikely.

While these thermodynamic considerations make unlikely the hypothetical steps of Scheme 12, a clear rejection of them can be made on kinetic grounds. As discussed earlier in this review, the rate of decay of the reduced enzyme-pyruvate complex (2.5 min^{-1} = $4 \cdot 10^{-2} s^{-1}$), produced as an intermediate in the reduction of the enzyme by lactate, is the same as the value of k_{off} determined by rapid reaction kinetics studies of the formation of the same complex by mixing reduced enzyme with different concentrations of pyruvate.[6] In these studies there was no experimental evidence that would indicate a two-step mechanism of the type shown in Scheme 12. If the mechanism summarized in Scheme 12 did apply, then the spectral changes associated with formation and decay of the complex would have to be due either to protonation/deprotonation determined by pK_2 or to the initial formation of the complex from the predominant, reduced lactate oxidase ~N(5)-H form. If the color change were controlled by the K_2 step, then the experimentally observed decay rate of $4 \cdot 10^{-2} s^{-1}$ should equal $k_4 \cdot [H^+]$, i.e., since the rate was determined at pH 7, k_4 would equal $4 \cdot 10^5$ $M^{-1}s^{-1}$. However, if $K_2 = 10^{-15}$ M, then k_3 would be $4 \times 10^{-10} s^{-1}$, corresponding to a $t_{1/2}$ >50 years. This would have to be the step associated with the color changes accompanying formation of the complex from reduced enzyme and pyruvate, a clearly unlikely situation.

On the other hand, if the color changes were associated with the initial step defined by K_d, then the observed decay rate, $4 \cdot 10^{-2} s^{-1}$, would be due to k_2. If the two-step mechanism applied, and K_d was really $2.5 \cdot 10^{-11}$ M, then k_1 would be $1.6 \cdot 10^9$ $M^{-1}s^{-1}$. This is a theoretically feasible value, but one which does not fit with the experimentally measured value for the absorbance changes, 17 $M^{-1}s^{-1}$. Thus, the two-step mechanism of Scheme 12 is inconsistent with experimental results. The latter support strongly a simple one-step equilibrium between the catalytic intermediate and free reduced enzyme and pyruvate, yielding a kinetically determined K_d of $2.3 \cdot 10^{-3}$ M, in agreement with the thermodynamically determined K_d.[6]

Finally, if an N(5) anion would exist, it would be closest to the α-carbonyl of the product. In such a case very strong forces would have to be operative in order to prevent a nucleophilic attack on the latter. Furthermore, the hydrogen bridge between the pyruvate carbonyl and Tyr-152 would appear to be ideally set up to induce such a nucleophilic attack.

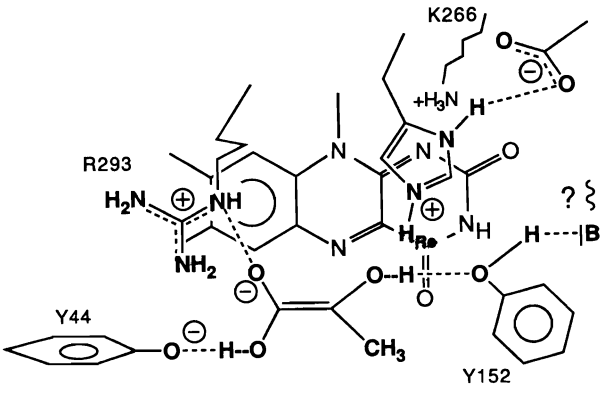


FIGURE 13. Active site description of the interactions which could stabilize the negative charge developing on the substrate α-position. Note that the substrate mesomeric structure shown is a canonical extreme, i.e., that delocalization towards Tyr-44 might be only partial. The existence of the base (?) to which a proton is being transferred from Tyr-152 is purely hypothetical.

G. ON THE MECHANISM OF TRANSIENT STABILIZATION OF THE SUBSTRATE α-CARBANION

Bruice[86] in a discussion of the detailed mechanism of α-OH-acid dehydrogenation has formulated the following requirement: "The formation of α-carbanion intermediate would require the delocalization of the electron pair to the undissociated carboxyl group." This delocalization will contribute to lower the pK of the lactate α-carbon (pK >18?). The presumed three-dimensional structure of the active center of lactate oxidase allows the formulation of a nice mechanism, in which the negative charge assumed to develop on the α-C is delocalized transiently to one of the carboxylate oxygens, from which, via interaction with Tyr-44, it can be transferred to the latter OH function (pK ~10). This would require that the transient (or intermediate) has a planar configuration. That this is possible is apparent from the planar configuration of pyruvate bound to the active center of flavocytochrome b_2 (see Chapter 7), from which representation the drawing of Figure 13 was adapted. The rupture of the substrate α-C-H would thus result in a separation of charges and their partial transfer to His-290 and Tyr-44, as shown on Figure 13. Subsequently, "charge" would "flow" to N(5) and end up at the function with the lowest pK, N(1), where it is stabilized by the interaction with Lys-266.

XIII. THE OXIDATIVE HALF-REACTION

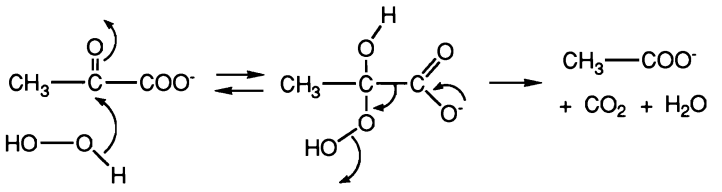
The second half of the catalytic cycle of lactate oxidase can be subdivided into two mechanistically different processes. First, oxygen must be activated by reaction with the reduced flavin to form presumably a peroxide, and second, the latter must react with the α-ketoacid and induce a decarboxylative fragmentation:

$$\text{a) } E\sim Fl_{red}H^- \sim R\text{–}C(=O)\text{–}COO^- + O_2 \rightleftarrows E\sim Fl_{ox}\sim H_2O_2\sim R\text{–}C(=O)\text{–}COO^-$$

$$\text{b) } E\sim Fl_{ox}\sim H_2O_2\sim R\text{–}C(=O)\text{–}COO^- \rightleftarrows E\sim Fl_{ox} + R\text{–}COO^- + CO_2$$

A. MECHANISM OF ACTIVATION OF OXYGEN

This process is generally thought to proceed via le^- oxidoreduction steps and an intermediate flavin-4a-hydroperoxide as summarized in some detail recently.[81] In the case of lactate oxidase, however, admission of oxygen to either free reduced enzyme or to its complex



SCHEME 13. Mechanism of oxidative decarboxylation of α-ketoacid by hydroperoxide.

with pyruvate leads to formation of oxidized enzyme flavin without any hint of the occurrence of intermediates of paramagnetic or diamagnetic nature and without formation of byproducts such as superoxide.[6] It should be noted that the reduced flavin is most probably in the anionic state prior to oxidation. The rate of reaction of free reduced enzyme with oxygen is $5 \cdot 10^5\ M^{-1}\text{min}^{-1}$ (Table 1) and thus considerably faster than that of free reduced flavin; this rate is further enhanced ~200-fold in the reduced enzyme-pyruvate complex (Table 1). The reasons for this rate enhancement are unknown; it could be speculated, that in the charge transfer complex between reduced enzyme and pyruvate, electronic effects favor the spin inversion steps required for reaction of triplet oxygen.

B. MECHANISM OF SUBSTRATE MONOOXYGENATION

The mechanism of flavin-mediated insertion of oxygen into substrate starting from a flavin-4a-hydroperoxide is still poorly understood.[81] The present case, however, is exceptional and the details of step b of the previous equation do not pose problems. Decarboxylation of α-ketoacids induced by peroxide is a reaction which proceeds also in the absence of enzymatic catalysis, albeit at a much slower rate.[6] The function of the enzyme in the present case is thus envisaged as consisting of the generation of H_2O_2 and ketoacid in close proximity and allowing the reaction to go to completion before dissociation of the components occurs.[6] The actual decarboxylation process can be formulated as shown in Scheme 13.[102]

C. GENERAL COMMENTS ON THE OXYGEN REACTIVITY OF α-HYDROXY ACID OXIDASES.

As pointed out earlier, the three-dimensional structures of the polypeptide chains of flavocytochrome b_2 and of glycollate oxidase are similar, in particular around the active center. These two enzymes are very different with respect to the oxygen reactivity, the former being a poor reactant with oxygen, while the second is an efficient oxidase. The three-dimensional structure of lactate oxidase is not yet available, but the similarities with the two other enzymes is such that a similar evaluation can easily be predicted. It is thus likely that a minor steric factor such as that discussed earlier for the case of Figure 11 is sufficient for governing access of oxygen to the reduced flavin. In other words, oxygen reactivity in this class of flavoproteins is likely to be governed by control of access of oxygen and not by influences on the chemistry of the reduced flavin. A particular case revolves around the factors which influence the reaction of the complex of the semiquinone anion of lactate oxidase and pyruvate with oxygen, which is extremely slow, as compared to that of fully reduced enzyme and the same keto acid, which is very fast (see earlier discussion). The two flavins are closely similar with respect to stereochemistry, location of charges, and most probably mode of binding of pyruvate (see Chapter 7). The main apparent difference between the two cases resides at N(5), which should have tetrahedral character in $Fl_{red}H^-$, and planar (sp_2) character in the anion radical as shown in Figure 14.

This difference alone does not appear to be sufficient to warrant such a large effect. In this case, the explanation might reside in a combination of thermodynamic and kinetic

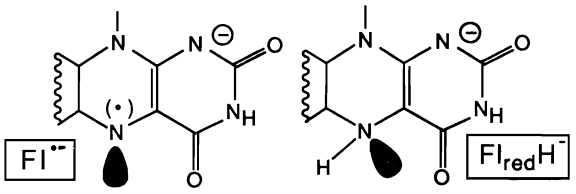


FIGURE 14. Comparison of the steric requirements of anionic flavin radical (left) and reduced flavin (right) around the flavin N(5).

effects. In the case of fully reduced enzyme, the thermodynamics clearly favor product formation:

$$[E{\sim}Fl_{red}H^{-}{\sim}Pyr] + O_2 \rightleftarrows [E{\sim}Fl_{ox} \sim H_2O_2 \sim Pyr] \longrightarrow \text{Products}$$

and the reaction is driven to the right also by the ensuing decarboxylation reaction. In contrast to this, as discussed in Section IV on redox properties, the tight binding of pyruvate to the anionic semiquinone form of the enzyme would be predicted to raise the potential of the E~FMN/E~FMN$^{\cdot -}$ couple to +80 mV, making the one-electron reduction of O_2 to $O_2^{\cdot -}$ by the flavin radical thermodynamically very unfavorable (E'_o of $O_2/O_2^{\cdot -}$ couple = −160 mV).[31]

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