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Mechanism of Inactivation of the Flavoenzyme Lactate Oxidase by Oxalate*

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SUMMARY

Lactate oxidase from *Mycobacterium smegmatis* is strongly inhibited by oxalate. The inhibition is reversible in the absence and irreversible in the presence of light. Oxalate is bound to the enzyme in a two-step process with an over-all K_d of 1.6×10^{-5} M. The first step is a fast second order reaction with $k_{-1}/k_1 = 8.3 \times 10^{-3}$ M, leading to a Complex I. This complex is then reversibly converted to a different Complex II in a slow first order reaction ($k_2 = 40$ min^{-1}; $k_{-2} = 0.07$ min^{-1}), which is accompanied by major spectral perturbations of the flavin spectrum. With oxamate, two steps could not be demonstrated, and its binding is described by a single step reversible process, which is second order in oxamate ($k_1 = 6.8 \times 10^3$ M^{-1} s^{-1}, $k_{-1} = 28$ s^{-1}). Upon illumination the enzyme-oxalate Complex II is converted very rapidly into a reduced flavoenzyme with carbonate covalently bound at position N(5) of the flavin. Slow hydrolysis in the dark under anaerobic conditions subsequently yields free reduced enzyme. The light reaction of the oxamate complex is, in contrast, very slow and yields a stable N(5) urea adduct of the reduced flavin.

Two recent publications have dealt with the mechanism of action of the flavoenzyme lactate oxidase from *Mycobacterium smegmatis* (1, 2), which catalyzes the oxidation of L-lactic acid to acetic acid and carbon dioxide under aerobic conditions. Under anaerobic conditions lactate is stoichiometrically oxidized to pyruvate and reduced enzyme is accumulated. Lockridge *et al.* (1) have also shown that this enzyme binds a large number of small anions, which behave as inhibitors competitive with lactate. During the course of our work we observed that oxalate is strongly bound to the enzyme, giving a marked perturbation of the flavin spectrum, and that it causes an unusual inhibition of the enzymatic activity. The extent of inhibition was found to be dependent on the time of incubation and on the presence of light.

The inhibition by oxalate (or oxamate) of NADH-dependent L- and D-lactic dehydrogenases from bacterial (3) and from mammalian sources (4-6) has long been recognized, and its mechanism has been investigated extensively. Oxalate has also been reported to inhibit flavoproteins that oxidize lactate (7, 8), but no mechanistic details have been published. It has been reported that some oxalic acid oxidases from plant sources, which utilize oxygen in the oxidation of oxalate to CO_2 and H_2O_2, are flavoproteins (9), or require riboflavin or FMN as cofactors (10). This claim has been questioned by Halliwell (11), who showed that oxalate is oxidized in the absence of enzyme by free flavins, such as riboflavin or FMN, and that this reaction requires light. Blankenhorn (12) similarly reported that flavins react with oxalate on illumination.

The above mentioned observations prompted us to investigate in some detail the mechanism of inhibition of the flavoprotein lactate oxidase by oxalate and to study the sensitivity of the interaction to light.

EXPERIMENTAL PROCEDURE

Materials—Lactate oxidase from *Mycobacterium smegmatis* was prepared as previously described (1). [^{14}C]Oxalic acid was purchased from Amersham-Searle and diluted with unlabeled oxalate. β-Chlorolactate was purchased from Sigma and purified from substantial oxalate impurities by precipitation of the latter as the calcium salt and subsequent recrystallization to constant melting point (79-81°) as described earlier (2). Commercial oxamic acid was found to contain significant amounts (up to 0.5%) of oxalic acid, and was purified as follows. A 0.1 M solution of $KMnO_4$ in water was added dropwise with stirring to a saturated aqueous solution of oxamic acid at approximately 60° until the rate of disappearance of the permanganate color had markedly decreased. A slight excess of permanganate was then added, the mixture was cooled immediately in an ice-brine mixture, and the precipitate was collected by vacuum filtration and washed with cold water and a small amount of acetone. The oxamic acid obtained by this procedure is oxalate-free and can be purified from trace MnO_2 contaminations by filtration of its solutions in the presence of small amounts of Celite-charcoal or by recrystallization from water. Lumiflavin-N(3)-acetate was synthetized from lumiflavin as described elsewhere (13). All other chemicals and reagents were the best commercially available grade.

Methods—Absorption spectra, slow kinetic studies, and the spectroscopic determination of dissociation constants were carried out with Cary 17, 14, or 118 recording spectrophotometers at 25°, when not otherwise indicated. For anaerobic experiments Thunberg-type cells were used.

The rates of the enzyme-catalyzed reaction (L-lactate + $O_2 \rightarrow$ acetate + CO_2 + H_2O) were measured by following oxygen consumption with an oxygen electrode (Yellow Springs Instruments, model 53). The rate of catalytic pyruvate production from β-chlorolactate ($Cl-CH_2-CHOH-COOH \rightarrow CH_3-CO-COOH + Cl^-$) was measured as described earlier (2).

The rapid kinetics and the relaxation experiments were carried out at 25° with the stopped flow apparatus of Gibson and Milnes

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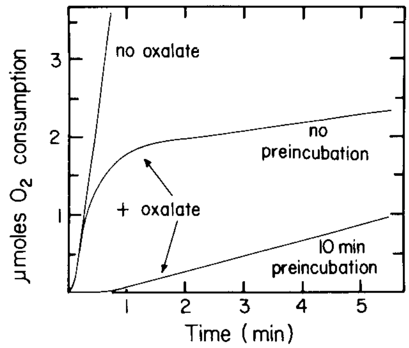


FIG. 1. Effect of oxalate on the oxidation of L-lactate to acetate followed by measurement of the oxygen consumption. The reaction was initiated by addition of the enzyme to the reaction mixture (3.0-ml final volume), containing 6.6×10^{-2} M L-lactate and, when indicated, 6.6×10^{-3} M oxalate in 0.01 M imidazole-HCl buffer, pH 7.0, at 25°. For the *lower curve* the enzyme was preincubated at 0° with a 20-fold molar excess of oxalate in the dark for 10 min. The assays in the presence of oxalate were carried out in the dark. Illumination of the reaction vessel caused progressive loss of activity and, when the enzyme was preincubated with oxalate in the light, practically no activity was observed (results not shown).

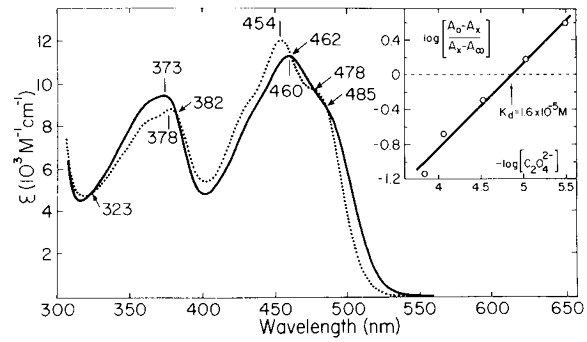


FIG. 2. Effect of oxalate on the absorption spectrum of lactate oxidase. ——, $2{,}4 \times 10^{-5}$ M enzyme (with respect to FMN) in 0.01 M imidazole-HCl buffer, pH 7.0, at 25°. ·····, in the presence of 5×10^{-4} M oxalate. The *arrows* indicate the isosbestic points of the spectral changes. *Inset*, Hill-type plot of complexed-uncomplexed enzyme *versus* oxalate concentration. (A_0 = absorbance of free enzyme, A_∞ = absorbance of enzyme in the fully complexed state, A_x = absorbance at the oxalate concentrations shown.) A_∞ was obtained by addition of a large excess of oxalate (10^{-2} M), and A was measured at 504 nm.

TABLE I

Dissociation constants of some carboxylic acids, which form complexes with lactate oxidase

Anion of	K_d	T
	M	°*C*
Acetic acid, CH_3COO^-	1.1×10^{-2}	25
Formic acid, $HCOO^-$	4.0×10^{-3}	25
Carbonic acid, $OCOO^{2-}$	5.0×10^{-3}	4
Oxamic acid, $H_2N\text{-}COO^-$	2.8×10^{-3}	25
Oxalic acid, $(CO_2)_2^{2-}$	1.6×10^{-5}	25

(14) which was modified as will be described elsewhere.[1] The light irradiations were carried out with the apparatus described previously (15), and the course of the reaction was followed spectrophotometrically by measuring the decrease of absorption at the λ_{max} in the 445 to 454 nm region. (The light intensity was of the order of 10^4 to 10^5 ergs per cm² per s as measured with a Yellow Springs model 65 radiometer, but the position of the cuvette was varied empirically in order to obtain optimal experimental conditions, which were then kept constant for each set of experiments.) In the experiments described in Fig. 8, the sample cuvette, immersed in a water bath thermostated at 20°, was placed in the focal plane of the reflector of a 650-watt tungsten-iodine lamp (model Q 1U, Smith-Victor Corp., Griffith, Ind.).

In the ^{14}C incorporation experiment, the radioactivity was measured with a Packard Tri-Carb model 3320 scintillation counter in toluene-Triton X-100 containing 2,5-diphenyloxazole (PPO) and *p-bis*-(*o*-methylstyryl)benzene (MSB) with an average efficiency of 80%. The photoreaction was followed by the decrease of absorption at 454 nm and stopped when the rate of the photoreaction had markedly decreased.

The reaction mixture was passed through a Sephadex G-25 column (40 × 1.5 cm), which was found to separate efficiently labeled enzyme, free oxalate, and carbonate (*cf.* Fig. 10).

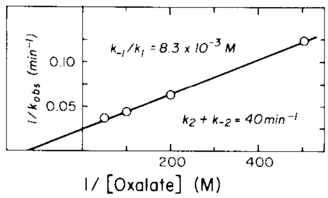
RESULTS

Effects of Oxalate on Catalytic Activity of Lactate Oxidase—Whereas most inorganic anions (*e.g.* phosphate, sulfate, chloride (1)) and some monocarboxylic organic anions (*e.g.* formate, acetate, oxamate) inhibit lactate oxidase in a typical competitive manner, the effect of oxalate on the enzymatic activity was found to be dependent on the time of incubation and on exposure to light. When lactate oxidase is added to an assay mixture containing lactate and oxalate (Fig. 1), the initial rate of oxygen consumption is similar to the rate observed in the absence of oxalate, but it rapidly decreases and finally, in the dark, converges to a second linear phase. The rate of the second phase is similar to the rate observed when the added enzyme is preincubated with oxalate in the dark. When the reaction vessel is illuminated with a strong light source, the rate of the second slow phase gradually diminishes to zero. Similarly, after preincubation of the enzyme with oxalate (*cf.* Fig. 1) in the light, practically no activity is observed. A similar inhibition pattern is exhibited by oxalate in the lactate oxidase catalyzed anaerobic elimination reaction, which forms pyruvate and chloride from β-chlorolactate (2).

Spectral Effects on Binding of Oxalate to Lactate Oxidase—When oxalate is added to lactate oxidase the characteristic, nonresolved flavin spectrum of the uncomplexed enzyme (in 0.01 M imidazole-HCl, pH 7.0) changes to a spectrum of the resolved type (16) (Fig. 2), in a way similar, but not identical, to that observed upon addition of inorganic anions (1). The difference spectrum between the uncomplexed and fully complexed forms shows positive maxima at 483, 450, 425, and 391 nm, negative maxima at 504 ($\Delta\epsilon$ = 2000 M^{-1} cm^{-1}), 470, 370, and 355 nm, and the isosbestic points indicated in Fig. 2. A K_d value of 1.6×10^{-5} M for the binding of oxalate to the enzyme can be estimated by several different methods from these spectral changes. At high oxalate concentrations (10^{-2} M), no further change in the absorption spectrum of the flavin chromophore is observed. The linearity and the slope (n = 1) of a Hill plot of the absorption changes are consistent with the reasonable assumption that only 1 molecule of oxalate per FMN unit causes the spectral perturbation in the concentration range covered. It should be noted that the approach to equilibrium is slow in the range of oxalate concentration used (10^{-5} to 5×10^{-4} M). In contrast to this, with the other small carboxylic acids investigated (*cf.* below and Table I) the process of binding is comparatively very fast. These anions cause similar, but less pronounced, spectral changes upon binding.

Kinetics of Binding of Oxalate to Lactate Oxidase—The time dependence of the oxalate inhibition illustrated in Fig. 1 suggests a slow step in the equilibrium between oxalate and enzyme.

[1] D. Ballou and G. Ford, in preparation.



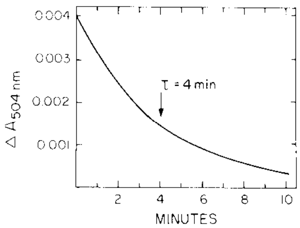


FIG. 3 (*left*). Reaction of lactate oxidase with oxalate followed at 504 nm in the stopped flow apparatus. Conditions: enzyme 3.25×10^{-5} M and variable oxalate concentrations in 0.01 M imidazole-HCl, pH 7.0, at 25°. See text for further explanation.

FIG. 4 (*right*). Relaxation of the lactate oxidase oxalate Complex II in a concentration jump experiment. The enzyme, 3.47×10^{-5} M, and oxalate, 1.0×10^{-4} M, were preincubated for >30 min in 0.01 M imidazole-HCl buffer, pH 7.0, and then diluted in the stopped flow apparatus with 1 volume of 0.01 M imidazole-HCl buffer at 25°. The spectral changes were recorded at 504 nm.

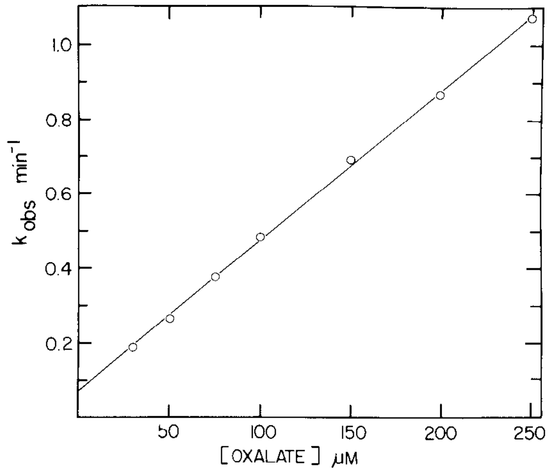


FIG. 5. Dependence of the reaction rate of complex formation on oxalate concentration at low oxalate concentrations. Lactate oxidase, 1.5×10^{-5} M (final concentration after mixing) was reacted with the concentrations of oxalate shown, and the decrease in absorbance was monitored at 504 nm in the stopped flow apparatus. Conditions: all reagents in 0.01 M imidazole-HCl, pH 7.0, 25°. It should be noted that such a plot is linear only at low concentrations, curving toward the horizontal axis as the oxalate concentration is increased (see the reciprocal plot of Fig. 3 at higher oxalate concentrations).

Accordingly, it was of interest to follow the kinetics of binding by rapid reaction spectrophotometry. When the formation of the enzyme-oxalate complex was monitored at 504 nm, it was found that the spectral change could be accurately described by a first order rate constant, k_{obs}, whose value depended on the oxalate concentration. Fig. 3 shows a plot of the reciprocal of k_{obs} *versus* the reciprocal of the oxalate concentration. Clearly the rate of complex formation approaches a maximum as the oxalate concentration is increased. A theoretical treatment of such plots[2] shows that the results are compatible with a two-step equilibrium process,

$$\mathrm{E} + \text{oxalate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(Complex I)}}{\mathrm{E}\cdot\text{oxalate}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{(Complex II)}}{\mathrm{E}^{*}\cdot\text{oxalate}}$$

in which the spectral change is due to Complex II, and in which the intercept on the $1/k_{obs}$ axis is equal to $1/(k_2 + k_{-2})$ and provided that $k_{-2} \gg k_2$, as will be shown later, the slope divided by intercept approximates closely to k_{-1}/k_1. From the data, the dissociation constant of the first complex, k_{-1}/k_1 can be estimated as 8.3×10^{-3} M, and $k_2 + k_{-2}$ equal to 40 min^{-1}. As the over-all equilibrium between enzyme, oxalate, and Complex II should be defined by a dissociation constant, $K_d = k_{-1} \cdot k_{-2}/k_1 \cdot k_2$, it was of obvious interest to determine k_{-2} directly. This was done by setting up the equilibrium with suitable concentrations of enzyme and oxalate, allowing sufficient time for full establishment of the equilibrium, and then perturbing the equilibrium rapidly by dilution in the stopped flow apparatus by mixing with an equal volume of buffer. Fig. 4 shows the results of such a concentration jump experiment. Such experiments were used to estimate k_{-2}, according to the method described by Gutfreund (17),

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_2}{1 + K_1/(\bar{C}_E + \bar{C}_{oxalate})}$$

where $K_1 = k_{-1}/k_1$, and $\bar{C}_E$ and $\bar{C}_{oxalate}$ are the concentrations of free enzyme and oxalate after perturbation of the equilibrium. From such results k_{-2} is estimated as approximately 0.05 min^{-1}. Thus, from the rapid reaction studies, the over-all dissociation constant, $k_{-1} \cdot k_{-2}/k_1 \cdot k_2$ can be calculated as 8.3×10^{-3} M $\times$ $0.05/40 = 1.05 \times 10^{-5}$ M. This is in reasonable agreement with the value determined by the equilibrium dependence of spectral change with oxalate concentration shown in Fig. 2, where a value of 1.6×10^{-5} M was obtained.

An alternative method of determining k_{-2}, which does not rely on estimates of the equilibrium concentrations of free enzyme and oxalate, has also been employed. The theoretical basis for this method will be reported separately,[2] and relies on the fact that even for a two-step equilibrium reaction, *at low concentrations* of the ligand (oxalate in this case), and when $k_2, k_{-2} \ll k_1, k_{-1}$ plots of k_{obs} *versus* ligand concentration should be linear, extrapolating at zero ligand concentration to a value of k_{obs} equal to k_{-2}. The results of such a study are shown in Fig. 5 where enzyme at a final concentration of 1.5×10^{-5} M in respect to bound flavin was reacted in the stopped flow apparatus with the concentrations of oxalate shown. Under all conditions the approach to equilibrium was fitted closely by a first order rate constant, k_{obs}, even when the oxalate concentration was approximately the same as that of the enzyme. From the intercept of Fig. 5, k_{-2} is determined as approximately 0.07 min^{-1}. Combined with the independently determined values of k_{-1}/k_1 and k_2, this would correspond to an over-all dissociation constant of 1.45×10^{-5} M. Thus, within the limits of experimental error of determination, the binding of oxalate to lactate oxidase is very satisfactorily accounted for by a two-step equilibrium process, where the primary binding is comparatively weak, as it is with other anions, and where the over-all tighter binding is determined largely by the forward and reverse rate constants of the second step.

Binding of Oxamate to Lactate Oxidase—In contrast to the tight binding found with oxalate, its half-amide oxamate was found to bind only weakly to lactate oxidase. The spectral perturbations of the flavin chromophore resulting from oxamate addition were much smaller than with oxalate, and the difference spectrum between free and complexed enzyme was significantly different, with positive ΔA_{max} at 488, 456, 427, and 399 nm and negative ΔA_{max} at 510, 474, and 350 nm.

From the spectral perturbations, the K_d for the enzyme-

[2] S. Strickland, G. Palmer, and V. Massey, in preparation.

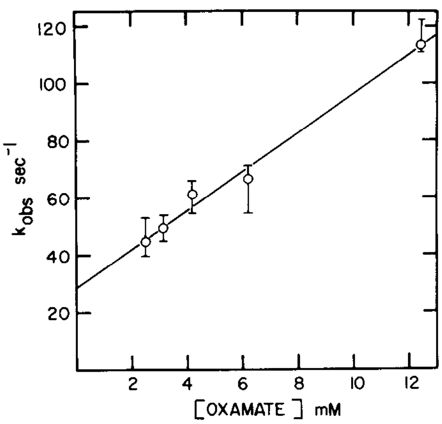


FIG. 6. Reaction of oxamate with lactate oxidase followed in the stopped flow apparatus. Conditions: enzyme, 2.9×10^{-5} M in 0.01 M imidazole-HCl buffer, pH 7.0, and variable concentrations of oxamate in the same buffer at 25°. The *circles* represent the average of at least five measurements, and the *bars* indicate the range of the individual measurements. The *line* was determined by a least square analysis of all points.

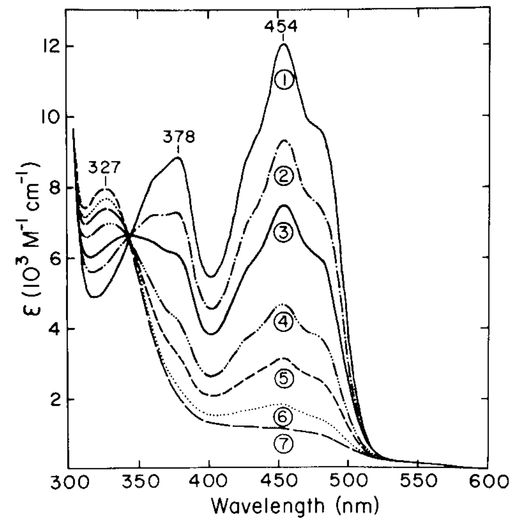


FIG. 7. Effect of light on the lactate oxidase-oxalate complex. A 2.5×10^{-5} M solution of lactate oxidase in 0.01 M imidazole-HCl buffer, pH 7.0, was preincubated for 20 min with 2.5×10^{-4} M oxalate in the dark (*Curve 1*). *Curves 2, 3, 4, 5,* and *6* after 5, 10, 20, 30, and 50 s of illumination, respectively, in the apparatus as described elsewhere (11). *Curve 7*, end of the reaction after a total of 8 min of illumination.

oxamate complex at pH 7.0, 25°, was estimated as 2.8×10^{-3} M. It should be emphasized that it was necessary to purify rigorously the oxamate from trace contamination of oxalate since, owing to the much tighter binding of the latter, small extents of contamination with oxalate alter significantly the observed results.

Also, in contrast to the results with oxalate, oxamate appears to bind to lactate oxidase in a rapid one-step equilibrium process, with no subsequent slower spectral changes. Fig. 6 shows a plot of the observed first order rate constants *versus* oxamate concentration obtained in a stopped flow study, where the kinetics of binding was determined either by following the absorbance decrease at 510, 350, or 358 nm, or by the absorbance increase at 394 nm. Identical kinetic behavior was found at all wavelengths. From Fig. 6, k_{-1} can be determined from the intercept as 28 s^{-1} and k_1 from the slope as 6.8×10^3 M^{-1} s^{-1} (18). Thus, the K_d determined kinetically, k_{-1}/k_1 of 4.1×10^{-3} M, is in reasonable agreement with that obtained from equilibrium spectral changes. It should be pointed out that the kinetic data do not rule out the possibility of oxamate binding in a two-step process, but with very different rate constants than those found for oxalate. However the data obtained are satisfactorily explained by a single step equilibrium binding, in contrast to the results with oxalate.

Reaction of Lactate Oxidase-Oxalate Complex with Light—When lactate oxidase is exposed to light in the presence of excess oxalate, a very fast reaction ($t_{1/2} \sim 12$ s) leads to disappearance of the typical spectrum of the oxidized flavin chromophore and, in the presence of O_2, to isosbestic formation of a new, nonfluorescent species with an absorption maximum at 327 nm (Fig. 7). No formation of semiquinones was observed during the reaction. The action spectrum of the photoreaction must approximate that of the oxidized enzyme, since similar rates were obtained on irradiation with monochromatic light at 380 and 455 nm (8 nm half-bandwidth).

If the photoreacted enzyme is treated with 80% methanol at 50–60° for a few minutes in the presence of oxygen, the denaturation of the protein results in immediate and complete liberation of oxidized FMN, which is detected by its fluorescence (enzyme bound FMN is nonfluorescent (19)) and absorption spectrum. In contrast to this, the photoproduct is relatively stable while it is bound to the apoenzyme; on standing at 25° aerobically in the dark, a slow decay takes place and the characteristic spectrum of the oxidized enzyme (*cf.* Fig. 7, *Curve 1*) is slowly restored ($t_{1/2} \sim 3.8$ hours). In the absence of oxygen a similar reaction occurs ($t_{1/2} \sim 4$ hours) which leads to a species having the characteristic spectrum (absorbance λ_{max} = 360 nm) and fluorescence (emission λ_{max} = 507 nm) of the reduced enzyme (19). The similarity of the rates of these decay reactions indicates that they probably constitute an oxygen-independent hydrolysis which leads primarily to enzyme-bound reduced FMN, which, in the presence of oxygen, immediately forms the oxidized enzyme (*cf.* Scheme 1).

It should be pointed out that the spectrum of the photoproduct is very similar to the spectrum of the released photoproduct obtained from inactivated enzyme reported by deKok *et al.* (20) upon illumination of D-amino acid oxidase in the presence of α-keto acids. These authors demonstrated that a covalent N(5)-acyl-reduced flavin was formed on the enzyme, which was stable to reoxidation with oxygen. The illumination of oxidized lactate oxidase in the presence of pyruvate also leads to the formation of a stable adduct with an absorption maximum at 327 nm ($t_{1/2} \sim 2.2$ min under the same conditions as for the irradiation of the enzyme-oxalate complex). Its spectral and chemical properties, while protein-bound and after release from the protein, parallel the properties of the D-amino acid oxidase adducts (20), and therefore the adduct formed from the lactate oxidase-pyruvate complex most probably is an N(5)-acetyl derivative of reduced FMN. Model flavins have been shown to yield N(5)-acyl derivatives ($\lambda_{max} \sim 300$ nm) upon irradiation in the presence of α-keto acids in the free system (20, 21).

When the photoreaction of lactate oxidase with oxalate is carried out under anaerobic conditions, the intermediate spectra obtained are not clearly isosbestic as they are under aerobic conditions. The photoreaction is similarly fast ($t_{1/2} \sim 10$ s), but results in the production of approximately 15% (protein-bound) 1,5-dihydro-FMN and 85% adduct. This is readily determined from the very rapid reoxidation of the former with O_2; reformation of oxidized flavin from the adduct is very slow and, as detailed above, probably arises through hydrolysis to 1,5-dihydro-FMN ($t_{1/2} \sim$ 4 hours).

The very facile photoreaction between oxalate and lactate oxidase suggests that this unusually fast reaction is due to a preformed complex. For example the photoreduction of this enzyme with EDTA is very slow and leads to semiquinone production. By comparison, D-amino acid oxidase, for which no evidence for complex formation with oxalate could be found, reacts photochemically with oxalate at about 1/10 the rate with which it reacts with EDTA, in both cases leading to substantial semiquinone formation. That the photoreaction between lactate oxidase and oxalate arises largely, if not solely, from Complex II is indicated by the following experiments (Fig. 8). When enzyme was mixed with an equimolar concentration of oxalate and exposed to light without prior incubation, the rate of photoreduction was slow (Fig. 8, *top line*) and very similar to the rate of Complex II formation observed spectrophotometrically ($\Delta A_{504\ nm}$) under the same conditions. On the other hand, when a 30-min preincubation was carried out to assure that equilibrium was reached, the photoreaction was rapid (Fig. 8, *middle curve*) for almost 50% of the reaction, as would be expected if only the enzyme in the form of Complex II were photoreactive. Furthermore, the rate of the first fast phase of the photoreduction is closely similar to the rate observed when enzyme is preincubated with high concentrations of oxalate. In this case the photoreaction is fast for at least 95% of the total (Fig. 7, and Fig. 8, *lower curve*).

Reaction of Lactate Oxidase-Oxamate Complex with Light—When lactate oxidase (4×10^{-5} M in 0.01 M imidazole-HCl, pH 7.0) is illuminated in the presence of 0.1 M oxamate under the conditions described in Fig. 7, a very slow reaction occurs ($t_{1/2} \sim$ 4.5 hours) which leads to isosbestic formation of a product having an absorption spectrum very similar (λ_{max} = 327 nm) to the spectrum of the species obtained upon illumination of the lactate oxidase-oxalate complex (*cf.* Fig. 7). In contrast to the latter compound, which is practically nonfluorescent, the product obtained from lactate oxidase, oxamate, and light exhibits a blue fluorescence (emission $\lambda_{max} \sim$ 440 nm, excitation $\lambda_{max} \sim$ 325 nm), which is stable for several days while bound to the apoenzyme. Upon denaturation of the protein with 50% methanol at 60° for 2 min and centrifugation, the absorption spectrum of the supernatant indicated formation of only a small amount of FMN and the presence of another species absorbing at 300 to 320 nm. This latter compound slowly decays over a period of hours at 25° to yield FMN (total yield of FMN $\sim$ 95%).

Photoreaction of Free Flavin with Oxalate—The reaction of flavins with oxalate in the presence of light has been reported to produce CO_2 under aerobic conditions (11), and it is reasonable to assume that it proceeds via reduction of the flavin either in a direct step or through formation of covalent intermediates as reported for several other carboxylic acids (22). Under the conditions of Fig. 9 this photoreduction is isosbestic in the absence of oxygen and yields the anion of the reduced flavin. The formation of intermediate species or radicals was not observed, and oxidized flavin is immediately restored quantitatively upon admission of oxygen.

Incorporation of [^{14}C]Carbonate in Lactate Oxidase—The experiments described above suggest that the photoreaction of the lactate oxidase-oxalate complex results in the addition of oxalate or, more probably, of carbonate to the flavin to form a relatively

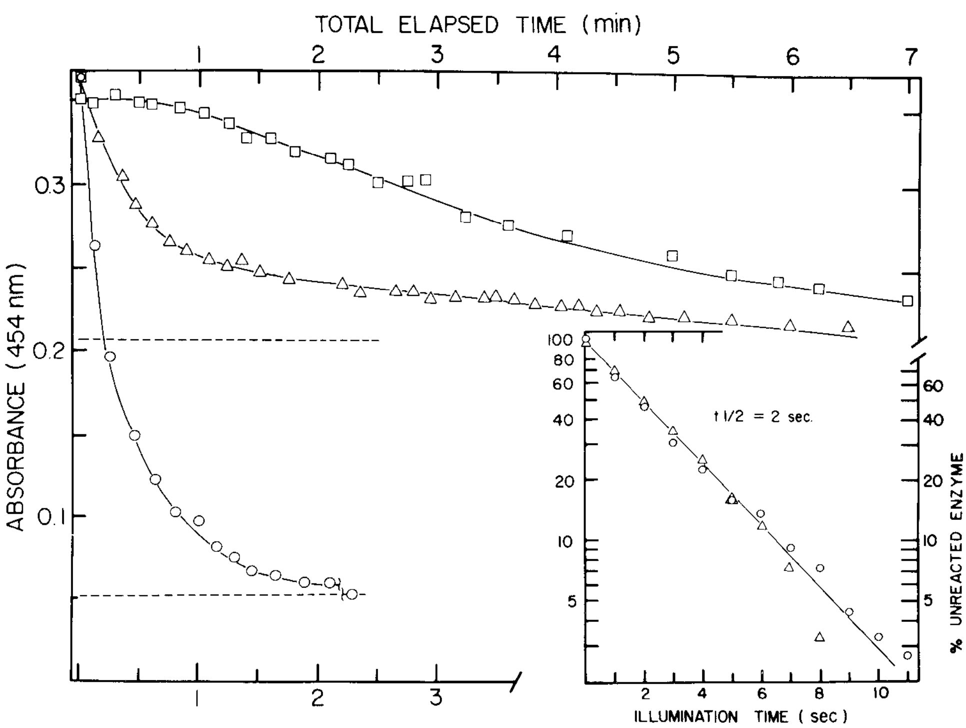


FIG. 8. Demonstration that Complex II of oxalate and lactate oxidase is photoactive. The enzyme, 3.1×10^{-5} M in 0.01 M imidazole-HCl buffer, pH 7.0, was irradiated as described under "Methods." Each point represents one second of illumination. ○—○, the enzyme was preincubated in the dark with 10^{-3} M oxalate for 20 min. The experimental end point of the photoreaction (*lower broken line*) was obtained by illumination for a total of 30 s. △—△, the enzyme was preincubated with an equimolar amount of oxalate in the dark for 30 min. The *upper broken line* represents the end point calculated for photoreaction with the amount of enzyme present in complexed form under the conditions given at the beginning of the irradiation and based on a K_d value of 1.6×10^{-5} M. □—□, irradiation of the sample was started immediately after mixing with 1 eq of oxalate. *Inset*, first order plot of the photoreduction of the preincubated samples calculated using the end point values shown by the *dotted lines*. The points of curve (△—△) are corrected for the second slow phase of photoreduction ($t_{1/2} \sim$ 17 s of illumination).

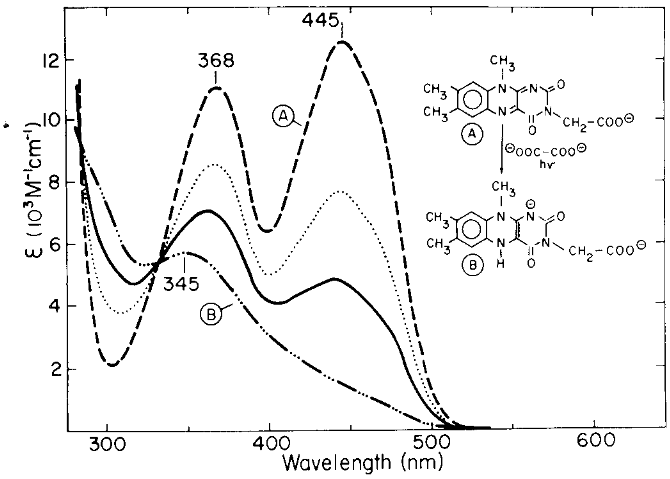


FIG. 9. Photoreaction of free flavin in the presence of oxalate. Lumiflavin-N(3)-acetate, 3.10^{-5} M in 0.001 M pyrophosphate buffer, pH 8.5, containing 0.01 M oxalate (*A*) was illuminated anaerobically for a total of 5 min at 25° to yield the anion of the reduced flavin (*B*). The two intermediate curves were obtained at 25 and 50 s, respectively. Upon admission of air the spectrum of the oxidized flavin (*A*) is restored immediately and quantitatively.

stable covalent adduct. In order to differentiate between these possibilities, the photoreaction was carried out with [^{14}C]oxalate, and the reaction mixture was separated by gel filtration.

The elution profile (Fig. 10) shows three distinct peaks of radioactivity, the first of which is clearly due to an enzyme-bound form. Separate experiments with [^{14}C]oxalate and [^{14}C]carbonate identify the two successive peaks as oxalate and carbonate, respectively. Integration of the radioactivity shows that approximately 90% of the total radioactivity initially used is accounted for, with approximately equal amounts in the enzyme and carbonate fractions (15.3 and 12%, respectively). This result indicates very strongly that the photoreaction results in the covalent addition of a carbonate moiety to the flavin of lactate oxidase, with the other half of the oxalate molecule eliminated as CO_2. Further substantiation of this reaction is obtained by the quantitative analysis of the incorporation of radioactivity in individual fractions of the protein peak, where radioactivity and protein concentration can be measured independently. Under the experimental conditions of Fig. 6, where a molar ratio of oxalate to enzyme-bound flavin of 3.1 was employed, 32.4% of the total counts originally present in the oxalate would be expected to be enzyme flavin bound, in the case of addition of intact oxalate, and half of this amount if carbonate is incorporated.

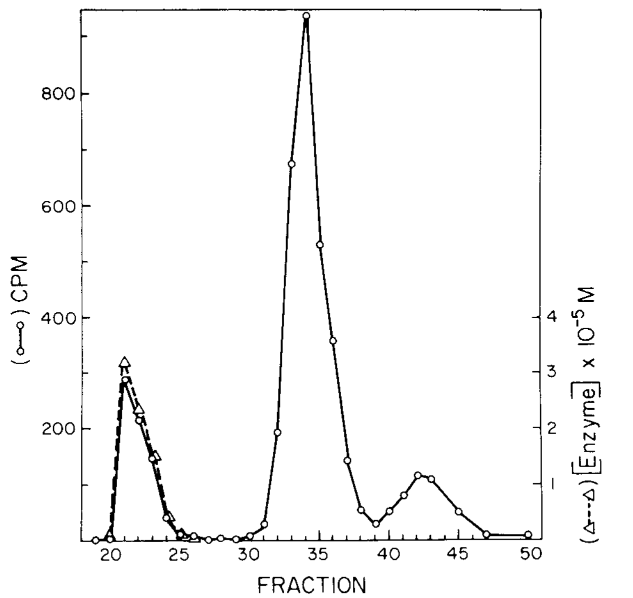
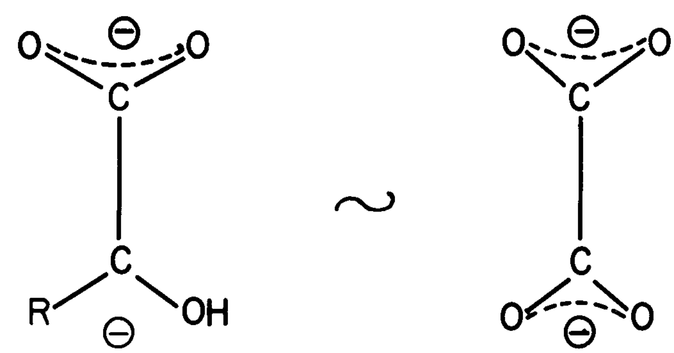


FIG. 10. Incorporation of [^{14}C]carbonate into lactate oxidase. In a typical experiment 1.5 ml (6.0×10^{-5} M) of lactate oxidase (9.0×10^{-8} mol) in 0.01 M imidazole-HCl buffer, pH 7.0, was incubated with 2.5×10^{-7} mol of [^{14}C]oxalate (26,925 cpm) at 0° in the dark until the absorption change at 504 nm was complete (approximately 15 min). The complex was then illuminated at 0° until 90.2% of the enzyme (8.1×10^{-8} mol) had reacted, as estimated by the absorption decrease of its oxidized form at 454 nm (see Fig. 7). The solution (1.4 ml) was then immediately passed through a Sephadex G-25 column at 0–4° which was equilibrated with the same buffer. Fractions (1.07-ml) were collected and 0.2-ml aliquots were counted. The enzyme concentration in the eluate was determined spectrophotometrically from the absorptions at 327 and 454 nm. The enzyme was eluted between Fractions 20 and 25, which contained 15.3% of the total radioactivity (3,840 cpm). Excess oxalate is eluted next, followed by carbonate (or CO_2) as was determined in separate experiments using [^{14}C]oxalate and [^{14}C]carbonate, the latter formed from oxalate by oxidation with $KMnO_4$.

DISCUSSION

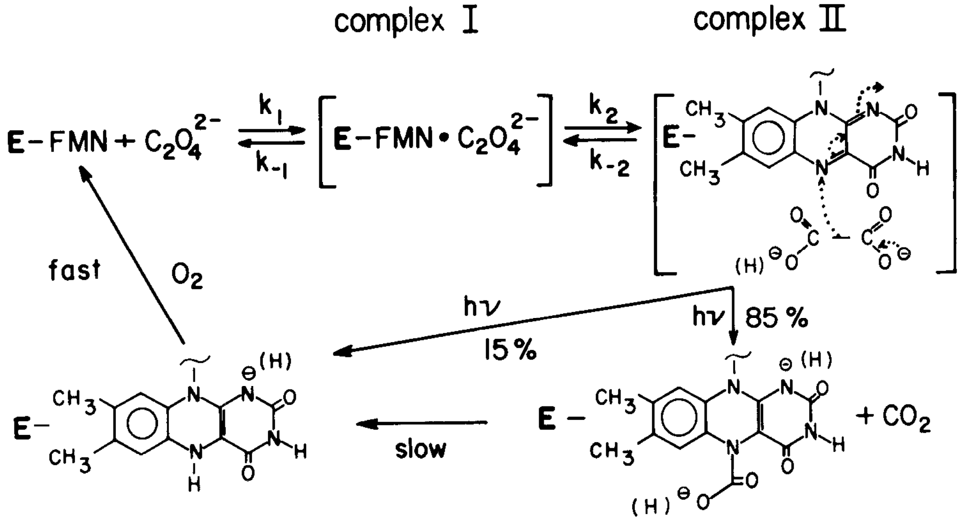
The results presented above for the reactions of oxalate with lactate oxidase can be summarized with the following scheme.[3]

The binding of oxalate to the enzyme is unusual in that it clearly involves two spectrally and kinetically different steps. The other anions investigated so far (1) and in particular, oxamate, the half-amide of oxalate, bind to the enzyme in a fast second order reaction, which is not followed by spectrally detectable slow processes. The different behavior of oxalate as compared to oxamate (and also to other anions) is likely to originate from its dianionic structure. Recently Walsh *et al.* proposed that the initial step of the oxidation of amino acids by D-amino acid oxidase (23) and of lactate by lactate oxidase (2) is the abstraction of the α-hydrogen of the substrate as a proton to form a (transient) carbanion. This reaction would be catalyzed by a group (base) on the protein which exchanges its proton(s) relatively slowly with the solvent (2). Comparison of the chemical structures of oxalate and of the α-carbanion of lactate indicate that the negative charge(s) is located in similar positions in both compounds. Oxalate, although not strictly isosteric, would therefore be similar (analog) to the proposed transient carbanion of lactate:



We consider it an attractive hypothesis that the slow step leading from Complex I to Complex II parallels the formation

[3] The *dotted arrows* are not meant to represent a specific mechanism; they rather indicate the formation of new bonds.



SCHEME 1

of the carbanion in the catalytic reaction, in that it would involve a slow solvent protonation of the same base which rapidly abstracts a proton from the enzyme-lactate complex.[4]

$$\underset{\text{(Complex I)}}{[E\text{-}B \cdot {}^{-}OOC\text{-}COO^{-}\ (H^{+})]} \;\underset{H^{+}}{\overset{H^{+}}{\rightleftharpoons}}\; \underset{\text{(Complex II)}}{[E\text{-}BH^{+} \cdot {}^{-}OOC\text{-}COO^{-}\ (H^{+})]}$$

Such a slow protonation is consistent with the observed stability of the flavin-carbonate adduct while bound to the protein, as compared with its instantaneous hydrolysis upon release. These results suggest that the accessibility of the solvent to the active center is restricted. Previous studies (2) have indeed indicated that the protonated active center base exchanges protons only slowly with the solvent.

The structure of the photoproduct resulting from the illumination of the lactate oxidase-oxalate complex (II) was ascertained to be the N(5)-carbamate of 1,5-dihydroflavin (*cf.* Scheme 1) from the following observations. (*a*) Its absorption spectrum closely resembles the spectrum of the sulfite N(5)-adduct to lactate oxidase (24), and the spectrum of the N(5)-adduct formed upon irradiation of the lactate oxidase-pyruvate complex. It is also similar to the spectra of N(5)-acyl-1,5-dihydroflavin adducts, which are formed on D-amino acid oxidase upon illumination in the presence of α-keto acids (20), and to the spectra of synthetic N(5)-acyl-dihydroflavins ($\lambda_{max} \sim 300$ nm) (21). (*b*) Its inertness toward oxygen on the protein strongly suggests a covalent substitution (19). (*c*) The spontaneous hydrolysis upon release from the protein to form oxidized FMN and the slow anaerobic decay of the enzyme-bound adduct to form reduced lactate oxidase indicates substitution at a nitrogen center of the isoalloxazine molecule with a function such as carbonate, which would be expected to hydrolyze easily. (*d*) The photoreaction and the subsequent slow decay of the adduct to yield reduced enzyme suggest an oxidation-reduction reaction involving an oxidation of oxalate. (*e*) Covalent addition of oxalate can be excluded in favor of carbonate from the radioactivity incorporation experiments (*cf.* Fig. 10).

Similar considerations indicate that the product obtained upon illumination of the lactate oxidase-oxamate complex is the N(5)-carbamoyl derivative ($H_2N-\overset{\overset{O}{\|}}{C}-$) of 1,5-dihydro-FMN. The spectra of the enzyme-bound adducts obtained on illumination with oxalate and oxamate are very similar, suggesting a related structure. The oxamate adduct exhibits a greatly enhanced stability towards hydrolysis as compared to the oxalate adduct. This is consistent with the greater stability toward hydrolysis of ureas ($R_2N-\overset{\overset{O}{\|}}{C}-NR_2$) as compared to carbamates ($R_2N-\overset{\overset{O}{\|}}{C}-OH$).

[4] Recent experiments to test this hypothesis indeed indicate that a proton is taken up from the solvent in the formation of Complex II. Thus, using phenol red as indicator in lightly buffered solutions, the uptake of approximately one proton per enzyme flavin has been detected on addition of oxalate; the rate of proton uptake observed being close to that found previously for the conversion of Complex I to Complex II.

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