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Stopped-Flow Kinetic Studies on the D-Lactate Dehydrogenase from *Megasphera elsdenii*[†]

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ABSTRACT: The reaction of oxidized D-lactate dehydrogenase with D-lactate and reduced D-lactate dehydrogenase with pyruvate and oxygen was studied in a stopped-flow spectrophotometer. Oxidized enzyme was reduced in a triphasic manner by D-lactate at a variety of pH values. The fastest phase is thought to represent charge-transfer complex formation and is equal to an increase in long wavelength (510–700 nm) absorbance. The two slower phases seem to be due to flavin reduction. At pH 6 the proportion of the flavin reduction which proceeds by either phase is dependent upon the concentration of D-lactate. The reaction between reduced enzyme and oxygen is a simple second-order reaction. However, the oxidation of reduced D-lactate dehydrogenase by pyruvate is biphasic at pH 6 and monophasic at pH 8. At pH 8 there is a rapid increase in absorbance above 510 nm which is again ascribed to formation of a charge-transfer complex. These results are taken to indicate that D-lactate dehydrogenase may exist in two active conformations. At high pH the enzyme operates mainly through only one form while at low pH both forms participate in catalysis.

The flavoenzyme D-lactate dehydrogenase from *Megasphera elsdenii* has recently been purified to a homogeneous state (Olson & Massey, 1979). It is a dimer consisting of two apparently identical subunits. Each subunit contains one tightly bound FAD and zinc.

In a previous paper (Morpeth & Massey, 1982), we have described the unusual steady-state kinetics of D-lactate dehydrogenase. We found that at pH 8 the enzyme displays Michaelis–Menten behavior but that at pH 6 the linear double-reciprocal plots show downward curvature. This behavior we have suggested is due to negative (anticooperative) interactions rather than to kinetic complexities in the reaction mechanism.

The purpose of the present paper is to investigate in detail the individual half-reactions of D-lactate dehydrogenase under various conditions in an attempt to understand more fully the basis of the nonlinear steady-state behavior.

Many simple flavoenzymes have been studied with respect to their individual half-reactions by using the same stopped-flow techniques employed in this study (e.g., Gibson et al., 1964; Lockridge et al., 1972; Matthews et al., 1977). Thus, the experimental rationale for simple systems has been well developed, and so it should be possible to apply the standard approaches [see Bright & Porter (1976) for a review] to a more complex system such as D-lactate dehydrogenase.

Experimental Procedures

Materials and Methods. D-Lactate dehydrogenase from *Megasphera elsdenii* was purified and assayed as previously described (Olson & Massey, 1979). D-Lactate was purchased from Sigma. Sodium dithionite was from MCB.

Spectrophotometric measurements were performed with Cary 17, 118, or 219 double-beam spectrophotometers, and rapid reaction studies were carried out in a stopped-flow apparatus designed for anaerobic work which was coupled to a Nova minicomputer system (Beaty & Ballou, 1981).

All rapid reaction studies were done in potassium phosphate buffer, ionic strength = 0.23, at the stated pH and temperature. In reductive half-reactions at high concentrations of substrate, the ionic strength of the solution was held constant by varying the concentration of phosphate buffer. The pH of all solutions of D-lactate was checked and corrected to the desired value before use.

Protein samples were made anaerobic by careful evacuation and equilibration with oxygen-free nitrogen. Residual oxygen was removed from the prepurified nitrogen (Matheson Co., Inc.) by passage over activated Ridox (Fischer).

All reaction traces which were not first order were analyzed as a sum of exponential phases as described by Mastrigt (1977).

Results

Studies at pH 8. When oxidized enzyme is rapidly mixed with varying concentrations of D-lactate at pH 8 in phosphate buffer, ionic strength = 0.23, at 4 °C, the visible absorbance of the enzyme flavin is bleached in a triphasic fashion (Figure 1). The slowest phase is independent of the substrate concentration between 5 and 60 mM D-lactate with a rate constant of 0.07 ± 0.015 s^{-1}. This phase accounts for no more than 10% of the total absorbance change at any wavelength.

The rate of the fastest phase of flavin reduction is also independent of D-lactate concentration with a rate constant of 11 ± 3 s^{-1}. This phase accounts for about 15% of the total absorption change in the near-UV absorbance maximum, 10% in the main visible peak. Because of the errors involved in the logarithmic stripping technique used for estimation of k_{fast} and the amplitude of this phase, it is not possible to determine with any accuracy whether there is a concentration dependence of the amplitude of the fast phase at 450 nm. Most of the decrease in flavin absorption (70–80%) takes place in the intermediate phase which is substrate concentration dependent.

On reaction with D-lactate there is also a transient increase in long wavelength absorbance (510–700 nm) similar to that seen with many other flavoproteins and generally ascribed to charge-transfer complex formation (Massey & Ghisla, 1974). The transient long wavelength absorbance is formed with a substrate-independent rate constant of 11 s^{-1}, which is the same as that for the fast decrease of the flavin peak. Though the rate of charge-transfer formation is independent of the substrate concentration, the amount formed is not. A similar situation was seen by Schopfer & Massey (1979) in the charge-transfer complex formation on mixing the complex of reduced melilotate hydroxylase and 2-OH phenyl propionate

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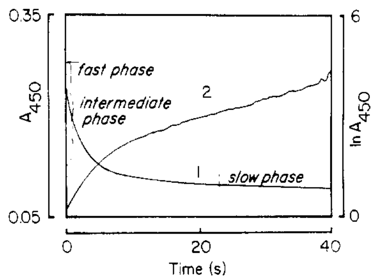


FIGURE 1: Change in absorbance at 450 nm with time when oxidized D-lactate dehydrogenase (22 μM) is mixed anaerobically with an equal volume of D-lactate (80 mM) in potassium phosphate buffer, pH 8, ionic strength = 0.23, at 4 °C. The reaction was followed in a stopped-flow spectrophotometer equipped with a 2-cm path-length cell. Curve 1 shows the bleaching at 450 nm, and curve 2 is the natural logarithm of curve 1. The arrow indicates the absorbance of oxidized enzyme, and the various phases in the reductive reaction are clearly marked.

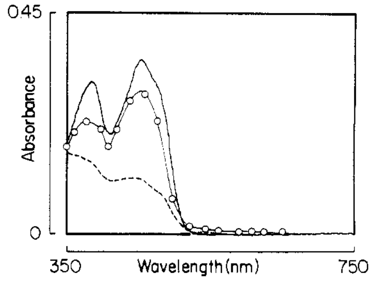
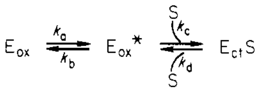


FIGURE 2: Transient species seen during the reduction of D-lactate dehydrogenase by 20 mM D-lactate at 4 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23. (—) Initial oxidized enzyme mixed with an equal volume of buffer; (○–○) spectrum 0.5 s after mixing with D-lactate; (---) final reduced spectrum 5 min after mixing.

Scheme I



with NAD$^+$. By analogy with their analysis, it can be concluded that the phenomenon requires an enzyme isomerization step such as shown in Scheme I, with k_a being equal to k_d, thus making $k_{obsd} = k_a$.

A spectrum of the intermediate charge-transfer complex seen on reaction with D-lactate after 0.5 s is shown in Figure 2. No definite long wavelength peak is seen. A double-reciprocal plot of the maximum amount of transient absorbance at 540 nm at varying D-lactate concentrations is linear and yields a molar extinction coefficient of 900 M^{-1} cm^{-1} and an apparent K_d of 20 mM. The disappearance of the long wavelength species is substrate concentration dependent and within an acceptable error (25%) is equal to the intermediate rate of flavin bleaching (k_{int}).[1]

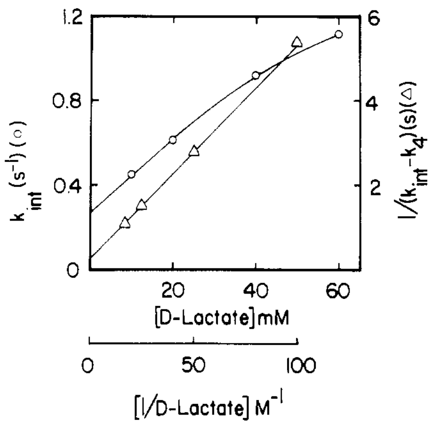
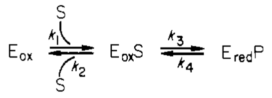


FIGURE 3: Variation of the rate constant for the intermediate phase of flavin reduction (k_{int}) (○) with D-lactate concentration. Each point is the average of at least eight determinations carried out at various wavelengths from 350 to 500 nm. The rate constants in Scheme II were estimated from a double-reciprocal plot (△) of the same data corrected for a significant k_4 [analysis by the method of Strickland et al. (1975)].

Scheme II



The plot of k_{int} vs. D-lactate concentration (Figure 3) and the corresponding double-reciprocal plot (not shown) are curved. This is typical of a two-step equilibrium mechanism of the type shown in Scheme II where k_4 is significant (Strickland et al., 1975).

An estimate of 0.25 s^{-1} for k_4 can be made from the intercept of the direct plot of k_{int} vs. D-lactate. When $1/(k_{int} - k_4)$ vs. 1/[D-lactate] is plotted, a straight line is seen (Figure 3). From this intercept we can estimate k_3 = 3.3 s^{-1} and from slope/intercept K_d^{app} = 150 mM.

Reoxidation of Reduced Enzyme with Pyruvate. Olson & Massey (1979) showed that the reductive half-reaction with D-lactate dehydrogenase is freely reversible, and they used this to determine the oxidation–reduction potential of the enzyme-bound FAD.

When reduced enzyme is mixed anaerobically at 4 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23, with varying concentrations of pyruvate from 1 to 200 mM, a monophasic rise in flavin absorbance at 450 nm is seen, due to formation of oxidized enzyme. When the variation of the apparent rate constant with pyruvate was analyzed according to Strickland et al. (1975), the data were found to fit a two-step scheme similar to Scheme II, in which k_4 is significant. From the direct plot and a corrected double-reciprocal plot, values for the kinetic constants were determined: k_3' = 2.5 s^{-1}, k_4' = 0.16 s^{-1}, and K_d^{app} = 45 mM (the primes on k_3 and k_4 are to distinguish them from rate constants involved in lactate oxidation).

Transient long wavelength absorbance (510–700 nm) is also seen during reaction of the reduced enzyme with pyruvate. Its formation is fully saturated at 1 mM pyruvate and occurs with a first-order rate of 15 s^{-1}. A molar absorption coefficient at 540 nm of 400 M^{-1} cm^{-1} for the transient long wavelength absorption at saturation can be calculated. The rate of disappearance of the long wavelength band is roughly the same as the rate of appearance of oxidized flavin at any given pyruvate concentration, i.e., ~2.5 s^{-1}.

Studies at pH 6: Reductive Half-Reaction. On mixing varying concentrations of D-lactate with anaerobic oxidized

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[1] It was suggested in editorial review that the substrate concentration independent formation of the long wavelength species and the substrate concentration dependent decay could be explained by an $A + B \rightleftharpoons C \rightleftharpoons D$ type of equilibrium in which the fast phase was due to the conversion of C to D and the slow phase the formation of C, which would of course be dependent on the concentration of B (substrate). We consider this possibility to be unlikely, since with k_{fast} of 11 s^{-1} and k_{int} of 3.3 s^{-1}, the maximum formation of the intermediate would be less than 20% of the total enzyme species. With a measured ϵ_{540} of 900 M^{-1} cm^{-1}, this would require that the true ϵ_{540} value of the intermediate be ≥4500 M^{-1} cm^{-1}. This seems unreasonably large; the corresponding value found with L-lactate oxidase, where quantitative formation of a reduced enzyme–pyruvate charge-transfer complex is observed, is 840 M^{-1} cm^{-1} (Lockridge et al., 1972). In addition this formulation is inconsistent with the results obtained on reoxidation of the reduced enzyme by pyruvate where the principle of microscopic reversibility for such a scheme is not obeyed (cf. Discussion).

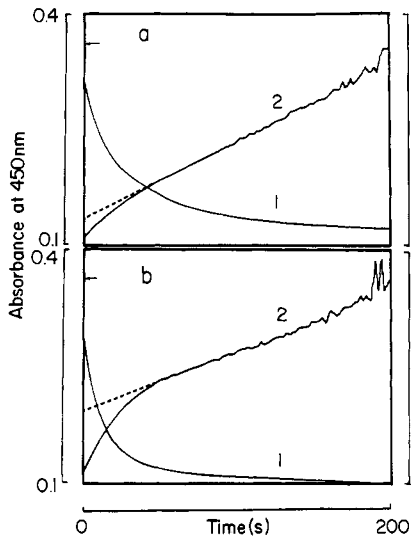


FIGURE 4: Change in absorbance at 450 nm with time when anaerobic oxidized D-lactate dehydrogenase (28 μM) is mixed with an equal volume of D-lactate in potassium phosphate buffer, pH 6, ionic strength = 0.23, at 4 °C. The reaction was followed in a stopped-flow spectrophotometer equipped with a 2-cm path-length cell. (Panel a) The reaction on mixing with 20 mM D-lactate and (b) 100 mM D-lactate. In all cases curve 1 shows a trace of the bleaching at 450 nm, and curve 2 is the natural logarithm of curve 1.

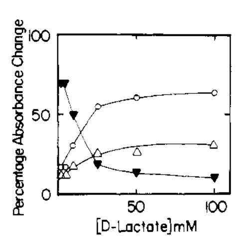


FIGURE 5: Proportion of total flavin bleaching which goes by the fast (△), intermediate (○), and slow (▼) phases on mixing anaerobic enzyme (28 μM) with various concentrations of D-lactate in potassium phosphate buffer, pH 6, ionic strength = 0.23, at 4 °C.

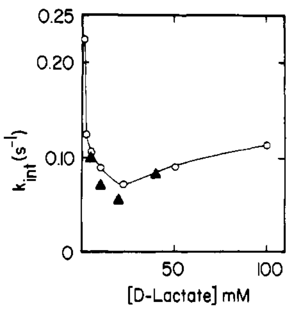


FIGURE 6: Variation of the rate constant of the intermediate phase (k_{int}) with D-lactate at 4 °C in potassium phosphate buffer, pH 6, ionic strength = 0.23. The symbols ○ and ▲ represent two different experiments performed on two different preparations of enzyme of varying activity to flavin ratio (AFR): (○) 120; (▲) 98.

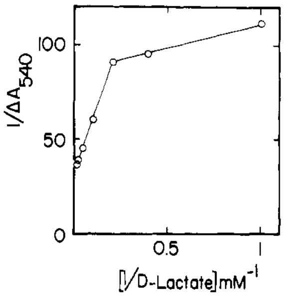


FIGURE 7: Double-reciprocal plot of the amount of transient absorption seen at 540 nm on mixing anaerobic D-lactate dehydrogenase (28 μM) with varying concentrations of D-lactate. The reaction was followed in a stopped-flow spectrophotometer in potassium phosphate buffer, pH 6, ionic strength = 0.23, at 4 °C.

enzyme at pH 6 in potassium phosphate buffer, ionic strength = 0.23, there is a triphasic bleaching of the flavin absorption, as is observed also at pH 8. The rate of the fastest phase is equal to the rate of appearance of a long wavelength (510–700 nm) charge-transfer absorbance. The spectrum after the fast phase at pH 6 is identical with that seen for this reaction at pH 8. Thus, the fast portion of the reaction appears to be similar at pH 6 and 8. There are, however, significant differences between the reductive half-reactions at pH 8 and 6. Figure 4 shows the most striking difference. Panel a shows the time course of reduction of the enzyme (and the corresponding logarithmic plot) with 10 mM D-lactate; panel b shows similar plots for 50 mM D-lactate. In all cases the reaction is triphasic. However, as the concentration of D-lactate is raised, the proportion of the reaction which goes by the intermediate phase quite clearly increases relative to the portion which goes by the slow phase. Such results are seen at all wavelengths examined in the flavin peak and are summarized in Figure 5.

Two important points should be mentioned. First, since the amount of reduction due to the slow and intermediate phases is independent of wavelength, the possibility of a spectrally distinct intermediate in flavin reduction is unlikely. Second, it is unlikely that the slow phase is due to some portion of the enzyme lacking an essential catalytic component, as is seen with xanthine oxidase (Morrell, 1952; Edmonson et al., 1972), since the proportion of the reaction in the slow phase would be expected to be independent of substrate concentration. When the rate constant for the intermediate phase of flavin reduction (k_{int}) is plotted against D-lactate concentration (Figure 6), a curve exhibiting a minimum is seen. This can obviously not be explained by any of the simple schemes considered. This point will be discussed in a later section.

At the lowest concentrations of lactate, due to the unfavorable equilibrium constant for the lactate/pyruvate couple, the full absorption change was not seen. Thus, rate measurements at these concentrations will be subject to the greatest error. All the points shown in Figure 6 are an average of duplicates which agreed within 20%. Also, as Figure 6 shows, the same results are obtained with two separate preparations of enzyme of different AFR values. Thus, we believe Figure 6 is a true representation of the substrate dependence of k_{int}.

It is not possible to study the dependence of k_{slow} at pH 6 at low substrate concentrations, due to the decreased amount of flavin reduction which was seen when the lactate concentration was lowered. The saturating value for k_{slow} at pH 6 is 0.017 ± 0.004 s^{-1}.

Unlike the situation at pH 8, the rate of formation of the charge-transfer complex (k_{fast}) at pH 6 seems to be slightly dependent upon D-lactate concentration. Analyzing the substrate dependence as described by Strickland et al. (1975), using the model in Scheme II, we estimate k_4 = 9.5 s^{-1}, k_3 = 33 s^{-1}, and K_d^{app} = 68 mM. From these numbers we can estimate an overall K_d^{app} = 19.6 mM.

When the maximal amount of long wavelength absorbance at 540 nm, produced on reacting oxidized enzyme with D-lactate, is plotted as a double-reciprocal plot, a biphasic binding curve is seen. This suggests that the binding of D-lactate is complex. The double-reciprocal plot is shown in Figure 7, and the apparent dissociation constants for the two sites are 8 mM and 0.3 mM. Since the total change in long wavelength absorbance is small, these values are subject to a large amount

Table I: Variation of the Kinetic Parameters Describing Flavin Reduction with pH[a]

pH	k_4 (s^{-1})	k_3 (s^{-1})	K_d^{app} (mM)	k_{fast} (s^{-1})	k_{slow} (s^{-1})
7	0.3	0.6	46	12.2	0.014
7.5	0.27	1.43	67	12.3	0.024
8	0.26	3.3	150	11	0.07
8.5	0.25	3.3	98	13.4	0.034
9	0.065	ND	ND	ND	0.009

[a] The parameters are as defined in the text.

of error. However, in two separate experiments we observed similar behavior and so believe the biphasic binding is a real phenomenon.

Reaction of Reduced Enzyme with Pyruvate at pH 6. There is a biphasic increase in flavin absorbance at 4 °C and pH 6 when reduced D-lactate dehydrogenase (18 mM) is reacted anaerobically with varying concentrations of pyruvate (5–200 mM). The slow phase is independent of the pyruvate concentration, $k_{slow} = 0.017\ s^{-1}$, and is responsible for 15–20% of the absorbance change at all concentrations of pyruvate. The faster phase is, however, dependent on the concentration of pyruvate, and when this variation is analyzed, the data fit Scheme II (a two-step reversible reaction), with a significant value for k_4. Values for the kinetic constants of Scheme II are $k_3' = 5.9\ s^{-1}$, $k_4' = 0.26\ s^{-1}$, and $K_d^{app} = 120$ mM.

Unlike the reaction of pyruvate with reduced enzyme at pH 8, at pH 6 no transient long wavelength absorbance was seen at any pyruvate concentration used.

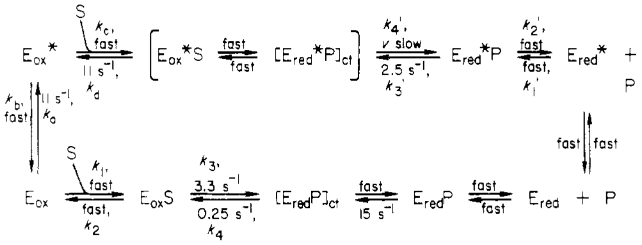
Reductive Half-Reaction at Other pH Values. Reductive half-reactions were repeated at pH 7, 7.5, and 8.5, in potassium phosphate buffer, ionic strength = 0.23. An experiment was also carried out at pH 9 in glycine/NaOH buffer, ionic strength = 0.23. At pH values greater than 9, D-lactate dehydrogenase rapidly loses activity.

In all these studies the results are essentially the same as those seen at pH 8. An exception is that in the reductive half-reaction at pH 7, where at low lactate concentrations the slow phase accounts for a significant proportion of the flavin bleaching.

The variation of k_{slow} and k_{fast} with pH is shown in Table I. When the apparent rate constant describing the intermediate phase (k_{int}) at these varying pH values is analyzed by the methods described in Strickland et al. (1975), the results in Table I are obtained. Most striking is that k_4 (Scheme II) is independent of pH in the pH range 7–8.5, decreasing only at pH 9. The apparent dissociation constant (K_d^{app}) and k_3 from Scheme II both increase with increasing pH. The rate constant k_3 represents the conversion of the binary enzyme–lactate complex to the enzyme–pyruvate complex. It is possible that the increase in k_3 with pH represents the deprotonation of a base on the protein responsible for the initial proton abstraction. However, due to the complexities of the reductive half-reaction and inherent errors involved in the calculations when k_4 is significant, the value of k_3 will be only a rough estimate. Thus we are unable to use its variation with pH to estimate a pK for any essential base.

No estimate for k_3 at pH 9 is included in Table I. A combination of the increase in slope of the corrected double-reciprocal plots and a decrease in intercept prevented any reliable estimate of k_3. Also, there is no estimate for k_{fast} at pH 9 since as the pH increases the change in flavin absorbance due to k_{fast} and the amount of charge-transfer complex formed decrease. At pH 9 the changes were too small for a reliable estimate to be made.

Scheme III[a]



[a] The values shown represent our interpretation of the results obtained at pH 8, 4 °C (see text for details). "k_{fast}" referred to in the text is here interpreted as the isomerization rate $k_a \approx k_d$ (cf. Scheme I). "k_{int}" is largely determined by k_3 (cf. Scheme II). "k_{slow}" ≈ k_4' of Scheme IV.

Oxidation by Molecular Oxygen. When reduced enzyme was rapidly mixed with 0.1 M potassium phosphate buffer, pH 7, equilibrated with varying concentrations of oxygen at 25 °C, a monophasic rise in the flavin absorption was seen at all wavelengths (350–480 nm).

There was no evidence of any transient long wavelength absorbance or of flavin–C(4a)–oxygen intermediates such as those seen with the flavoprotein hydroxylases (Entsch et al., 1976). A simple second-order reaction occurs with a rate constant of $1.5 \times 10^4\ M^{-1}\ s^{-1}$. This may be compared with rate constants of $0.9 \times 10^4\ M^{-1}\ s^{-1}$ for the reaction of free reduced L-lactate oxidase and $1.8 \times 10^6\ M^{-1}\ s^{-1}$ for the reaction of the reduced enzyme–pyruvate complex of lactate oxidase with oxygen (Lockridge et al., 1972).

Discussion

We believe that the oxidation–reduction of D-lactate dehydrogenase by the D-lactate/pyruvate couple at pH 8 is consistent with a situation such as that shown in Scheme III. In Scheme III there are two catalytically competent conformations of D-lactate dehydrogenase (represented by E_{ox} and E_{ox}*) in equilibrium as defined in Scheme I. One form, E_{ox}*, is envisaged to form rapidly a reduced enzyme–pyruvate charge-transfer complex ($[E_{red}*P]_{ct}$) on reaction of oxidized enzyme with D-lactate. If this charge-transfer complex can only break down slowly, then the reduction of D-lactate dehydrogenase will go predominantly through the enzyme form E_{ox}. The limiting step in the reaction of E_{ox} with D-lactate from the analysis of k_{int} would be 3.3 s^{-1}. If we assume that the breakdown of the E_{red}P charge-transfer species is rapid, then the disappearance of the absorbance at 540 nm at the same rate as k_{int} is readily explained. The same argument requires that the observed charge-transfer species be $[E_{red}*P]_{ct}$. Its substrate concentration independent rate of formation is then defined by the conversion of the predominant species E_{ox} to the reactive species E_{ox}* with the rate constant k_a, and the dependence of the observed concentration of this intermediate on the substrate concentration is explained by $k_a \approx k_d$ (Schopfer & Massey, 1979).

When reduced enzyme is reoxidized by pyruvate, a charge-transfer complex is rapidly formed. This charge-transfer complex would correspond to $[E_{red}P]_{ct}$ in Scheme III and would again only slowly undergo further reaction (due to the low value of k_4). Thus the main path of flavin oxidation will be through E_{red}* with a limiting rate of 2.5 s^{-1} at pH 8 (k_3' of Scheme III).

In our model we envisage that E_{ox}* will have a higher affinity for D-lactate than E_{ox}. This seems to be a reasonable assumption since K_d for lactate from the charge-transfer data (representing E_{ox}*) is 20 mM while the K_d for lactate from analysis of k_{int} (representing E_{ox}) is 150 mM. The possible

Scheme IV

$$\mathrm{E_{ox}} + \text{D-lactate} \xrightleftharpoons[k_2]{k_1} \mathrm{E_{ox}}\text{-D-lactate} \xrightleftharpoons[k_4/k_3']{k_3/k_4'} \mathrm{E_{red}}\text{-pyruvate} \xrightleftharpoons[k_1']{k_2'} \mathrm{E_{red}} + \text{pyruvate}$$

importance of this difference in affinity for lactate will be considered later.

Scheme III enables us to account for several apparent discrepancies in the data at pH 8 which are not consistent with any simple system. First, in the basic model for lactate/pyruvate oxidation–reduction represented by Scheme IV, where rate constants with primes represent those measured for pyruvate reduction and those without are for lactate oxidation, the principle of microscopic reversibility predicts that $k_3 = k_4'$ and $k_4 = k_3'$. However, $k_3 = 3.3\ \mathrm{s^{-1}}$ and $k_4 = 0.24\ \mathrm{s^{-1}}$, yet $k_3' = 2.5\ \mathrm{s^{-1}}$ and $k_4' = 0.16\ \mathrm{s^{-1}}$. Thus Scheme IV cannot be a true representation of the situation. In Scheme III, k_3 and k_4 represent the interconversion of $\mathrm{E_{ox}S}$ and $\mathrm{E_{red}P}$ while k_3' and k_4' represent interconversion of $\mathrm{E_{red}{*}P}$ and $\mathrm{E_{ox}{*}S}$. Thus $k_3 \neq k_4'$ and $k_4 \neq k_3'$.

Second, from the steady-state results (Morpeth & Massey, 1982) at 4 °C in the same buffer, $1/\phi_0$ ($V_{\max}$) = 370 $\mathrm{min^{-1}}$. When the possible errors involved in the graphical determination of k_3, estimated from Figure 5 as $\sim$200 $\mathrm{min^{-1}}$, are considered, these results suggest, with potassium ferricyanide accepting electrons, that k_3, the rate constant describing the reduction of $\mathrm{E_{ox}}$–S, is rate limiting. The slope of the plot $1/(k_{\mathrm{int}} - k_4)$ vs. 1/[D-lactate] (Figure 3) should be equal to the steady-state parameter ϕ in any simple situation. However, there is an 11-fold difference between the two parameters, slope of Figure 3 = 0.45 mM min and ϕ(lactate) = 0.071 mM min (Morpeth & Massey, 1982).

In Scheme III at pH 8, we suggest that most of the reduction by lactate is through the path involving $\mathrm{E_{ox}}$. However, in steady state some reduction may also occur through $\mathrm{E_{ox}}$*. This would predict nonlinear reciprocal plots such as those seen at pH 6 (Morpeth & Massey, 1982). At pH 8 the double-reciprocal plots with varying lactate concentrations are linear from 100 to 0.1 mM D-lactate. Pettersson (1969, 1972) has shown that reciprocal rate plots may appear asymptotically linear when the rate equation is nonlinear. Thus it could be that the linearity at pH 8 is really the linear asymptote of a curve in which curvature becomes apparent only at impractically high concentrations of D-lactate. In this case the ϕ(lac) will also contain terms involving the high-affinity form ($\mathrm{E_{ox}}$*) of D-lactate dehydrogenase and so would not be expected to be equal to the slope of Figure 3 which only involves $\mathrm{E_{ox}}$.

The results at pH 6 are even more complex than those at pH 8. However, we believe they are consistent qualitatively with Scheme III, but now with significant enzyme reduction by D-lactate occurring with both forms $\mathrm{E_{ox}}$ and $\mathrm{E_{ox}}$*, and not just $\mathrm{E_{ox}}$. Evidence to support this is presented in Figure 5, which shows the dependence on lactate concentration of the percentage reduction proceeding by k_{slow} or k_{int}. If k_{slow} represents reduction via $\mathrm{E_{ox}}$* and $\mathrm{E_{ox}}$* has a high affinity for D-lactate, then at low lactate concentrations the bulk of the reaction should proceed with this rate constant. As the lactate concentration is raised, if $\mathrm{E_{ox}}$ (represented by k_{int}) has a lower affinity for D-lactate, then the proportion of enzyme reduced by the faster rate constant (k_{int}) should increase. Inspection of Figure 5 shows this type of inverse relationship between k_{int} and k_{slow}; as reduction due to one increases, the reduction due to the other decreases and vice versa.

The substrate dependence of k_{int} is also consistent with the idea that more than one form of D-lactate dehydrogenase is being reduced at pH 6. The variation of k_{int} with D-lactate shown in Figure 6 cannot be explained by any simple scheme. The data in Figure 6 require that k_{int} contains higher order terms in D-lactate concentration. In Scheme III, if k_{int} represents the limiting step in conversion of $\mathrm{E_{ox}S}$ to $[\mathrm{E_{red}P}]_{\mathrm{ct}}$, the complex dependence on substrate concentration of k_{int} is readily explained.

Scheme III predicts that on reduction of D-lactate dehydrogenase by D-lactate two charge-transfer complexes can be formed. At pH 8 only the complex $[\mathrm{E_{red}{*}P}]_{\mathrm{ct}}$ is seen since $[\mathrm{E_{red}P}]_{\mathrm{ct}}$ is assumed to break down very rapidly. However, at pH 6 with both $\mathrm{E_{ox}}$ and $\mathrm{E_{ox}}$* being reduced, then $[\mathrm{E_{red}{*}P}]_{\mathrm{ct}}$ must be broken down at an increased rate, and if $[\mathrm{E_{red}P}]_{\mathrm{ct}}$ breakdown is slower, then we would expect to see evidence for the existence of both charge complexes. Such evidence is presented in Figure 7. The curve is clearly biphasic, suggesting that there are binding sites of different affinity for D-lactate. The simplest explanation is that the biphasic curve is due to the two charge-transfer complexes of Scheme III, both being observable transients at pH 6.

In summary, we believe that the evidence presented in this paper is consistent with D-lactate dehydrogenase being able to exist in more than one catalytically competent form in solution. That this is more evident at low pH raises the possibility that a proton may be acting as an allosteric effector. It would be of considerable interest to determine the nature of this ionizing residue or residues. Possibilities include histidine, thiol, or water liganded to the essential zinc, all of which may have a pK in the range pH 6–8. The ionizing residues responsible for mediating any conformational change need not of course be in the vicinity of the active site.

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References

Beaty, N., & Ballou, D. P. (1981) *J. Biol. Chem. 256*, 4611–4618.

Bright, H. J., & Porter, D. J. T. (1976) *Enzymes, 3rd Ed. 13*, 89–173.

Edmonson, D., Massey, V., Palmer, G., Beacham, L. M., III, & Elion, G. P. (1972) *J. Biol. Chem. 245*, 2837–2844.

Entsch, B., Ballou, D. P., & Massey, V. (1976) *J. Biol. Chem. 251*, 2550–2563.

Gibson, Q. H., Swoboda, B. E. P., & Massey, V. (1964) *J. Biol. Chem. 239*, 3927–3934.

Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 248*, 7012–7017.

Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446–465.

Mastrigt, R. (1977) *Comput. Biol. Med. 7*, 231–247.

Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem. 252*, 3199–3207.

Morpeth, F. F., & Massey, V. (1982) *Biochemistry* (preceding paper in this issue).

Morrell, D. B. (1952) *Biochem. J. 51*, 657–666.

Olson, S. T., & Massey, V. (1979) *Biochemistry 18*, 4714–4724.

Pettersson, G. (1969) *Acta Chem. Scand.*, 2717–2729.

Pettersson, G. (1972) *Biochim. Biophys. Acta 276*, 1–11.

Schopfer, L. M., & Massey, V. (1979) *J. Biol. Chem. 254*, 10634–10643.

Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048–4052.