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Studies on the Mechanism of Action of the Flavoenzyme Lactate Oxidase

PROTON UPTAKE AND RELEASE DURING THE BINDING OF TRANSITION STATE ANALOGS*

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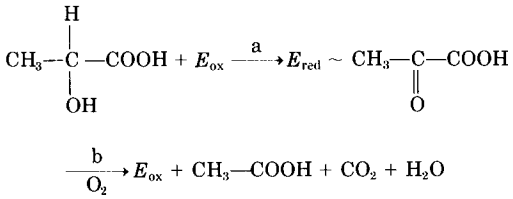
Oxalate and malonate have been found to bind to the flavoenzyme, lactate oxidase, in a two-step equilibrium process. The dissociation constant for the first step is of the order of 10^{-2} M, and is independent of pH. The second step equilibrium is on the other hand directly proportional to $[H^+]$ due to the second forward step, which is a second order reaction involving proton uptake. The reverse of this step is a first order reaction involving proton release, and consequently is independent of pH. Simultaneous measurement of proton uptake and formation of the enzyme·oxalate complex show that the rate of proton uptake is coincident with the large spectral changes occurring on binding, with the stoichiometry of one proton uptake per equivalent of enzyme-bound flavin. These results give strong support to the concept that oxalate and malonate are transition state analogs of a carbanion form of the substrate, formed in an early step in the enzyme reaction mechanism. The enzyme base responsible for abstraction of the proton from the α-carbon of the substrate is postulated to be the same one which is protonated (from the medium) in the second step of binding of oxalate and malonate, to form the transition state equivalent to that occurring with the substrate carbanion in catalysis. The pK of this group has been determined as 4.7, and is shifted to 8.2 and 9.8 on binding with malonate and oxalate, respectively. These pK shifts represent stabilizations of ~5 and ~7 kcal/mole of the enzyme·inhibitor complexes. These findings offer strong support for the intermediate involvement of a substrate carbanion in catalysis.

Flavin monooxygenases and oxidases catalyze generally the oxidation (dehydrogenation) of C-H functions (Step a) below. In the case of lactate monooxygenase (lactate oxidase) two separate steps occur during catalysis; the primary oxidation step (a) being followed by an oxidative decarboxylation process involving insertion of molecular oxygen into the product, acetate (1):



While the mechanism of Step b appears analogous to that of the oxidative decarboxylation of α-keto acids by peroxide (1), the elucidation of the substrate dehydrogenation Step a only recently has shown progress. Extensive reviews discuss this mechanism and seem to agree on the oxidation process being initiated by abstraction of substrate α-hydrogen as a proton (2–6). Such a carbanion mechanism had first been suggested by Cornforth (7) for the oxidation of fatty acid CoA derivatives by butyryl-CoA dehydrogenase. The concept was subsequently revived by the work by Walsh *et al.* (8, 9), who demonstrated that the enzymes D-amino acid oxidase and lactate oxidase catalyze the elimination of halide from β-halogeno substrate analogs. Lactate oxidase was subsequently shown to be irreversibly inactivated by the suicide substrate α-hydroxybutynoic acid (10–12). This inactivation reaction, leading to formation of a covalent flavocoenzyme adduct, is similarly thought to be initiated by proton abstraction and to proceed through intermediate formation of highly reactive allene carbanions (11).

In brief, the experimental evidence presently available points toward a carbanion mechanism, although the conclusiveness of the arguments supporting it has been questioned (4). The transition state theory (13–15) predicts that molecules which have structures analogous to that of the transition state, will have a higher affinity to the enzyme active center than substrate or substrate analogs. This concept has been

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verified within a series of hydrolytic enzymes (13), but has not yet, to our knowledge, been applied in the study of flavin-catalyzed oxidation-reduction reactions. It might be expected to help distinguish between mechanistic alternatives and to offer independent verification of the carbanion mechanism. It predicts that dianionic molecules being structurally similar to the postulated transient α-carbanion of lactate will bind very tightly to the enzyme lactate oxidase.

Recently oxalate was found to inhibit very strongly this enzyme, and to bind in a two-step process, the first step being a fast second order reaction leading to a primary complex, which then reversibly converted to a different complex in a slow first order reaction (16). The hypothesis was put forward (16) that oxalate would constitute a transition state analogue, and that the slow secondary process would involve a proton uptake/release from the protein (Scheme 1). The validity of the hypothesis can be verified experimentally by testing the

$$E + \text{oxalate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \sim \text{oxalate} \underset{H^+}{\overset{H^+}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}}} EH^+ \sim \text{oxalate}$$

Complex I Complex II

SCHEME 1

following requirements. (*a*) The proton concentration in the medium should decrease at the same rate as Complex I is converted to Complex II. (*b*) The stoichiometry should be the uptake of one proton for each molecule of Complex II formed. (*c*) The rate of conversion of Complex I to Complex II (k_2) should depend linearly on $[H^+]$. (*d*) The overall dissociation constant K_d, measured in static experiments, should depend linearly on $[H^+]$.

In the present work, we describe studies supporting this hypothesis and describing the detailed binding kinetics of the two transition state analogues, oxalate and malonate.

EXPERIMENTAL PROCEDURES

Materials—Lactate oxidase from *Mycobacterium smegmatis* was prepared as previously described (1). Malonic acid, succinic acid, tartronic acid, and imidazole were recrystallized before use. All other chemicals and reagents were the best commercially available grade. The universal buffer system employed consisted of 10^{-2} M piperazine, 10^{-2} M Tris, 10^{-2} M imidazole $4 \cdot 10^{-2}$ M Cl^-. The desired buffer pH values were obtained by titrating with HCl and adjusting the final chloride concentration to $4 \cdot 10^{-2}$ M with KCl. This system has a fairly linear buffering capacity in the region pH 5 to pH 10.

Methods—Spectroscopic determination of dissociation constants and absorption spectra were carried out with Cary 17 or 118 recording spectrophotometers at 25° in 1-cm path quartz cuvettes in the pH range 5 to 7 and in 10-cm path quartz cells at pH <5. The rapid kinetics experiments were carried out at 25° with the stopped flow apparatus of Gibson and Milnes (17) or with a newly designed stopped flow spectrophotometer (18).

For the proton uptake experiments, solutions of lactate oxidase $3.2 \cdot 10^{-5}$ M in 10^{-2} M NaCl containing 10^{-4} M imidazole·HCl, pH 7.0, and phenol red, $8 \cdot 10^{-6}$ M, and catalytic amounts of carbonic anhydrase were equilibrated with CO_2-free nitrogen until the pH of the mixture was constant (measured from the 560 nm absorbance of the indicator phenol red). The mixture was then mixed in the stopped flow apparatus with varying concentrations of sodium oxalate in 10^{-2} M NaCl, which had been freed of CO_2 by the same procedure. The pH of the solution after mixing was obtained from the absorbance of the indicator at 560 nm and was 7.60 ± 0.10 in the stopped-flow experiments. The total pH changes occurring during the reaction (in stopped flow and static experiments) were 0.16 ± 0.02 pH units. The stoichiometry of proton uptake was obtained by comparison of the absorbance changes at 560 nm with a standard curve. The latter was determined by titration of a CO_2-free sample of exactly the same composition as that used for stopped flow and static experiments in the range pH 6.8 to 8.2 with known amounts of HCl and NaOH in an apparatus similar to that described elsewhere for anaerobic titration experiments (19). Phenol red was shown not to interact with lactate oxidase in the pH range used; the spectrum of the enzyme is in fact not appreciably altered by additions of the indicator. Its pK determined spectrophotometrically with a $1.8 \cdot 10^{-5}$ M solution in 10^{-2} M NaCl in the presence of 3.3×10^{-5} M lactate oxidase was 7.84 and thus not significantly different from the pK value determined under the same conditions in absence of the enzyme (pK = 7.84, literature value (20), pK = 8.03). In contrast to this, addition of lactate oxidase to similar concentrations of bromthymol blue caused its pK to shift from 7.1 to 7.7 (literature pK = 7.3 (20)).

RESULTS

Spectral Effects Generated by Binding of Oxalate and Malonate—Very pronounced changes in the absorption spectrum of lactate oxidase result from the binding of oxalate and malonate. These changes are shown in Fig. 1, both as absolute and as difference spectra. The major difference with oxalate is at 504 nm, with an extinction change of -2100 M^{-1} cm^{-1}. Malonate produces even more marked changes; the most dramatic of these is an extinction decrease of 3500 M^{-1} cm^{-1} at 498 nm. Both compounds also result in extinction changes of the order of 1000 M^{-1} cm^{-1} in the region 350 to 370 nm. These characteristic changes were used subsequently to measure overall dissociation constants in static titration experiments, and to monitor the kinetics of binding in stopped flow experiments.

It should be noted that experiments involving both oxalate and malonate have to be performed in dim light, as both enzyme·inhibitor complexes are very sensitive to photoreactions, to yield covalent adducts with the enzyme-bound flavin. With oxalate the photoreaction product has been shown to be the *N*-5-carbonate adduct of reduced flavin (16); with malonate the product is the corresponding *N*-5-CH_2COOH adduct.[1]

Stoichiometry and Kinetics of Proton Uptake by Lactate Oxidase Occurring upon Binding of Oxalate—The binding of oxalate to the enzyme was investigated in the range pH 7.0 to 7.8 and in lightly buffered solutions in the presence of the pH indicator phenol red. This dye does not interfere measurably with the enzyme. In static experiments, in which the formation of Complex II from uncomplexed enzyme and oxalate is monitored (Scheme 1), addition of a 4-fold molar excess of unbuffered oxalate to the enzyme (4×10^{-5} M) caused a pH increase corresponding to uptake of 0.9 ± 0.1 protons per enzyme molecule (*cf.* "Experimental Procedures" for details).

That the rate of conversion of Complex I to Complex II is identical with the rate of decrease of $[H^+]$ concentration in the medium is demonstrated in experiments such as those of Fig. 2. The rate of proton uptake was measured at 560 nm, the λ_{max} of dissociated phenol red, and where the enzyme does not absorb (Fig. 2*A*). This rate is closely similar to the rate of conversion of Complex I to Complex II, which was measured by the decrease of absorbance at 365 nm (*cf.* Fig. 1), where the neutral and anionic forms of phenol red have an isosbestic point (Fig. 2*B*). The same correspondence of rates was found over the range of oxalate concentrations shown in Fig. 3. The estimated value of k_{-1}/k_1 obtained from the slope/intercept of Fig. 3 is 2×10^{-2} M (22). The conversion of Complex I to Complex II represents an approach to equilibrium; the k_{obs} value extrapolated at infinite oxalate concentrations (Fig. 3) should therefore correspond to $k_2 + k_{-2}$ (22). The value of k_{-2}, which had been determined earlier as 0.06 min^{-1} (16), under the present conditions will be $\ll k_2$ and should be independent

[1] S. Ghisla and V. Massey, unpublished observations.

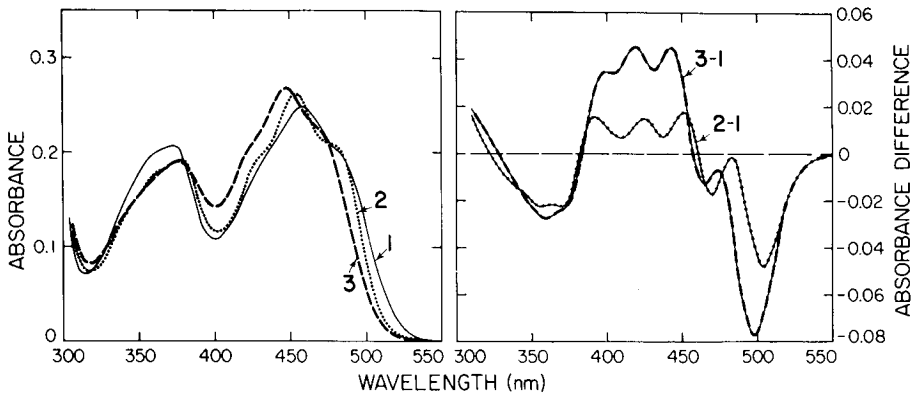


FIG. 1. Spectral perturbations induced by the binding of oxalate and malonate to lactate oxidase. *Curve 1*, enzyme, 2.22×10^{-5} M with respect to bound FMN in 2 mM imidazole·HCl, pH 7.0. *Curve 2*, plus excess solid sodium oxalate. *Curve 3*, plus excess solid sodium malonate. The corresponding difference spectra are shown on *right*. The line (— –) represents the base-line difference spectrum recorded before the addition of oxalate or malonate.

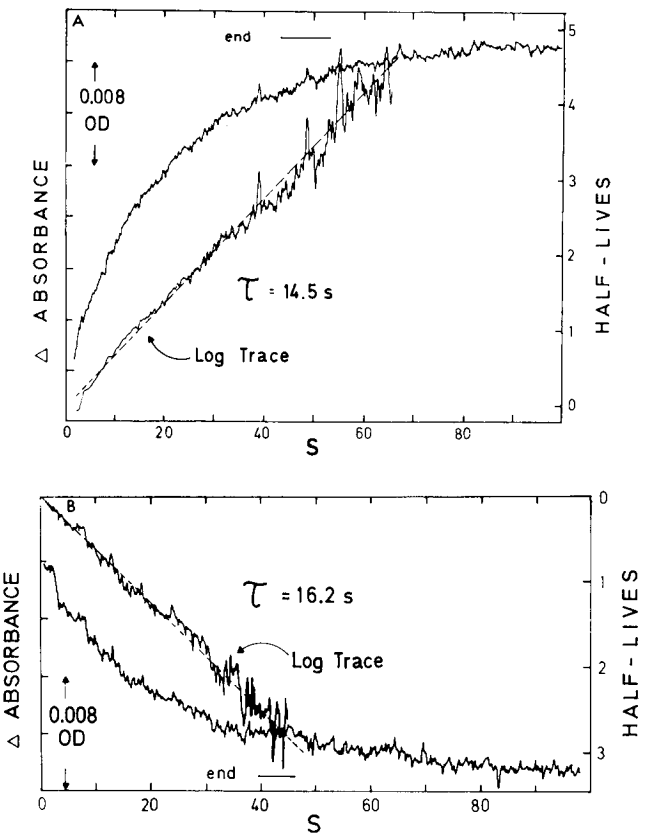


FIG. 2. Binding of oxalate to lactate oxidase. *A*, pH increase in the medium followed by the ionization of the indicator phenol red at 560 nm. *B*, course of the flavocoenzyme spectral changes induced upon binding of oxalate followed at 365 nm. This corresponds to conversion of Complex I to Complex II. Lactate oxidase, at a final concentration of $1.6 \cdot 10^{-5}$ M was reacted at 24.5° in a CO_2-free medium containing $5.0 \cdot 10^{-5}$ M imidazole·HCl buffer, pH 7, and 10^{-2} M Cl^- with a final concentration of $5.0 \cdot 10^{-3}$ M oxalate (*cf.* text for further details). The data shown are a readout from a transient recorder onto an X-Y recorder. For details about the generation of the log trace see Ref. 21. The half-time of the reaction is calculated from the slope of the log plot.

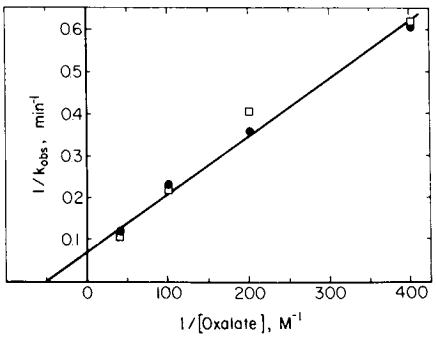


FIG. 3. Rates of formation of Complex II and of H^+ uptake measured as a function of oxalate concentration. Same conditions as in Fig. 2 (for further details see "Experimental Procedures"). Formation of Complex II from Complex I was measured by the absorbance decrease at 365 nm (●). The rate of H^+ uptake measured under identical conditions (□) was recorded at 560 nm. The points represent the average of at least two measurements.

of oxalate concentration and pH. It should be noted that the absorbance changes reflecting pH increase and conversion of Complex I to Complex II (Fig. 2) obeyed first order decay curves for up to three half-lives. The pH change occurring during conversion of Complex I to Complex II as estimated from the absorbance changes such as in Fig. 2*A* corresponds to uptake of 1.0 ± 0.15 protons per molecule of enzyme-bound flavin; no change of pH was observed upon formation of Complex I, which occurs in the deadtime of the stopped flow instrument (Fig. 2*A*).

The proton uptake postulate also requires that the conversion of Complex I to Complex II is first order in Complex I and proton concentration. This was found to be the case for the binding of oxalate in the range pH 5.2 to 7.6 (Fig. 4), when the rate of conversion of Complex I to Complex II was measured in experiments similar to those of Figs. 2*B* and 3. From the slope of the dependence of log k_{obs} on pH, k_2 is thus shown to be a second order rate constant with the value of $4 \cdot 10^8$ M^{-1} min^{-1}. This value of k_2 yields, together with the value of k_{-2} determined earlier (16), the pK of the lactate oxidase complex:

$$p\frac{k_{-2}}{k_2} \simeq p\frac{0.06}{4.10^8} \simeq 9.8$$

Tartronate (α-hydroxymalonic acid), which bears a structural resemblance both to the transition state (dianion) as well as to substrate (α-hydroxy function), in fact binds better than malonate to the enzyme ($K_d = 2.4 \cdot 10^{-5}$ M at pH 6.0, and $2.3 \cdot 10^{-4}$ M at pH 7.0 (1)). 6-Hydroxyhexylmalonic acid binds with K_d values similar to those of malonate (*e.g.* $6.3 \cdot 10^{-4}$ at pH 7.0, universal buffer) and shows a comparable pH dependence in the range pH 5.5 to 7.0 (not shown).

Kinetics of Binding of Malonate to Lactate Oxidase—As expected, the results of stopped flow studies showed that malonate binds to lactate oxidase in a similar manner as does oxalate. However, both k_2 and k_{-2} are considerably greater

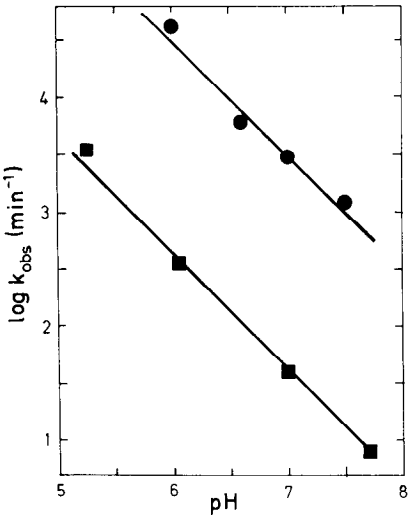


FIG. 4. Dependence of the rate of conversion of Complexes I to Complexes II (k_2) on H^+ concentration. Lactate oxidase, 2.10^{-5} M in the universal buffer described under "Experimental Procedures," was reacted in the stopped flow spectrophotometer with varying concentrations of oxalate (■) and malonate (●) in the same buffer at the pH values shown. The rate of formation of the enzyme oxalate Complex II was measured by the decrease of the 505 nm absorption, the formation of the enzyme malonate Complex II similarly by the decrease of the 500 nm absorption. The values of k_{obs} were obtained by secondary plots such as those of Fig. 3 and Fig. 5. The lines put through the experimental points have unit slopes.

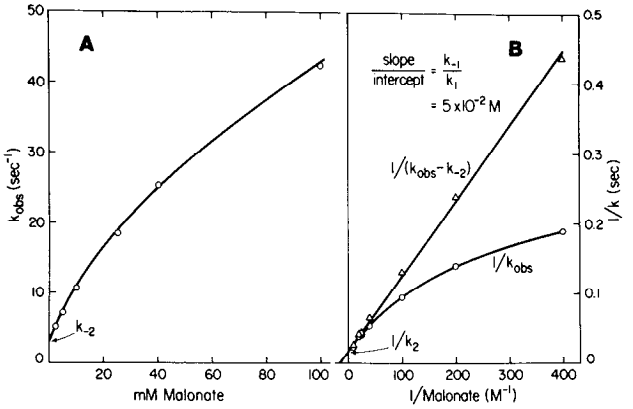


FIG. 5. Graphical determination of rate constants for the binding of malonate to lactate oxidase. Lactate oxidase, in the universal buffer system, pH 7.0, 25°, at a final concentration of 1.24×10^{-5} M, was reacted in the stopped flow apparatus with the concentrations of malonate shown. The absorbance decrease at 498 nm due to complex formation (Complex II) was monitored, and the pseudo-first order rate constant, k_{obs}, determined as shown in Fig. 2. Each point shown is the average of at least four such determinations. *A* shows the direct plot of the primary data yielding a value for k_{-2} of 3 s^{-1}. *B* shows reciprocal plots of the data; the linear plot is obtained from $1/(k_{obs} - k_{-2})$ *versus* 1/[malonate]. The intercept yields the value of $1/k_2$ (k_2 = 56 s^{-1}); the slope/intercept yields $k_{-1}/k_1 = 5 \times 10^{-2}$ M.

with malonate than they are with oxalate. Fig. 5 shows the results at pH 7.0, and their analysis by the method of Strickland *et al.* (22). The observed first order rate constant for malonate binding (monitored at 498 nm) varies with malonate concentration as shown in Fig. 5*A*. The fact that the plot is not linear demonstrates that a two-step equilibrium is involved (22). The extrapolated value of k_{obs} at zero malonate concentration is quite finite, 3 s^{-1}, and should equal k_{-2} (22). This value should be compared to the corresponding one determined for oxalate, 10^{-3} s^{-1} (16). As expected for two-step equilibria when k_{-2} is finite, the reciprocal plot of $1/k_{obs}$ *versus* 1/[malonate] is markedly curved (Fig. 5*B*). When $1/(k_{obs} - k_{-2})$ is plotted *versus* 1/[malonate], a linear plot is obtained. According to Strickland *et al.* (22) the intercept of this plot yields $1/k_2$ and the slope/intercept yields the value of k_{-1}/k_1. From Fig. 5 it can be seen that at pH 7.0 $k_{-1}/k_1 = 5 \times 10^{-2}$ M, k_2 = 56 s^{-1}, and k_{-2} = 3 s^{-1}.

Similar results to those of Fig. 5 were found at all pH values studied. Within experimental error a constant value of k_{-2} of 3 s^{-1} was obtained, and a constant value of k_{-1}/k_1 of 5×10^{-2} M. However, the value of k_2 varied markedly with pH as shown in Fig. 4. As in the case with oxalate, the value of k_2 for malonate is directly proportional to $[H^+]$, but is nearly two orders of magnitude greater. The average value of k_2 for malonate is 5×10^8 M^{-1} s^{-1}; that for oxalate is 6.7×10^6 M^{-1} s^{-1}.

The overall K_d for malonate binding, like that for oxalate, is thus pH-dependent, and made up of a pH-independent first step, and a pH-dependent protonation of the primary complex (*cf.* Scheme 1). Kinetically, the overall $K_d = k_{-1}/k_1 \cdot k_{-2}/k_2$. The pK value for the $E \cdot$malonate complex is given by the pH at which $k_{-2}/k_2 = 1$. Thus $3/[H^+] \times 5 \times 10^8 = 1$ at the pK, corresponding to $[H^+] = 6 \times 10^{-9}$ M or pK 8.2. This value agrees very well with that calculated by extrapolation of the statically determined K_d values to that of the pH-independent K_d shown in Fig. 6. Indeed it should be emphasized that there

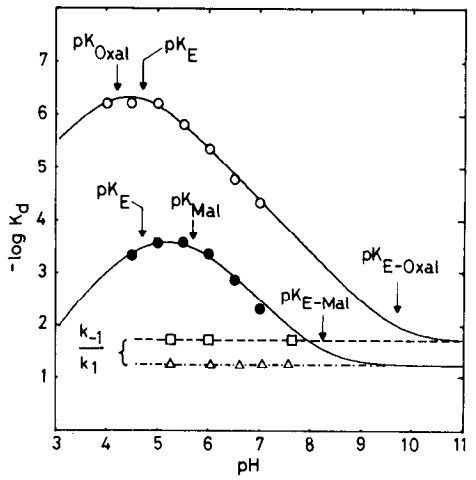


FIG. 6. pH dependence of the overall dissociation constant K_d for the binding of oxalate (○) and malonate (●) to lactate oxidase. The K_d values were determined spectrophotometrically as described previously (16) at constant Cl^- concentrations in the universal buffer system described under "Experimental Procedures" at the pH values indicated. The *lower horizontal lines* (□) and (△) represent the values of k_{-1}/k_1 for oxalate and malonate obtained from stopped flow experiments such as shown in Fig. 3. The *solid lines* represent the calculated curve obtained for best fit to the experimental points using literature values for the ionizations of oxalate (pK_{Oxal}) and malonate (pK_{Mal}), the values indicated by the *arrows* for the ionization of the enzyme (pK_E) and the enzyme·inhibitor complexes (pK_{E-Oxal}) and (pK_{E-Mal}), and the values of k_{-1}/k_1 extrapolated at pH $\gg pK_{E-Oxal}$ and pK_{E-Mal}. The theoretical lines were obtained with a Hewlett Packard 9820A calculator provided with a type 9862A plotter.

is excellent agreement throughout the entire pH range studied, with both oxalate and malonate, between K_d values determined by static titration experiments and ones determined kinetically.

pH Dependence of Oxalate and Malonate Binding to Lactate Oxidase—The overall dissociation constant K_d for the binding

of malonate and oxalate was determined spectrophotometrically (*cf.* Fig. 1) in the pH range 4 to 7 and plotted according to Dixon and Webb (23) (Fig. 6). At pH values >7, static experiments are not possible, as the uncomplexed enzyme undergoes slow and irreversible spectral changes. Similarly no reliable data could be obtained at pH values <4.5, with the exception of oxalate binding, as solutions of uncomplexed enzyme denature at these pH values. The binding of malonate shows monophasic kinetics (*cf.* Fig. 2) also in the region of pK_{a2} (5.69); the absorbance changes associated with the conversion of Complex I to Complex II corresponding in their magnitude to the spectral changes obtained in static experiments at the same inhibitor concentrations. Thus A^{2-} most probably will be the only species binding to the enzyme in the first reaction step. The system would be then described by the following equilibria:

$$\begin{array}{ccc} A^{2-} + E & \xleftrightarrow{K_1} & E - A^{2-} \\ K_E \;\Updownarrow\; H^+ & & H^+ \;\Updownarrow\; K_{EA} \\ A^{2-} + E - H^+ & \xleftrightarrow[K_2]{} & E - H^+ - A^{2-} \end{array} \qquad (1)$$

$$AH_2 \xleftrightarrow{K_{a1}} AH^- \xleftrightarrow{K_{a2}} A^{2-} \qquad (2)$$

Where AH_2, AH^-, and A^{2-} are the ionization forms of the dicarboxylic acid, K_{a1} and K_{a2} the corresponding ionization constants; K_E and K_{EA} represent the ionization constants of uncomplexed and complexed enzyme and K_1, K_2 the dissociation constants for binding of A^{2-} to unprotonated and protonated enzyme. Thus the pH dependence of K_d according to Dixon and Webb (23) will be:

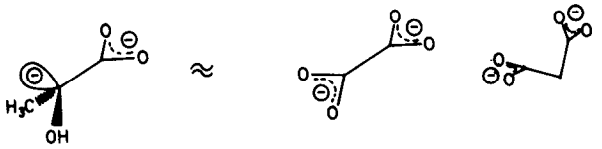
$$pK_d = pK_1 + pK_{EA} - pK_E + \log\left(1 + \frac{K_{EA}}{[H^+]}\right) - \log\left(1 + \frac{K_E}{[H^+]}\right) - \log\left[1 + \frac{[H^+]}{K_{a2}}\left(1 + \frac{[H^+]}{K_{a2}}\right)\right] \qquad (3)$$

In Fig. 6 the theoretical curves fitting the experimental points are shown. They were obtained by using the following values: for the binding of oxalate: $pK_{a1} = 1.23$ (20), $pK_{a2} = 4.19$ (20), $pK_E = 4.7$, $pK_{EA} = 9.7$ and $pK_1 = 1.7$. For the binding of malonate: pK_{a1} 2.83 (20), $pK_{a2} = 5.69$ (20), $pK_E = 4.7$, $pK_{EA} = 8.25$ and $pK_1 = 1.25$. ($K_1 = 5.5 \times 10^{-2}$ M.) The values of pK_1 ($K_1 = k_{-1}/k_1$) were obtained from stopped flow experiments such as those of Fig. 2 and are independent of pH; they would correspond to the extrapolated value of K_d at $pH \gg pK_{EA}$. The values of pK_{EA} estimated from the Dixon plot (Fig. 6) are in very good agreement with those determined kinetically.

The next homologue of malonate, succinate, also binds to lactate oxidase, but much more weakly. The induced spectral changes are qualitatively similar to those obtained with malonate and oxalate (Fig. 1), but of much smaller magnitude. An approximate K_d of 4×10^{-2} M which is not dependent on pH can be estimated in the pH range 6 to 7.

DISCUSSION

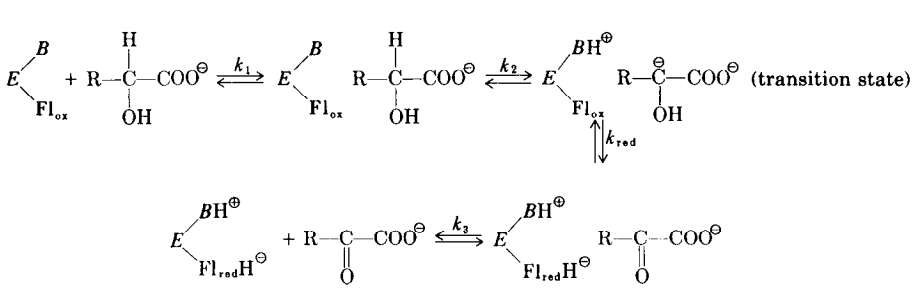
Small anionic molecules are competitive inhibitors of lactate oxidase and can be subdivided in two classes. Monoanionic acids and dianionic acids in which the negative charges are located at one site (phosphate, sulfate, carbonate) bind with K_d values typically around 10^{-2} M (1, 16). Dianionic acids such as oxalate, malonate, and its derivatives, in which the negative charges are separated, show a much tighter binding, the K_d values being minimal with oxalate and increasing again with increasing separation of the negative charges. With the flavoenzyme glycollate oxidase the dicarboxylic acids oxalate, malonate, and succinate were found to be competitive inhibitors, the K_i values increasing in that order (24). The presence of two positively charged groups at the active center was proposed, but no studies as a function of pH were carried out (24). The specific dependence of K_d on the distance separating the negative charges of the lactate oxidase inhibitors suggest that they are transition state analogs. A comparison of their structures with that of the postulated transient substrate α-carbanion reveals in fact a similar location of the charges:



Clearly upon binding to the protein the negative charge of the carboxylate function of the substrate will be neutralized by a protein counterion, this effect possibly going as far as to result in a formal protonation of the group. ($-COO^{\ominus}\cdots H\overset{\oplus}{B} \rightleftarrows -COOH\cdots B-$). Such an effect would contribute to the lowering of the energy (pK) of the transient α-carbanion. A similar effect could be expected on binding of carboxylate inhibitors.

A primary binding step with a K_d of the order of 10^{-2} M appears to be common for the binding of simple competitive inhibitors, for the formation of the Michaelis complex of substrate (1), and for the formation of Complex I with transition state analogues (Scheme 1). In the case of substrate, the subsequent step would be the abstraction of the α-proton to form a formal carbanion (8, 9) (see Scheme 2).

It should be noted that in the electron transfer step the negative charge would be transferred from the substrate carbanion to the reduced flavocoenzyme. In the reduced enzyme·product complex, which is known to dissociate very slowly (k_3, Scheme 2) (1), again two negative charges would be present at the active center. Thus the reduced flavin is proposed to function as a "charge sink;" its pK, which is ~6.7 in the free system (5), being lowered to <5 in the enzyme lactate oxidase.[1] The step k_2 of α-proton removal clearly will require the largest activation energy ΔG (Scheme 2). Williams and Bruice (25) report a value of $\Delta G_{expt} = 23$ to 26 kcal/mole



SCHEME 2

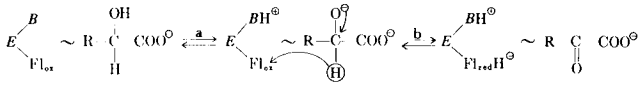
for the oxidation of lactic acid by oxidized flavin in the free system and state that the process is compatible with transient formation of an α-carbanion. The Arrhenius activation energy for the oxidation of L-lactate by lactate oxidase is 10 to 12 kcal/mole (26).[1] The difference in activation energies of ~12 kcal/mole might be attributed to the lowering of activation energy brought about by the enzyme, *i.e.* to the amount of stabilization of the enzymatic transition state as compared to that in the free system. As pointed out by Jencks (13), a transition state analogue will, at the best, only approach the structure of the transition state. Hence, it appears reasonable that the enzyme will bring about a stabilization of an α-carbanionic transition state of the order of 10 to 12 kcal/mole, in view of the magnitude of the effects induced by oxalate and malonate corresponding to stabilizations of ΔG ~7 kcal/mole and ~5 kcal/mole, respectively (Fig. 6). Such a stabilization could be brought about by the presence of appropriately distributed counterions at the enzyme active site.

The proton uptake step (k_2, Scheme 1), occurring after binding of the transition state analogs, is thus proposed to parallel the substrate proton abstraction Step k_2, Scheme 2; the same enzyme base being involved in both processes. In the uncomplexed enzyme, this base will be unprotonated at pH > pK_E = 4.7; its pK is shifted to 8.2 and 9.7 upon binding of malonate and oxalate, respectively (Fig. 5). This predicts that substrate turnover at pH < 4.7 would require proton release to the medium concomitant with the binding Step k_1 (Scheme 2); *i.e.* prior to abstraction of the α-proton from the substrate, deprotonation of the protonated enzyme base would have to occur. In fact, with the enzyme D-amino acid oxidase, which catalyzes a similar set of reactions as lactate oxidase, proton release is observed upon binding of the substrate D-valine, and prior to reduction of the enzyme flavin (27). This has been interpreted by Porter and Bright (27) as deprotonation of the amino acid α-nitrogen, but a more attractive interpretation would represent deprotonation of a base functioning in abstraction of the α-proton of a neutral amino acid ($R\text{-}CH(NH_3^+)\text{-}COO^-$). Clearly such a distinction might be merely a formal one in a closed system, where H^+ interchange will be fast.

The extreme slowness of the proton uptake and release rates (Steps k_2 and k_{-2}, Scheme 1) is to our knowledge unprecedented for enzyme functional groups. The fact that both these rates decrease by a factor 10^2 to 10^3 on going from malonate to oxalate, suggest that in the case of normal substrate carbanions, still slower proton exchange processes with the solvent might occur. This set of data requires that the active site must be effectively shielded from the environment. Such a shielding has been invoked in order to explain the lack of proton exchange during the β-elimination reactions catalyzed by lactate oxidase and D-amino acid oxidase (9, 28). In these cases, substrate α-hydrogen is transferred to position β of the product in the course of the reaction. A strong shielding would also help explain the seemingly paradoxical fact, that nonexchangeable substrate hydrogens which are involved in oxidation-reduction reactions of flavoproteins, are quantitatively transferred to position C-5 of enzyme-bound 5-deazaflavocoenzyme; the "carbanion mechanism" would predict instead incorporation of solvent or of exchangeable protons (29).

Even if the observed pK shifts of 5 and 3.5 units are taken as reflecting the pK values of the active center system as a whole, their magnitude appears rather unusual. A pK shift of 4 units has been reported for an active center lysine of acetoacetate decarboxylase (30). In a recent study, Lederer and Mulet (31) succeeded in the alkylation of an active center base of the flavoenzyme yeast L-lactate dehydrogenase with bromopyruvate, a product analog. Interestingly the enzyme is alkylated in the oxidized state, but is protected against electrophilic attack in the reduced state, where most probably the active center base is protonated forming an ionic pair with the reduced flavocoenzyme anion (*cf.* Scheme 2). The base in question is most probably not the oxidized flavocoenzyme position N-5 itself, as its basicity should be much too low (5). From the stoichiometry of ^3H incorporation in position β of eliminating substrates, Walsh *et al.* suggested that the base is monofunctional (9), while Lederer and Mulet (31) based on their alkylation experiments propose a cysteine sulfhydryl group as the active center base. Our results, although compatible with the above proposals, do not allow predictions about the nature of the base, although histidine and cysteine might be reasonable candidates.

Finally it should be stated that the above results are also compatible with the formation of a transient dianion in which the second negative charge is located at the oxygen function in position α of the substrate (Step *a*):



This species would constitute an intermediate preceding hydride abstraction from the α position and its transfer to the oxidized flavocoenzyme (Step *b*). In disfavor of the hydride mechanism is however the fact, that thiolactic acid, α-methyllactic acid, and oxamic acid, while they might be expected to be reasonable transition state analogs for a hydride transfer mechanism, behave as simple competitive inhibitors of the enzyme. The unlikeliness of a hydride mechanism in flavoenzyme-catalyzed dehydrogenation reactions has been discussed in detail elsewhere (6, 8–12).

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REFERENCES

1. Lockridge, O., Massey, V., and Sullivan, P. A. (1972) *J. Biol. Chem.* **247**, 8097–8106
2. Massey, V., and Flashner, M. S. (1974) in *Molecular Mechanism of Oxygen Activation* (Hayaishi, O., ed) pp. 245–283, Academic Press, New York
3. Massey, V., and Hemmerich, P. (1975) in *The Enzymes* (Boyer, P. D., ed) pp. 191–252, Academic Press, New York
4. Bright, H. J., and Porter, D. J. T. (1975) in *The Enzymes* (Boyer, P. D., ed) pp. 421–505, Academic Press, New York
5. Bruice, T. C. (1976) in *Progress in Bioorganic Chemistry* (Kaiser, E. T., and Kezdy, E. J., eds) Vol. 4, pp. 1–87, J. Wiley & Sons, New York
6. Hemmerich, P. (1976) in *Progress in the Chemistry of Organic and Natural Products* (Grisebach, H., ed) Vol. 33, pp. 451–526, Springer Verlag, Berlin
7. Cornforth, J. W. (1959) *J. Lipid Res.* **1**, 3–28
8. Walsh, C. T., Schonbrunn, A., and Abeles, R. H. (1971) *J. Biol. Chem.* **246**, 6855–6866
9. Walsh, C., Lockridge, O., Massey, V., and Abeles, R. (1973) *J. Biol. Chem.* **248**, 7049–7054
10. Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H., and Walsh, C. T. (1976) *Biochemistry* **15**, 1791–1797
11. Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H., and Massey, V. (1976) *Biochemistry* **15**, 1798–1807
12. Massey, V., Ghisla, S., Ballou, D. P., Walsh, C. T., Cheung, Y. T., and Abeles, R. H. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed) pp. 199–212, Elsevier, Amsterdam
13. Jencks, W. P. (1975) *Adv. Enzymol.* **43**, 219–410
14. Lienhard, G. E. (1973) *Science* **180**, 149–154

15. Wolfenden, R. (1972) *Accts. Chem. Res.* **5**, 10–18
16. Ghisla, S., and Massey, V. (1975) *J. Biol. Chem.* **250**, 577–584
17. Gibson, Q. H., and Milnes, L. (1964) *Biochem. J.* **91**, 161–171
18. Entsch, B., Ballou, D. P., and Massey, V. (1976) *J. Biol. Chem.* **251**, 2550–2563
19. Foust, G. P., Burleigh, B. D., Mayhew, S. G., Williams, C. H., and Massey, V. (1969) *Anal. Biochem.* **27**, 530–535
20. Sober, H., ed (1970) *Handbook of Biochemistry*, 2nd Ed, Section J, CRC Press, Cleveland, Ohio
21. Lambeth, D. O., and Palmer, G. (1973) *J. Biol. Chem.* **248**, 6095–6103
22. Strickland, S., Palmer, G., and Massey, V. (1975) *J. Biol. Chem.* **250**, 4048–4052
23. Dixon, M., and Webb, E. C. (1964) *Enzymes*, 2nd Ed, pp. 116–135, Academic Press, New York
24. Schuman, M., and Massey, V. (1971) *Biochim. Biophys. Acta* **227**, 521–537
25. Williams, R. F., and Bruice, T. C. (1976) *J. Am. Chem. Soc.* **98**, 7752–7768
26. Sullivan, P. A. (1968) *Biochem. J.* **110**, 363–371
27. Porter, D. J. T., and Bright, H. J. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed) pp. 225–237, Elsevier, Amsterdam
28. Walsh, C. T., Krodel, E., Massey, V., and Abeles, R. H. (1973) *J. Biol. Chem.* **248**, 1946–1955
29. Spencer, R., Fisher, J., Laura, R., and Walsh, C. T. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed) pp. 349–361, Elsevier, Amsterdam
30. Schmidt, D. E., and Westheimer, F. H. (1971) *Biochemistry* **10**, 1249–1253
31. Lederer, F., and Mulet, C. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed) pp. 238–243, Elsevier, Amsterdam