Mechanism of Action of the Flavoenzyme Lactate Oxidase*

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SUMMARY

Lactate oxidase from Mycobacterium smegmatis forms a spectrally detectable intermediate during reaction with each of the substrates, L-lactate, β-phenyl L-lactate, L-α-hydroxy-β-methylvalerate and L-α-hydroxyisovalerate. This intermediate, while present in greater amounts during anaerobic reactions, is also seen in the course of aerobic catalysis. The intermediate formed from each substrate has a molar extinction coefficient at 540 nm of about 800 M⁻¹ cm⁻¹ and its rate of formation, kₚ, and rate of reaction with oxygen, kₔ, are fast enough to account for turnover. A spectrally identical intermediate can be made by mixing reduced enzyme with an excess of the corresponding keto acid. The intermediate produced by mixing reduced enzyme and pyruvate, on letting in air, forms the normal catalytic product, acetate. Free hydrogen peroxide does not react with free pyruvate at the concentrations and in the short time interval in which the enzymatic reaction occurs, thus eliminating the possibility that the pathway for acetate production includes the nonenzymatic reaction of these two substances. We conclude that (a) the reaction product with lactate is acetic acid only if oxygen is present from the start of the reaction; (b) in the absence of oxygen a reaction still takes place but it gives a different product, namely, pyruvate; (c) pyruvate is not a substrate. Both Cousins (2) and Sutton (3) noted that the catalytic conversion of lactate to acetate apparently does not involve free pyruvate or free hydrogen peroxide since the reaction is not inhibited by the pyruvate-fixing reagent, hydroxylamine, nor by catalase, a peroxide scavenger. Sutton suggested that the enzyme forms an intermediate with pyruvate which decarboxylates in the presence of oxygen but dissociates in its absence. Our mechanism agrees with this hypothesis.

Takemori et al. (4) observed what appeared to be two intermediates on reacting enzyme with lactate anaerobically. We have looked at the reaction of enzyme with various substrates in the stopped flow apparatus and have seen just one intermediate. This difference is due to the fact that we used 0.01 M imidazole-HCl buffer, while Takemori et al. worked in the presence of high concentrations of phosphate, a competitive inhibitor of the enzyme. Evidence is presented that the intermediate participates in catalysis and that it is a complex made up of reduced enzyme and ketoacid. The mechanism is like that of ω-amino acid oxidase, with product dissociation being the rate-determining step (5).

MATERIALS AND METHODS

L-α-Hydroxyisovaleric acid was purchased from Calbiochem, sodium [2-¹⁴C]pyruvate from Nuclear-Chicago. All other reagents were obtained from Sigma Chemical Company.

Enzyme was purified essentially according to the method of Sullivan (6) with the modification that the bacteria were broken in a Manton-Gaulin homogenizer at 6000 pounds per square inch and then treated with 20 mg of crude DNase per liter to reduce viscosity. All experiments were done at 25°C in 0.01 M imidazole-HCl, pH 7.0, unless otherwise indicated.

Separation and Identification of Acetic, Pyruvic, and Lactic Acids—The reaction mixture (2.6 ml) was applied to the top of a 12-m1 Dowex 1-X8 (Cl⁻) column in the cold room and eluted with an acid gradient, 250 ml of 10 mM HCl versus 250 ml of water. Acetic acid came off first at 140 to 144 ml, always in just one tube, followed by lactic acid, and lastly by pyruvic acid (400 to 500 ml). In control runs, made to establish the size column and volume of eluant needed to get good separation of 10⁻² moles of each reagent, acetic acid was identified by gas chromatography. A glass column (4 feet × ½ inch, inner diameter) packed with Chromosorb 102 (mesh size 100 to 120, Johns-Manville) was used. This packing has the advantage that acids can be applied in water solution. F & M 402 gas chromatograph was operated at 200°C. Pyruvate and lactate were assayed with lactic dehydrogenase (7).

In the enzyme reaction with [2-¹⁴C]pyruvate, acids were identified by comparing the elution pattern of the radioactive peaks to that of controls. Radioactive pyruvate was also chromatographed alone to determine the elution pattern of contaminants.

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Contaminants accounted for about 1 to 2% of the total pyruvate content and came off in two small broad peaks between acetic and pyruvic acids and clearly separated from both of them. The seinitilization fluid for aqueous samples (10 ml of fluid plus 1 ml of sample per vial) consisted of 100 mg of 1,4-bis[2-(5-phenyl-

absorbance at one wavelength were done with a Beckman-

Gilford spectrophotometer.

Flux measurement—The rate of the lactate oxidase-catalyzed reaction of oxygen with L-lactate, or one of its analogs, was measured by following oxygen consumption in the oxygen electrode (Yellow Springs Instruments, model 53) and by stopped flow measurements of absorbance change in the enzyme itself. The oxygen electrode data were obtained in a simple straightforward manner from initial velocities of oxygen consumption at 10%, 21%, 50%, and 100% oxygen saturation at various lactate concentrations. The reaction generally dropped off after the first minute, having by then consumed about 20% of the dissolved oxygen. Enzyme concentration is expressed in terms of FMN absorbance at 450 nm with a determined molar extinction coefficient of 10,600 M⁻¹ cm⁻¹ (11). A stock solution of 10 mM o-dianisidine in 3 mM imidazole-HCl buffer, pH 7.0, 25°, is 2.5 min⁻¹. The variation of the apparent first order rate constant for the formation of the intermediate as a function of lactate concentration is shown as a reciprocal plot in Fig. 1. Saturation kinetics are obtained, with a limiting velocity at infinite lactate concentration of 14,000 min⁻¹. Such results are consistent with the existence of an equilibrium reaction between enzyme and lactate prior to the formation of the intermediate (12).

\[ E + \text{L-lactate} \rightarrow E \text{L-lactate} \rightarrow \text{intermediate} \]

The dissociation constant for the enzyme-lactate complex can be obtained from the slope/intercept of this plot and is \( \frac{5 \times 10^{-2} \text{ M} \text{M}^{-1}}{\text{M}} \). The value of \( K_d \) is obtained from the intercept and is 14,000 min⁻¹.

The above results were obtained in the presence of 0.01 M imidazole buffer, pH 7.0. This buffer was chosen because of the sensitivity of lactate oxidation to inhibition by a wide variety of anions. The use of phosphate buffer in a previous rapid reaction study of this enzyme led to erroneous interpretation of the results (4, 13). Phosphate is an inhibitor of the lactate oxidase reaction, competitive with lactate and with a \( K_i \) of 1.0 to 1.6 \( \times 10^{-2} \text{ M} \). The formation of an enzyme-phosphate complex can be monitored readily by the rather extensive spectral changes which occur on the addition of phosphate (Fig. 2), characterized by an increase in absorbance in the region of 340 to 487 nm and a decrease in absorbance at wave lengths greater than 487 nm. The inset of Fig. 2 shows absorbance changes at 450 and 510 nm as a function of phosphate concentration, plotted by the method of Benesi and Hildebrand (14). The results give a value for the dissociation constant of the enzyme-phosphate complex of \( 1.95 \times 10^{-2} \text{ M} \), very similar to that of the \( K_i \) value obtained from inhibition studies.

The effect of added phosphate on the course of anaerobic reduction of the enzyme by lactate is shown in Fig. 3. In the absence of phosphate a simple biphasic reaction is observed. The changes at 540 nm have already been described above; these changes are reflected by a biphasic decay of absorbance at 460 nm, with the same rate constants. When 0.01 M phosphate is present, the results become much more complicated. The absorbance changes are now triphasic in nature. Approximately half of the rapid absorbance changes which occur in the absence of phosphate still occur, with approximately the same apparent rate constant. At 540 nm this is followed by a slow further increase in absorbance, which, however, never reaches the same level as in the absence of phosphate, presumably because its rate of formation is comparable with the normal slow decay of the intermediate. When the phosphate concentration is increased to 0.1 M, less than 10% of the reaction goes in the fast phase, as monitored either at 540 or 460 nm. On the assumption that the extent of rapid reduction is due to uncomplexed enzyme, the fraction of enzyme in the form complexed with phosphate
Fig. 1 (left). Comparison of the observed first order rates of conversion of lactate oxidase to intermediate with turnover number. The bottom line (○) shows the rates for intermediate formation when 1.94 × 10⁻⁸ M lactate oxidase was reacted anaerobically with the given concentrations of L-lactate. The upper line shows the results of analysis of stopped flow turnover experiments such as shown in Figs. 8 and 9. The points (□) were obtained from experiments in which 6.36 × 10⁻⁸ M enzyme was reacted with L-lactate at various concentrations and an initial O₂ concentration of 6.6 × 10⁻⁴ M. The points (△) were obtained can be determined. The inset in Fig. 3b shows treatment of the data in this way, yielding a value for Kₐ of 0.9 × 10⁻⁸ M. The rate of the slow formation of intermediate observed in the presence of phosphate is independent of phosphate concentration and is 7.2 min⁻¹. The simplest interpretation of these results is that lactate cannot reduce the enzyme flavin when phosphate is bound, and that the rate of the slow formation of the intermediate in the presence of phosphate is in fact merely the rate at which phosphate is released from the enzyme-phosphate complex (kₐ), kₐ = kₜₐ + k₉ₕ (15). From these results, the second order rate constant, k₉ₕ was determined to be ~600 M⁻¹ min⁻¹. On the assumption that k₉ₕ is equal to the rate of formation of the intermediate in the presence of phosphate (7.2 min⁻¹), Kₐ is therefore calculated on kinetic grounds to be 1.2 × 10⁻² M, in good agreement with the values obtained by spectrophotometric titration and from inhibition studies.

While not investigated in the same detail, analogous results have been found with a variety of inorganic and organic anions which have been found to be inhibitors of lactate oxidase. These all produce detectable, and sometimes marked perturbations of the visible absorption spectrum of the enzyme. Values of dissociation constants, obtained in the same way as shown in Fig. 2, are given in Table II for some of these compounds. Chloride ion is also found to bind weakly to lactate oxidase. For this reason we have worked at a low concentration of imidazole-HCl buffer (0.01 M), in order to minimize Cl⁻ inhibition, while at the same time buffering the system.

Demonstration that Intermediate Is Complex of Reduced Enzyme and Pyruvate—The identification of the nature of the long wavelength-absorbing intermediate observed transiently on anaerobic reduction of the enzyme by L-lactate is of obvious importance, since on kinetic grounds it is the only observable species which could be an intermediate in catalysis. The spectrum of the enzyme found after disappearance of the intermediate is typical of the anionic form of fully reduced flavin (17) and is quantitatively indistinguishable from that found on reductive titration with dithionite or by anaerobic reduction with NaB₄H₄ (18).
FIG. 3 (left). Effect of phosphate on the reduction of lactate oxidase by L-lactate under anaerobic conditions. Lactate oxidase, 1.43 x 10^{-6} M with respect to bound FMN, was reacted with 0.01 M L-lactate at 25° under anaerobic conditions, in 0.01 M imidazole (pH 7.0), alone, or in the presence also of 0.01 M potassium phosphate or 0.1 M potassium phosphate, pH 7.0. A, changes monitored at 540 nm. The initial A540 before mixing with L-lactate was zero. In the absence of phosphate, the appearance of 540 nm absorbance is monophasic and complete within 200 ms. B, changes monitored at 460 nm. The results are expressed as absolute absorbance values for 1-cm observation; the values actually measured were double since the stopped flow observation path was 2 cm. The arrows on the left ordinate of B show the initial A460 values in the presence and absence of phosphate. The inset shows a Benesi-Hildebrand plot of the fraction complexed with phosphate versus the phosphate concentration, as described in the text.

FIG. 4 (right). The rate of absorbance change at 450 nm when 1.97 x 10^{-5} M lactate oxidase (in 0.01 M imidazole, pH 7.0, 25°) was reacted with the concentrations of phosphate shown. The inset shows a Benesi-Hildebrand plot of the equilibrium absorbance changes found.

TABLE I

| [phosphate] | k'obs | k'corr | k |
|-------------|-------|--------|---|
| 2.5 x 10^{-2} M | 22.5 min^{-1} | 15.3 min^{-1} | 610 M^{-1} min^{-1} |
| 5 x 10^{-2} M | 39.6 min^{-1} | 32.4 min^{-1} | 650 M^{-1} min^{-1} |
| 1 x 10^{-1} M | 64.9 min^{-1} | 57 min^{-1} | 570 M^{-1} min^{-1} |

It is therefore reasonable to conclude that the transient intermediate decays to fully reduced enzyme, and that because of its slow formation (2.5 min^{-1}), the fully reduced enzyme cannot be involved in catalysis. As previous studies (2, 3) have indicated that pyruvate is a product of anaerobic reduction of the enzyme by L-lactate, an obvious possibility is that the intermediate is a complex of reduced enzyme and pyruvate, this complex having long wave length absorption, and that the observed decay time represents the rate at which pyruvate dissociates from this complex (k3 in the scheme below).

That this formulation is correct is supported by several lines of evidence. In Fig. 5 it is shown that addition of pyruvate to reduced enzyme results in the appearance of a long wave length-absorbing species. High concentrations of pyruvate are required for maximal production of the intermediate. The inset of Fig. 5 shows the data plotted according to Benesi and Hildebrand (14) to determine the dissociation constant of the reduced enzyme-pyruvate complex, as well as its maximal absorbance (obtained by extrapolation of the Benesi-Hildebrand plot to the ordinate). The dashed line (Fig. 5, Curve 7) shows the extrapolated spectrum of the fully formed reduced enzyme-pyruvate complex. At selected wave lengths are also shown the absorbance values observed in stopped flow studies at the end of the fast stage of the reduction process. It is evident that the transient intermediate observed in anaerobic reduction with lactate is the same as that formed in an equilibrium reaction between reduced enzyme and pyruvate.

From the data of Fig. 5, the Kd value for the reduced enzyme-pyruvate complex can be calculated as 2.5 x 10^{-3} M at 25°. An

![Diagram](http://example.com/diagram.png)
FIG. 5 (left). Equilibrium formation of intermediate from reduced lactate oxidase and pyruvate. Curve 1, oxidized enzyme, $8.6 \times 10^{-5}$ M with respect to bound FMN, under anaerobic conditions in 0.01 M imidazole, pH 7.0, at 25°. Curve 2, 5 min after addition of $8 \times 10^{-4}$ M sodium L-lactate. Curves 3, 4, 5, and 6, after the sequential addition of $1 \times 10^{-5}$ M, $2 \times 10^{-5}$ M, $5.33 \times 10^{-5}$ M, and $1.535 \times 10^{-4}$ M sodium pyruvate, added from side arms of the anaerobic cuvette. The inset shows extrapolation of the absorbance changes at 540 nm to $[\text{pyruvate}]$ by the method of Benesi and Hildebrand (14); Curve 7 of the main figure shows the spectrum of the fully formed complex calculated in this way. The points (●) show the absorbance at various wave lengths obtained at the end of the rapid phase of reduction of enzyme by L-lactate, determined in separate experiments using the stopped flow spectrophotometer (cf. Fig. 3).

FIG. 6 (right). Determination of the second order rate constant for binding of pyruvate to fully reduced lactate oxidase ($k_d$). Enzyme, $1.84 \times 10^{-5}$ M with respect to FMN content, was reduced with a stoichiometric concentration of L-lactate, and reacted under anaerobic conditions with the concentrations of pyruvate shown. The absorbance increase at 540 nm was monitored with the stopped flow spectrophotometer and the apparent first order rate constant calculated. The points shown are corrected by subtraction of the value of $k_a$ (2 min$^{-1}$). Conditions, 0.01 M imidazole, pH 7.0, at 20°.

A striking difference in the rate of reaction with O$_2$ is displayed by free reduced enzyme and reduced enzyme in complex form with pyruvate. This is illustrated in Fig. 7, in which reduced enzyme titrated with various concentrations of pyruvate was mixed with air-equilibrated buffer and reoxidation monitored in the stopped flow apparatus at 460 nm. The reoxidation is markedly biphasic, the extent of the reaction occurring in the rapid phase of the reaction being markedly influenced by the pyruvate concentration. On varying the O$_2$ concentration it was found that the reaction with O$_2$ is second order for both the fast and slow phases. The reduced enzyme in complex form with pyruvate was found to react with O$_2$ approximately 200 times faster than the uncomplexed reduced enzyme. At pH 7.0, 25°, the second order rate constants were found to be $1.1 \times 10^8$ M$^{-1}$ min$^{-1}$ and $5.4 \times 10^6$ M$^{-1}$ min$^{-1}$, respectively. If the proportion of the reaction occurring in the fast phase is assumed to represent the proportion of reduced enzyme in complex with pyruvate, the data of Fig. 7 can also be used to calculate the $K_d$ for the reduced enzyme-pyruvate complex. Treatment of the data in this way is shown in the inset of Fig. 7; the $K_d$ value so obtained is $2.2 \times 10^{-3}$ M, in good agreement with the results already described, and obtained by different experimental methods.

Results from Stopped Flow Turnover Experiments—When lactate oxidase is mixed with excess reducing substrate in the presence of O$_2$, a steady state level of oxidized enzyme and intermediate is rapidly established, which persists for varying lengths of time depending on the initial concentrations of O$_2$ and reducing substrate used. Fig. 8 shows the results of such an experiment with lactate as substrate, at an initial O$_2$ concentration of 2.0 $\times$ $10^{-4}$ M, and monitoring the absorbance at 490 nm. When in the same experiments the absorbance changes were monitored at 540 nm very little intermediate was detected in the steady state, but gradually accumulated as the O$_2$ was depleted. It is evident from Fig. 8 that most of the enzyme is in the oxidized form during the steady state. This is in agreement with the findings already documented, that the reaction of intermediate with O$_2$ (see previous section) is much faster than the rate of formation of the intermediate from oxidized enzyme and lactate (see Fig. 1). Following the principle enunciated by Chance (8)
and as detailed by Gibson et al. (9), the data of Fig. 8 can be used to calculate turnover numbers as a function of \(O_2\) concentration at each lactate concentration. This is done by assuming that the area swept out from the ordinate to the plateau level is proportional to the \(O_2\) concentration employed. Knowing the enzyme flavin concentration one can easily calculate turnover numbers at various \(O_2\) concentrations. The validity of this assumption has been documented previously (5, 9) and found to apply also in the present study; within experimental error the same turnover numbers are obtained at a given lactate concentration by increasing the initial \(O_2\) concentration to \(6.5 \times 10^{-4}\) M or by increasing the enzyme concentration 4-fold. Analysis of the data of Fig. 8 yields the series of parallel Lineweaver-Burk plots shown in Fig. 9. A secondary plot of the intercepts at infinite \(O_2\) concentration (from Fig. 9) is given in Fig. 1, together with values obtained in similar experiments employing different initial concentrations of \(O_2\) and enzyme. While the turnover numbers so obtained are similar in value to the observed rate of intermediate formation they are in all cases smaller. Within the limits of error, the reciprocal plot of turnover number is parallel to that of the rate of formation of intermediate. Qualitatively similar results have been obtained with all substrates tested, although big differences in individual rate constants have been observed. These will be documented later in a table. Fig. 10 shows a companion experiment to that of Fig. 8 but employing \(\alpha\)-hydroxy-\(\beta\)-methylvalerate as substrate instead of \(L\)-lactate. Analysis of the data yielded a set of parallel Lineweaver-Burk plots similar to those of Fig. 9; the turnover numbers (obtained by extrapolation to infinite \([O_2]\)) are shown in the upper line of Fig. 11. In the same figure are shown the rates of conversion of oxidized enzyme to intermediate, obtained by anaerobic stopped flow studies. (The intermediate produced by \(\alpha\)-hydroxy-\(\beta\)-methylvalerate has similar spectral properties to...
that produced by lactate, and may be generated in static equilibrium by addition of α-keto-β-methylvalerate to reduced enzyme.) The phenomenon of the turnover numbers being consistently lower than the corresponding rates of formation of the intermediate, and the parallel nature of the reciprocal plots, is well illustrated with this substrate.

A striking feature of the turnover results with this substrate, compared to lactate, is that although it clearly leads to intermediate formation at a much slower rate, nevertheless, less enzyme is in the oxidized form in the steady state than when lactate is employed as substrate (compare Figs. 8 and 10). This would imply that the intermediate in this case must react with O₂ considerably more sluggishly than does the reduced enzyme-pyruvate complex. Indeed this was found to be the case.

When reduced enzyme was titrated with α-keto-β-methylvalerate and reacted with O₂ at 25°C, the second order rate constant was found to be $2.7 \times 10^6$ M⁻¹ min⁻¹. This value should be compared to that for the reduced enzyme-pyruvate complex of $1.1 \times 10^8$ M⁻¹ min⁻¹.

The finding of parallel Lineweaver-Burk plots such as shown in Fig. 9 (and found for all substrates) is that expected for a binary complex mechanism (19, 20) in which the first product dissociates from the reduced enzyme before reaction of the latter with the second substrate (O₂). However, as pointed out previously, there are limiting cases of ternary complex mechanisms which also yield parallel Lineweaver-Burk plots (21, 22). The results obtained here are very reminiscent of those obtained previously with d-amino acid oxidase, which was also shown to operate by a ternary complex mechanism in which dissociation of oxidized product from the reoxidized enzyme was the rate-limiting step in catalysis (5, 22). In the present study the reaction of O₂ with intermediate has been shown to be second order. The minimal (and undoubtedly oversimplified) mechanism consistent with the observed results is therefore

$$
\text{K} \quad \text{FMN} \quad \text{E}' \quad k_2 \quad / \quad \text{FMNH}_2 \quad ' \quad \text{RCOCH}_2 \quad + \quad \text{O}_2 \quad \downarrow \quad \text{CO}_2 + \text{H}_2\text{O} \\
\text{EFMN} + \text{RCOOH} \quad k_5 \quad / \quad \text{RCOCH}_2 + \text{H}_2\text{O} \\
0 \quad \text{EFMN} + \text{O}_2 \\
\text{EFMN} + \text{RCOOH} \quad k_5 \\
0 \quad \text{EFMN} + \text{O}_2 \\
\text{EFMN} + \text{RCOOH} \quad k_5 \quad / \quad \text{RCOCH}_2 + \text{H}_2\text{O}$$

While $k_5$ is different depending on whether lactate or α-hydroxy-β-methylvalerate is employed as substrate, the observed turnover numbers cannot be explained by the rate-limiting step being either $k_2$ or $k_4$; in both cases the expected turnover numbers would be practically indistinguishable from the observed rates of formation of intermediate. The results can be explained very satisfactorily, however, by assuming that the rate-limiting step is $k_4$. For the above reaction sequence, the initial rate equation following Palmer and Massey (22) is

$$
\text{V} = \frac{\text{V}_{\text{max}}}{1 + K_{\text{RCHCOOH}}} + \frac{\text{V}_{\text{max}}}{[\text{O}_2]} + \frac{K_{\text{O}_2}}{[\text{RCHCOOH}][\text{O}_2]} \\
\text{where} \quad \text{V}_{\text{max}} = \frac{k_2 k_5}{k_2 + k_5} \\
k_{\text{RCHCOOH}} = \frac{k_2 (k_2 + k_3)}{k_1 (k_2 + k_3)} \\
k_{\text{O}_2} = \frac{k_5 (k_2 + k_3)}{k_4 (k_2 + k_3)} \\
K_{\text{O}_2} = \frac{k_4 (k_2 + k_3)}{k_1 k_4 (k_2 + k_3)}$$

The conditions for obtaining parallel Lineweaver-Burk plots (such as shown in Fig. 9, and found for all substrates) is that the last term in the denominator of the initial rate Equation 7 be negligibly small. This is entirely in keeping with the large value
of $k_1$ compared to the other known or calculated rate constants which go to make up $K$ (in view of evident difficulty of reversing the anaerobic reduction sequence (cf. Fig. 5) it is also reasonable to assume that $k_{-1}$ has a very small value).

The above mechanism also accounts very satisfactorily for the observation that with all substrates, the reciprocal plot of catalytic turnover number and reducing substrate concentration is parallel to the similar reciprocal plot of observed rate of reduction to the intermediate. This is due to the dissociation of product from the reoxidized enzyme ($k_3$) being at least partly rate-limiting.

On the assumption that the above mechanism is correct, $k_3$ may be calculated from the known $V_{\text{max}}$ and the determined value of $k_2$ by the use of Equation 8. For lactate as substrate, the value of $k_3$ calculated in this way is 11,300 min$^{-1}$. This calculated value may then be used in Equations 9 and 10 to predict values for $K_{\text{R CHOR COOH}}$ and $K_{O_2}$. For Equation 9, absolute values for $k_1$ and $k_{-1}$ are not available, but their ratio is. Thus, the $K_d$ for lactate ($k_{-1}/k_1$) from Fig. 1, is $5 \times 10^{-2}$ M. The other rate constants in Equation 9 are available, either as directly observed or as calculated values. If one then assumes various values for $k_1$ (and hence $k_{-1}$) one can therefore determine the minimal values of $k_3$ and $k_{-3}$ required to obtain a reasonable fit to the observed Michaelis constant, $K_{\text{lactate}}$, of $2.22 \times 10^{-2}$ M. Values of $k_3$ of $10^6$, $10^7$, and $10^8$ M$^{-1}$ min$^{-1}$ substituted in Equation 9 yield $K_{\text{lactate}}$ values of $2.85 \times 10^{-3}$, $2.31 \times 10^{-3}$, and $2.26 \times 10^{-2}$ M, respectively. Thus the minimal value of $k_3$ required to fit the observed $K_{\text{lactate}}$ value is $10^5$ M$^{-1}$ min$^{-1}$, an entirely reasonable value for enzyme-substrate interaction and orders of magnitude lower than would be permitted in a diffusion-controlled process.

A more telling test of the mechanism is the prediction of $K_{O_2}$ from the kinetically determined rate constants. It should be remembered that the reaction of $O_2$ with the intermediate appears to be second order, i.e. there is no experimental evidence for $O_2$ actually forming a complex with the intermediate; if such a complex is formed it must have a very large dissociation constant. Nevertheless a Michaelis constant for $O_2$, $K_{O_2}$, is readily measured, and with lactate as substrate, is determined as 7.1 $\times 10^{-2}$ M. All kinetic constants in Equation 10 are known, except for $k_{-3}$. If it is assumed that $k_{-3}$ is small compared to the other rate constants, an assumption which has been discussed previously, then $K_{O_2}$ can be calculated to be $5.5 \times 10^{-2}$ M. Considering that this value is derived from three independently measured or calculated rate constants, the agreement between the calculated and experimental $K_{O_2}$ is regarded as being very satisfactory.

The calculation of $K_{O_2}$ requires knowing $k_4$. The only other substrate for which $k_4$ has been determined is $\alpha$-hydroxy-$\beta$-methylvalerate, where a much lower value was found than with lactate. In this case the calculated value of $K_{O_2}$ ($8.1 \times 10^{-4}$ M) was also in very good agreement with the experimentally determined value ($8.0 \times 10^{-4}$ M).

The values of rate constants, experimentally determined or calculated, for the four substrates investigated in some detail, are shown in Table III. It can be seen that in all cases the dissociation of product from the reoxidized enzyme, $k_3$, is the step which largely controls the rate of overall catalysis.

Products from Reaction of Reduced Enzyme and Intermediate with Oxygen—It is well known that $H_2O_2$ will decarboxylate keto acids in a nonenzymic reaction. Hence the possibility existed that the primary products of the lactate oxidase reaction were free $H_2O_2$ and pyruvate and that these reacted independently of the enzyme to give the final products of acetate, $CO_2$, and $H_2O$. That this was not the case was shown in several ways. We tested the rate of reaction of $H_2O_2$ with pyruvate under conditions even more favorable for nonenzymic reaction than those used for the enzyme-catalyzed reaction. With the $\alpha$-dianisidinediaperoxidase assay for $H_2O_2$, we could detect no reaction in 30 min when $10^{-4}$ M $H_2O_2$ and $10^{-3}$ M pyruvate were incubated at 0°C in 0.01 M imidazole, pH 7.0, and only 30% destruction of $H_2O_2$ over 30 min with $10^{-4}$ M $H_2O_2$ and $10^{-4}$ M pyruvate. At 25°C, the reaction between $10^{-4}$ M $H_2O_2$ and $10^{-4}$ M pyruvate was also very slow (13% destruction of $H_2O_2$ in 30 min). In contrast, when $10^{-4}$ M lactate oxidase is added to air-equilibrated $10^{-4}$ M lactate, the reaction is complete within 10 s (to yield stoichiometric amounts of acetate and $CO_2$). Clearly, the enzyme-catalyzed reaction is much faster than the reaction of free pyruvate with free $H_2O_2$; therefore this pathway is eliminated as a significant route of product formation.

A second approach was to reduce enzyme with $L$-lactate under anaerobic conditions, wait a few minutes for the intermediate to disappear (i.e. allow time for pyruvate to dissociate from the reduced enzyme), then mix with air and assay for pyruvate and $H_2O_2$. The results, in Table IV, show that pyruvate and $H_2O_2$ are produced in amounts stoichiometric with the enzyme flavin. These results support the conclusion stated above and add further weight to the conclusions derived from kinetic experiments.

**Table III**

*Kinetic constants of lactate oxidase with various substrates*

| Constant          | $L$-Lactate | $\beta$-Phenyl $L$-lactate | $L$-$\alpha$-Hydroxy isovalerate | $L$-$\alpha$-Hydroxy-$\beta$-methylvalerate |
|-------------------|-------------|---------------------------|----------------------------------|---------------------------------------------|
| $K_d - k_{-1}/k_1$| $5 \times 10^{-3}$ M | $0.34$ min$^{-1}$ | $9 \times 10^{-4}$ M | $3 \times 10^{-4}$ 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$^{-1}$ | $<0.2$ min$^{-1}$ |
| $k_{-3}$          | $1 \times 10^6$ M$^{-1}$ min$^{-1}$ | $5.7 \times 10^6$ M$^{-1}$ min$^{-1}$ (calculated) | $3.3 \times 10^6$ M$^{-1}$ min$^{-1}$ (calculated) | $2.7 \times 10^4$ M$^{-1}$ min$^{-1}$ (observed) |
| $k_4$             | $11,300$ min$^{-1}$ | $1,000$ min$^{-1}$ | $1,370$ min$^{-1}$ | $350$ min$^{-1}$ |
| $V_{\text{max}}$, catalytic | $6,250$ min$^{-1}$ | $910$ min$^{-1}$ | $1,000$ min$^{-1}$ | $220$ min$^{-1}$ |
| $K_{\text{R CHOR COOH}}$ | $2.53 \times 10^{-4}$ M | $5 \times 10^{-4}$ M | $2.5 \times 10^{-4}$ M | $1.13 \times 10^{-4}$ M |
| $K_{O_2}$         | $7.1 \times 10^{-4}$ M | $1.6 \times 10^{-4}$ M | $3 \times 10^{-4}$ M | $8 \times 10^{-4}$ M |

$k_4$ (free enzyme) $= 5.4 \times 10^4$ M$^{-1}$ min$^{-1}$.
TABLE IV

| Reactants | Products |
|-----------|----------|
| [Enzyme flavin] | [L-Lactate] | [O2] | [Pyruvate] | [H2O2] |
| 10.0 × 10^-3 M | 10.0 × 10^-5 M | 21% | 0 | 0 |
| 3.09 × 10^-5 | 10.2 × 10^-5 | 0 | 2.75 × 10^-5 M | 0 |
| 5.15 × 10^-5 | 10.2 × 10^-5 | 0 | 4.84 × 10^-5 | 4.43 × 10^-5 M |
| 7.14 × 10^-6 | 15.3 × 10^-6 | 0 | 5.50 × 10^-5 | 6.42 × 10^-5 |

that the form of the enzyme which reacts with O2 is the intermediate of reduced enzyme and pyruvate.

That it is indeed the intermediate which reacts with O2 to produce acetate and CO2 was shown as follows. In a typical experiment, 1.18 × 10^-4 M enzyme was reduced anaerobically at 25°C with a stoichiometric amount of L-lactate, then a 10-fold M excess of [14C]pyruvate (labeled in the C-2 position and containing 1.22 × 10^6 dpm) was added to produce the intermediate. Air was then admitted, and the entire volume of 2.6 ml immediately applied to a Dowex column as described under "Materials and Methods." The acetate neatly separated from other radioactive components with a total count of 74,500 dpm. The number of counts expected for a stoichiometric production of acetate from intermediate calculated on the basis that the K_d for the dissociation of intermediate to reduced enzyme and pyruvate is 2 × 10^-4 M, is 42,400 dpm. From the measured absorbance at 540 nm prior to O2 addition, it could be calculated that lactate would have to be accompanied by the highly visible oxidized enzyme. No evidence was seen for such oxidized enzyme (cf. Fig. 5). It was also shown that the [14C]pyruvate was not contaminated with [14C]lactate; no reaction was detected with lactate dehydrogenase in the presence of DPN^+. Similarly, radioactive contaminants were eliminated as the source of the [14C]acetate. The [14C]pyruvate chromatographed separately on the Dowex column revealed the presence of two minor radioactive components well separated from acetate; these contaminants were still present in the same quantity in the experiments where the reduced enzyme was reacted with [14C]pyruvate and O2.

DISCUSSION

From the results described, we feel that the following reactions as shown in Scheme 1 constitute a minimal mechanism for the reactions catalyzed by lactate oxidase. There are several lines of evidence which argue strongly that the long wave length-absorbing intermediate is the species of enzyme which reacts with O2 in the catalytic sequence. It is the only spectroscopically detectable intermediate produced sufficiently fast to be involved in catalysis. With all substrates, the rate of formation of free reduced enzyme (k_2) is orders of magnitude slower than catalysis, making it unlikely that free reduced enzyme is involved in catalysis.

The catalytic noninvolvement of free reduced enzyme is also emphasized by its comparatively slow rate of reaction with O2, which is also too slow to account for catalysis. Furthermore, as shown by product analysis, reaction of this form of the enzyme with O2 results in stoichiometric formation of H2O2 rather than H2O.
H₂O₂, the normal product of catalysis. In contrast, the intermediate, formed by mixing reduced enzyme with excess pyruvate, reacts with O₂ some 200-fold faster than the reduced enzyme, and acetate is the product of the reaction, together with H₂O and CO₂. Thus a highly favorable situation exists in the enzyme for the normal catalytic reaction to occur, rather than the production of free keto acid and H₂O₂ since the intermediate, produced rapidly, is also capable of rapid reaction with O₂, whereas free reduced enzyme and pyruvate are formed only slowly, and free reduced enzyme, once formed, is capable of only slow reaction with O₂.

The nature of the long wave length-absorbing intermediate is of obvious interest. Its spectral characteristics, with little absorbance at 450 nm, indicate that it is a form of reduced flavin. That it can be produced in an equilibrium reaction with keto acid argues that it may well be a charge transfer complex between reduced enzyme flavin and keto acid. Whatever its nature, it is evident that keto acid is bound to the reduced enzyme in close juxtaposition to the FMNH₂. Reaction of the latter with O₂ to produce H₂O₂ would then seem to be a reasonable postulate to account for catalysis, since the two products, H₂O₂ and keto acid, still bound to the enzyme, might be expected to react much more effectively than in free solution, to produce the oxidative decarboxylation products typical of this enzyme.

With all substrates tested (cf. Table III) a step in catalysis which is at least partly rate determining, is the dissociation of products (k₅). This step is responsible for the fact that in reciprocal plots such as illustrated in Figs. 9 and 11 the plot for turnover number is parallel to that for the rate of production of the intermediate. As discussed under “Results,” the determined and derived kinetic constants are fully consistent with this mechanism. The finding of distinctly different values of k₅ depending on the substrate used argues strongly for k₅ being the rate constant associated with the release of R-COOH from the oxidized enzyme; if the release of CO₂ or H₂O had been rate limiting, the same value of k₅ would have been found for all substrates. The finding of such a rate-limiting step is interesting in view of the similar phenomenon found previously with D- and L-amino acid oxidases (23), β-chlorolactate has been found to be a substrate for lactate oxidase under both aerobic and anaerobic conditions. The products of the anaerobic reaction are pyruvate and Cl⁻; in the presence of O₂ the products are H₂O₂, CO₂, and chloroacetate. Thus, as with D- and L-amino acid oxidases (23), it is probable that the primary step in the lactate oxidase reaction involves the abstraction of a proton from the substrate by the enzyme. This work will be reported in full in a separate communication.

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