

D-Lactate Dehydrogenase of *Peptostreptococcus elsdenii*¹

HOWARD L. BROCKMAN² AND WILLIS A. WOOD*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Received for publication 24 June 1975

D-Lactate dehydrogenase has been purified to near homogeneity from *Peptostreptococcus elsdenii*. As isolated, the enzyme contains flavine adenine dinucleotide and a tightly bound metal cofactor. Inactivation by *ortho*-phenanthroline occurs in two steps and is partially blocked by D-lactate. Reactivation by divalent metal ions occurs, with divalent zinc being the most effective. When ferricyanide is used as the electron acceptor, D-lactate has an apparent $K_{0.5}$ of $3.3 \text{ M}^{0.46}$; its binding is negatively cooperative with a Hill coefficient of 0.46. Replacement of ferricyanide by the other components of the electron transport system yields hyperbolic kinetics with an apparent K_m for D-lactate of 26 mM. The apparent K_m for ferricyanide is $2.2 \times 10^{-4} \text{ M}$. Phosphate and pyrophosphate compounds stimulate the D-lactate:ferricyanide activity. These properties suggest that interaction of this enzyme with other electron transport proteins in the chain may enhance D-lactate binding and, hence, the rate of electron transport.

The obligate anaerobe, *Peptostreptococcus elsdenii*, grows on D- or L-lactate as a sole energy source (5) with the production of acetate, propionate, butyrate, and valerate (6). Studies of lactate metabolism have established that L-lactate is racemized to D-lactate by a lactate racemase (D. L. Schneider, Ph.D. thesis, Michigan State University, East Lansing, 1969) and that D-lactate is then oxidized to pyruvate and acetyl-CoA (R. L. Baldwin, Ph.D. thesis, Michigan State University, East Lansing, 1962). An accompanying paper (1) shows that electrons generated in the oxidation of D-lactate are transferred to an unsaturated acyl-coenzyme A (CoA) via an electron-transferring flavoprotein and a butyryl-CoA dehydrogenase. To understand more clearly the role of each component in this electron transport chain, characterization of the proteins was undertaken. This paper describes the purification and properties of the D-lactate dehydrogenase of *P. elsdenii*; its relationship to the other enzymes of electron transport is discussed in the accompanying paper (1).

MATERIALS AND METHODS

Spectrophotometric measurements. All absorbance spectra were obtained on a Cary 15 spectrophotometer using 2-ml cells (light path, 1 cm). Spectrophotometric rates were measured with a Gilford 2000 spectrophotometer using 0.5-ml microcuvettes (light path, 1 cm).

¹Journal article no. 7287 of the Michigan Agricultural Experiment Station.

²Present address: The Hormel Institute, University of Minnesota, Austin, Minn. 55912.

Protein determination. Protein was measured spectrophotometrically using absorbance at 260 and 280 nm in conjunction with a nomograph (distributed by the California Corp. for Biochemical Research) based on extinction coefficients determined for nucleic acid and enolase by Warburg and Christian (13).

Oxygen determination. Oxygen consumption was measured with an oxygraph (Yellow Springs Instrument Co.) equipped with a Clark-type oxygen electrode.

Electrophoresis. Polyacrylamide gels (7.5% cross-linking) were run at pH 8.3 as described by Davis (3). Stacking and spacer gels were not used. After electrophoresis in quartz tubes, the gels were scanned directly at 280 and 450 nm with a Gilford recording spectrophotometer equipped with a linear transport attachment.

Assay for D-lactate dehydrogenase. Activity was determined by following the disappearance of ferricyanide at 420 nm upon reduction by D-lactate. The procedure was a modification of that described by Symons and Burgoyne (12). Each reaction mixture contained 160 μmol of D-lactate, 0.5 μmol of potassium ferricyanide, 10 μmol of potassium phosphate buffer (pH 7), and enzyme in a total volume of 0.2 ml. Assays were normally performed at 24 C. The molar extinction coefficient of ferricyanide was taken as 1,040 cm^2/mol relative to ferrocyanide. A unit of D-lactate dehydrogenase is that amount of enzyme which catalyzes the formation of 1 μmol of ferricyanide per min at 24 C.

Determination of kinetic constants. The saturation behavior of substrates is expressed as K_m when the response is hyperbolic. With sigmoidal kinetics indicating cooperative interaction among ligands, the Michaelis-Menten relationship in reciprocal form between reaction velocity and ligand concentration is $1/v = K^n/V_m(1/S^n) + 1/V_m$, where n is equivalent to

the Hill coefficient and is an expression of the degree of cooperativity. The K value so derived is more properly referred to as a $K_{0.5}$ for the amount of ligand giving half maximal velocity and has the dimension M^n . Using a computer, the value of n was varied until a plot of $1/v$ versus $1/S^n$ gave the best least-squares fit to a straight line as judged by the correlation coefficient (4).

Purification of D-lactate dehydrogenase. The purification procedure utilized two steps, a summary of which is presented in Table 1. A 193-fold purification was obtained with a 9.4% recovery of activity. All purification steps were performed in the presence of 1 mM dithiothreitol which minimized activity losses. *P. elsdenii* (strain B159, ATCC 17752) was grown anaerobically at 37 C with lactic acid as a primary carbon source (1).

Chromatography on DEAE-cellulose. Initial experiments indicated the presence of D-lactate dehydrogenase activity in crude extract. This activity was adsorbed by diethylaminoethyl (DEAE)-cellulose equilibrated at 0.3 M phosphate (pH 6.0), and was totally eluted by 1 M buffer. In subsequent purifications, the crude extract was mixed with 300 ml of moist DEAE-cellulose (equilibrated against water), stirred for 10 min at 4 C, and retrieved by vacuum filtration. The DEAE-cellulose was washed with 500 ml of 0.3 M potassium phosphate buffer (pH 6.0), and applied to the top of a column (5 by 30 cm) containing the same quantity of DEAE-cellulose equilibrated with the same buffer. D-Lactate dehydrogenase was eluted with potassium phosphate (pH 6.0), in a linear gradient (600 ml per chamber) from 0.3 to 0.8 M. The enzyme, which eluted at approximately 0.7 M phosphate, was then precipitated by adding ammonium sulfate to 2.7 M concentration.

Chromatography on hydroxyapatite. The resulting precipitate was dissolved in a minimum volume of water and the residual ammonium sulfate was removed by passage through a column (2.5 by 20 cm) of Sephadex G-25, which had been equilibrated against 0.1 M potassium phosphate buffer (pH 7.0). A sample of this G-25 eluate was then applied to a column (0.75 by 15 cm) of hydroxyapatite and equilibrated against 0.1 M potassium phosphate buffer (pH 7.0), and the enzyme was eluted with this buffer.

RESULTS

Figure 1 shows absorbance tracings of 80 μ g of 193-fold-purified D-lactate dehydrogenase after polyacrylamide gel electrophoresis. The figure shows one major protein band (280-nm trace) which comigrates with the major flavin band (450-nm trace). Staining of similar gels containing microgram quantities of the preparation with 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-tetrazolium chloride (see data on acceptor specificity) in the presence and absence of D-lactate showed that the enzyme activity was associated with the major protein band. In addition, there are one (or more) minor components in both 280- and 450-nm scans which appear as should-

ers on the main peak. The nature of the prosthetic group will be more fully discussed in a subsequent section.

Specificity and kinetics. The electron donor specificity of the enzyme was determined by measuring the rates of oxidation of α -hydroxy acids using ferricyanide as the acceptor. Table 2 shows the activities of other compounds relative to D-lactate. Only D-lactate, L-lactate, and D,L- α -hydroxybutyrate showed appreciable activity. In an end-point determination using an excess of D-lactate dehydrogenase, it was found that only 0.5% of the L-lactate added was oxidized under conditions affording complete oxidation of an equivalent amount of D-lactate. Therefore, it was concluded that the L-lactate preparation was contaminated with a trace of D-lactate and that the enzyme is specific for the D-isomer. The activity exhibited by D,L- α -

TABLE 1. Purification of D-lactate dehydrogenase from *P. elsdenii*

Step	Ac-tivity (units)	Sp act (units/mg of P)	Re-cove-ry (%)	Purifi-cation (fold)
Crude extract	60,500	1.44	100	1
DEAE-cellulose, 0.3-0.8 M (pH 6.0)	31,780	80.00	53	56
Hydroxyapatite eluate, 0.1 M (pH 7.0)	6,470	280.00	9.4	193

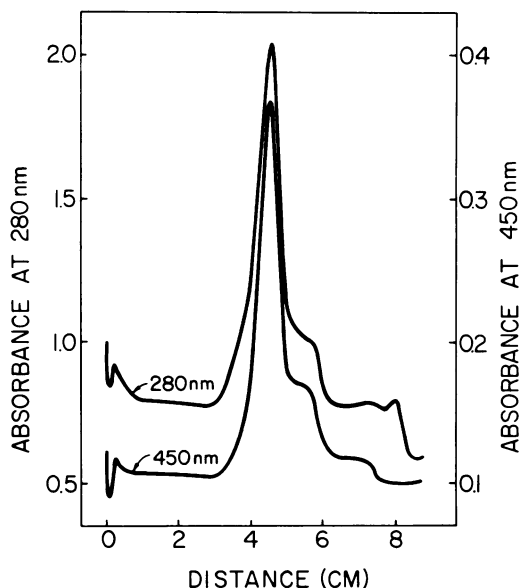


FIG. 1. Absorbance tracings of a polyacrylamide gel of a 0.08-mg sample of D-lactate dehydrogenase.

TABLE 2. Donor specificity of *D*-lactate dehydrogenase^a

Substrate	Relative activity (%)
Glycolic acid	1.7
<i>D</i> -Lactic acid	100.0
<i>L</i> -Lactic acid	10.0
<i>D,L</i> - α -Hydroxybutyric acid	57.0
<i>D,L</i> - α -Hydroxyisobutyric acid	0.2
<i>D,L</i> - α -Hydroxyvaleric acid	1.6
<i>D,L</i> - α -Hydroxycaproic acid	0.3
<i>D</i> -Malic acid	0.1
<i>L</i> -Malic acid	0.0
<i>D,L</i> -Glyceric acid	1.9

^a Each assay consisted of 20 μ mol of substrate, 5 μ mol of $K_3Fe(CN)_6$, and 20 μ mol of potassium phosphate buffer (pH 7.0) in a total volume of 0.2 ml. The assays were run at 24 C and were initiated by the addition of enzyme.

hydroxybutyrate probably reflects the oxidation of the *D*-isomer. The relative lack of activity exhibited by the lactic acid homologues, glycolic acid, *D,L*- α -hydroxycaproic acid, and *D,L*- α -hydroxyvaleric acid, shows that the enzyme is specific for three- and four-carbon α -hydroxy acids. Also, lactate analogues with substituents other than hydrogen on the α - or β -carbon atoms are oxidized only at very low rates. On the basis of these configurational and substituent specificities, the enzyme is properly referred to as a *D*-lactate dehydrogenase.

The velocity of *D*-lactate oxidation was measured as a function of the concentration of *D*-lactate at pH values of 6.0, 7.0, and 8.0 using ferricyanide as the acceptor. In each case, markedly nonlinear Lineweaver-Burk plots were obtained. Using a computer to determine the best least-squares fit to a straight line, as described in Materials and Methods, a value of $n = 0.46$ was obtained with a correlation coefficient of 0.999. This yielded an apparent $K_{0.5}$ for *D*-lactate of $3.3 M^{0.46}$.

To determine if the atypical kinetic behavior occurs under more physiological conditions, ferricyanide was replaced by the physiological acceptor, the electron-transferring flavoprotein (1). The rate of the reaction was monitored by following the reduction of butyryl-CoA dehydrogenase as described in the accompanying paper (1). Even at the highest *D*-lactate concentrations used, the velocity of the reaction was still a direct function of the *D*-lactate dehydrogenase concentration, thereby excluding the possibility that some other step in electron transfer was rate limiting. Figure 2 shows that, with this change in the assay system, the Lineweaver-

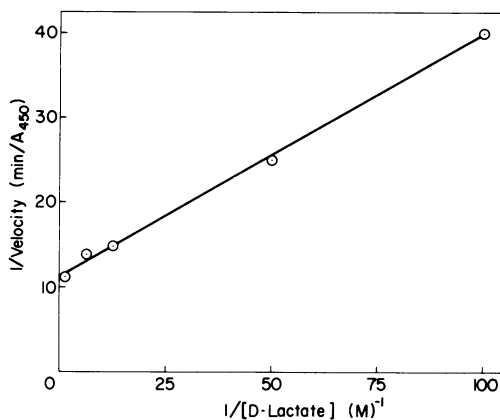


FIG. 2. Kinetics of *D*-lactate binding with electron-transferring flavoprotein as acceptor. Each cuvette contained 3.2 units of *D*-lactate dehydrogenase, butyryl-CoA dehydrogenase to give 0.125 OD_{480} , 0.26 units of electron-transferring flavoprotein, 50 μ mol of potassium phosphate buffer (pH 7.0), in a total volume of 0.20 ml. Assays were performed anaerobically at 37 C and were initiated by addition of electron-transferring flavoprotein.

Burk plot is linear, yielding an apparent K_m of 26 mM. The marked lowering of the apparent $K_{0.5}$ observed when ferricyanide is replaced by electron transport proteins suggests that the presence of one or both of these proteins is necessary for efficient *D*-lactate binding. It is also possible that the artificial acceptor, ferricyanide, is inhibitory in the usual *D*-lactate dehydrogenase assay and gives rise to the atypical kinetic behavior of the enzyme.

Although *D*-lactate dehydrogenase exhibits a narrow specificity for electron donors, its acceptor specificity is rather broad. Table 3 shows the relative rates obtained with assays containing *D*-lactate, *D*-lactate dehydrogenase, and various acceptors. Dyes, oxygen, and cytochrome *c* all served as acceptors to some degree. However, the lack of reactivity with 3-acetylpyridine-nicotinamide adenine dinucleotide (NAD) at pH 8.5 confirms that this enzyme is of the "NAD-independent" type as previously observed (R. L. Baldwin, Ph.D. thesis, Michigan State University, East Lansing, 1962). An attempt to demonstrate the reverse reaction with reduced NAD and pyruvate was also unsuccessful.

An apparent K_m for ferricyanide, the acceptor routinely used, was calculated from data obtained by varying its concentration in assays run at fixed *D*-lactate and enzyme concentrations. A Lineweaver-Burk plot was linear, yielding an apparent K_m of $2.2 \times 10^{-4} M$.

TABLE 3. Acceptor specificity of D-lactate dehydrogenase^a

Acceptor	Concn (M)	Wavelength (nm)	Rate ($\mu\text{mol}/\text{min}/\text{ml}$)
Ferricyanide	2.5×10^{-3}	420	266
2- <i>para</i> -iodophenyl-3- <i>para</i> -nitrophenyl-5-phenyltetrazolium chloride	2×10^{-3}	490	82
Dichlorophenol indophenol	4×10^{-5}	600	832
Cytochrome c	1.3×10^{-4}	550	200
3-Acetylpyridine NAD	2.9×10^{-4}	366 ^b	0
Oxygen	2.4×10^{-4}	O ₂ electrode	11

^a Each spectrophotometric assay consisted of 10 μmol of potassium phosphate buffer (pH 7.0), 160 μmol of D-lactate, and acceptor as indicated in a total volume of 0.2 ml. For measurements of oxygen consumption, the reagents were increased in proportion to a volume of 2.5 ml and endogenous oxygen was the acceptor. All assays were run at 24 C and were initiated by the addition of enzyme.

^b Run at pH 8.5.

pH Optimum. Assays performed at pH values ranging from 6 to 9 with a nonsaturating D-lactate concentration showed little pH dependency between pH values of 6 and 8 with a slight optimum at pH 7.9. Above pH 8, activity losses were observed before all of either the D-lactate or ferricyanide was consumed, suggesting that the enzyme is not stable in this region.

Activators. In the course of developing the D-lactate:ferricyanide assay, it was noted that the observed rate was a function of the phosphate buffer concentration in the assay. It was further shown that both sodium and potassium phosphate stimulated whereas sodium chloride did not, indicating that the stimulation was caused by the orthophosphate anion. The dependence of the velocity on the phosphate concentration was linear with a positive intercept. The non-zero intercept shows that, although phosphate stimulates, it is not required for activity, or, alternatively, that other anions in the assay mixture cause some activation.

In view of the unusually high K_m for D-lactate observed with the D-lactate:ferricyanide assay system and the phosphate stimulation of the activity observed with this assay, it was believed that the level of some phosphate-containing compound might regulate the activity of the enzyme. Accordingly, a series of phosphate-containing nucleotides, carbohydrates, and other compounds was tested for ability to alter the rate of D-lactate oxidation at a concentration of D-lactate which was well below its apparent K_m . Among 25 compounds tested, the relative rates varied from 52% for NAD to 140 and 148% for thiamine pyrophosphate and sodium pyrophosphate, respectively.

Cofactors. The yellow color exhibited by purified D-lactate dehydrogenase suggested that a cofactor is associated with the enzyme. Since

previously studied pyridine nucleotide-independent D-lactate dehydrogenases have been shown to be metalloflavoproteins, flavin analysis and metal chelator studies were undertaken.

The flavin cofactor was identified by thin-layer chromatography. A 0.5-ml volume of a D-lactate dehydrogenase preparation (specific activity 105) was heated to 100 C for 5 min and the resulting white precipitate was collected by centrifugation and discarded. Portions of the yellow supernatant were spotted on Silica Gel F₂₅₄ thin-layer plates and chromatographed in two different solvent systems with authentic standards, and the flavin spots were located by their yellow color. The solvent systems were water-saturated collidine and *t*-butanol-water (60:40, vol/vol) (7) and each system clearly separated authentic flavine adenine dinucleotide (FAD) and riboflavine 5'-phosphate. The enzyme cofactor was indistinguishable from FAD. Even when fluorescence under ultraviolet light was used to increase the sensitivity of detection, no riboflavine 5'-phosphate or any other fluorescing compound was found.

Curve 1 of Fig. 3 shows the spectrum of the oxidized, deaerated enzyme. It exhibits a spectrum typical of a flavoprotein with maxima at 450 and 378 nm. Upon the addition of 0.2 mmol of D-lactate, a substantial bleaching was observed at 450 nm with some increase in absorbance around 570 nm (Fig. 3, curve 2). This spectral bleaching by D-lactate shows that the addition of substrate to the enzyme solution causes a reduction of the flavin cofactor, suggesting, as might be expected, that the enzyme-bound flavin acts as an immediate electron acceptor in the oxidation of D-lactate.

To determine if the enzyme contains a divalent metal cofactor, inactivation by metal chelators was studied. Preliminary experiments

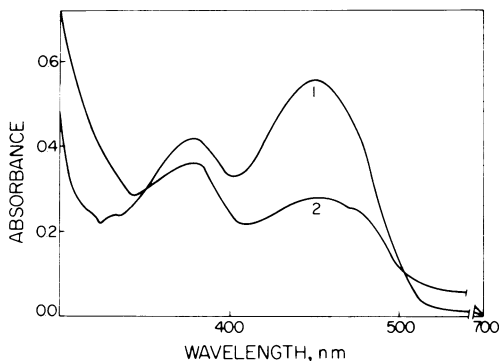


FIG. 3. Spectra of oxidized and substrate-reduced *D*-lactate dehydrogenase. Each cuvette contained 1.4 mg of enzyme and 1.5 mmol of potassium phosphate buffer (pH 6.0) in a total volume of 0.5 ml (curve 1). Sample 2 (curve 2) also contained 200 μ mol of *D*-lactate. Each cuvette was deaerated and maintained at 24 C. Each reference cuvette contained 1.5 mmol of potassium phosphate buffer (pH 6.0), and the reference cuvette for sample 2 also contained 200 μ mol of *D*-lactate.

indicated that ethylenediaminetetraacetic acid (EDTA), *N,N'*-bipyridyl, or *ortho*-phenanthroline would with time completely inactivate the enzyme. The kinetics of inactivation by phenanthrolines were studied in more detail. Equal amounts of enzyme in 0.05 M potassium phosphate buffer (pH 8.0) were incubated with the additions indicated in Fig. 4 and, at various times, portions were withdrawn and assayed for activity. Fig. 4 shows the \log_{10} of percent activity remaining in each sample as a function of time of incubation with the chelator. Each line shows a rapid loss of activity followed by a slower, pseudo-first order inactivation. Table 4 lists the pseudo-first order rate constant, k_1 , and the extent of the rapid inactivation as a percent of activity lost for each curve shown in Fig. 4. No loss of activity was observed in control samples containing 25% ethanol. Relatively rapid inactivation is observed with both *ortho*- and *meta*-phenanthroline. The extent of this inactivation had little dependence on the phenanthroline concentration, but it was significantly decreased by the presence of *D*-lactate. The slower, pseudo-first order inactivation has been shown to result in complete loss of activity (see experiment below), and it proceeds with a rate constant which is a direct function of *ortho*-phenanthroline concentration. In addition, the rate is decreased by the presence of *D*-lactate. Since this latter inactivation is not observed with the nonchelating analogue, *meta*-phenanthroline, the inactivation appears to depend upon the chelating ability of the com-

pound. This conclusion is supported by time-dependent inactivations observed with the chelators, EDTA and *N,N'*-bipyridyl.

To define more clearly the role of *ortho*-phenanthroline in inactivating the enzyme, reactivation by divalent metal ions was studied. Passage of inactivated enzyme through Sephadex G-25 to remove the chelator, followed by addition of divalent cation and/or FAD, caused no recovery of activity, although such a procedure was successfully employed with the *D*-lactate dehydrogenase from yeast (2). Accordingly, the gel filtration step was omitted and reactivation was studied by direct addition of metal ions to the incubation mixture. A sample of enzyme was inactivated to greater than 99% by incubation with 4 mM *ortho*-phenanthroline for 45 min

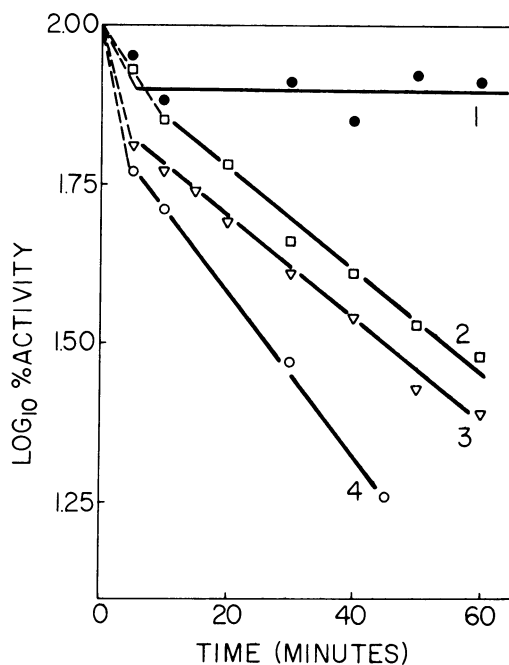


FIG. 4. Inactivation of *D*-lactate dehydrogenase by phenanthroline. Each incubation mixture contained a catalytic amount of enzyme in 0.05 M potassium phosphate buffer (pH 8.0), plus additions to give the following final concentrations: line 1, 1.9 mM *meta*-phenanthroline; line 2, 1.9 mM *ortho*-phenanthroline plus 0.5 M *D*-lactate; line 3, 0.91 mM *ortho*-phenanthroline, and line 4, 1.9 mM *ortho*-phenanthroline. *Meta*-phenanthroline was added as a solution in ethanol to give a final ethanol concentration of 2.5%. The incubation mixtures were maintained at 4 C and portions were withdrawn with time as indicated in the figure. Each assay for enzyme activity contained 20 μ mol of *D*-lactate, 0.5 μ mol of potassium ferricyanide, and 10 μ mol of potassium phosphate buffer (pH 8.0) in a total volume of 0.2 ml.

TABLE 4. Inactivation of D-lactate dehydrogenase by phenanthroline

Curve ^a	Additions	Rapid inactivation ^b	$k(s^{-1})^c$
1	Meta-phenanthroline, 1.91 mM	17	0
2	Ortho-phenanthroline, 1.91 mM	23	1.73
3	Ortho-phenanthroline, 0.91 mM	20	1.11
4	Ortho-phenanthroline, 1.91 mM, plus D-lactate, 0.5 M	14	1.13

^a See Fig. 4 for experimental details.

^b Rapid inactivation is that loss in relative activity shown by extrapolating each linear segment to zero time.

^c k is the first order rate constant calculated as the slopes in Fig. 4 multiplied by 2.303.

as described. When a volume of this enzyme was diluted with an equal volume of water and immediately assayed for D-lactate dehydrogenase activity, none was detected. Repetition of this procedure by dilution with an equal volume of divalent cation solution resulted in recovery of activity, the extent of which was dependent upon the nature and concentration of the divalent ion added.

Figure 5 shows the percent of enzyme activity restored as a function of the concentration of divalent cation. At any concentration, divalent zinc was a better reactivator than divalent cobalt, and divalent manganese was without effect. More importantly, at a zinc concentration 250-fold lower than the *ortho*-phenanthroline concentration, more than 25% of the activity is restored; total activity is restored at a zinc concentration 2.5-fold higher than that of *ortho*-phenanthroline. The ability of zinc to cause significant reactivation in the presence of an excess of chelator shows that the affinity of the enzyme for zinc must be much higher than that of the chelator. Further, relative to other metals, the higher total reactivation observed with zinc in slight molar excess over *ortho*-phenanthroline is consistent with zinc being the metal cofactor present in the purified enzyme.

DISCUSSION

The evidence indicates that, as isolated, the D-lactate dehydrogenase from *P. elsdenii* contains a flavin cofactor, FAD, and a tightly bound, divalent metal ion. The observed reduction of the flavin cofactor by D-lactate is consistent with the flavin being an intermediate elec-

tron carrier between D-lactate and the physiological acceptor, the electron-transferring flavoprotein (1). The inhibition of activity by phenanthrolines and its subsequent complete regeneration by divalent metal ions suggests that upon addition of *o*- or *m*-phenanthroline, equilibrium binding is rapidly established between chelator and enzyme. When *ortho*-phenanthroline is used, there follows a slow, pseudo-first order decay of the partially active enzyme-*ortho*-phenanthroline complex. The ability of D-lactate to impede both the rapid and slow inactivations is consistent with the presence of metal and phenanthroline binding near a D-lactate binding site. The reactivation by zinc shows the reversibility of these reactions. The scheme in Fig. 6 fits the data. Since reactivation by added divalent metal ions did not occur after separation of excess phenanthroline by gel filtration, it is not possible to eliminate a second alternative; i.e., that the metal *o*-phenanthroline complex is dissociated, yielding an enzyme species which is unstable under the conditions of the chromatography.

The Hill number of 0.46 obtained from D-lactate saturation studies is indicative, according

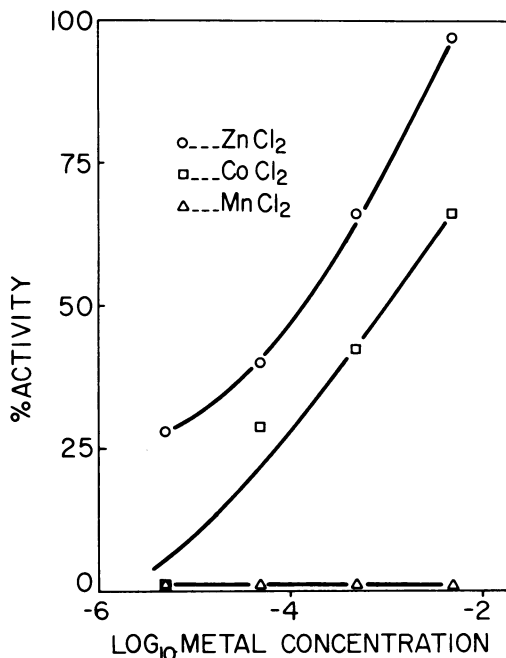


FIG. 5. Reactivation of D-lactate dehydrogenase by divalent metal ions. Each assay contained 0.5 μ mol of potassium ferricyanide, 160 μ mol of D-lactate, and 10 μ mol of potassium phosphate buffer (pH 7.0) in a total volume of 0.2 ml. Assays were run at 24 C and were initiated by the addition of enzyme.

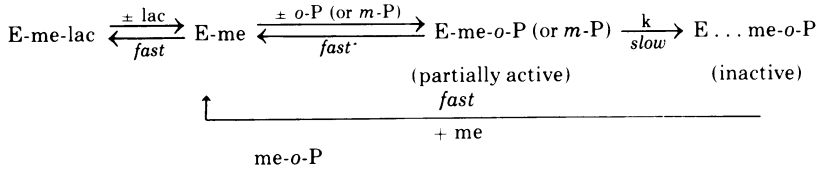


FIG. 6. Scheme for metal ion activation of D-lactate dehydrogenase. E, apodehydrogenase-flavin complex; me, the metal ion; lac, D-lactate; and o-P and m-P, ortho- and meta-phenanthroline, respectively. The dotted line indicates that, in the completely inactive species, the metal-ortho-phenanthroline complex may still be bound to the apoprotein-flavin complex.

to the sequential isomerization model of allosteric behavior of Koshland and associates (8, 9), that the binding of this substrate to the enzyme is negatively cooperative. This implies that each enzyme molecule possesses more than one binding site for D-lactate and that the binding of each D-lactate molecule greatly decreases the affinity of the enzyme for the next. At present, physical studies have not been performed to test the existence of subunits or multiple D-lactate binding sites for this enzyme.

The unusually high apparent $K_{0.5}$ of $3.3 \text{ M}^{0.46}$ for D-lactate and the observed phosphate stimulation, both with ferricyanide as the acceptor, prompted a search for a possible nonprotein effector which would lower the apparent $K_{0.5}$. Of the low-molecular-weight compounds tested, none showed significant activation or inhibition greater than 50% at a D-lactate concentration well below $K_{0.5}$. However, when the apparent K_m for D-lactate was determined using electron-transferring protein and butyryl-CoA dehydrogenase as acceptor, the dependence of velocity on substrate concentration was hyperbolic, yielding an apparent K_m of 26 mM.

In these studies, the D,L-lactate concentration of the growth medium was 0.15 M. Therefore, during most of the growth period, the D-lactate concentration would be at or above the K_m concentration. Nevertheless, this sensitivity of K_m for lactate to the acceptor used suggests that other conditions of either enzyme purification or assay may be found which decrease the K_m further. If the interaction of D-lactate dehydrogenase with the other electron transport proteins changes the apparent $K_{0.5}$ for lactate by 2 to 3 orders of magnitude under physiological conditions, then this interaction may well control the rate of lactate metabolism in this organism. The phosphate and pyrophosphate stimulations may weakly mimic the action of the electron-transferring flavoprotein and/or butyryl-CoA dehydrogenase in the D-lactate:ferricyanide assay, or they may reflect some other control phenomenon. Such polyvalent ion effects at high concentrations have been previ-

ously observed with other D-lactate dehydrogenases. The D-lactate dehydrogenase from *Propionibacterium pentosaceum* is stimulated 2.6-fold by 0.5 M ammonium sulfate with dichlorophenolindophenol as an acceptor (10) and the enzyme from *Butyribacterium rettgeri* is also stimulated by ammonium sulfate with this acceptor (14). With a D-lactate dehydrogenase from anaerobically grown yeast, Nygaard (11) observed a decrease in activity toward cytochrome c (the physiological acceptor) and an increase in activity toward ferricyanide in the presence of polyvalent cations such as protamine. The existence of such effects suggests that, under physiological conditions, polyvalent ions may be important in enabling or controlling protein-protein interactions in the electron transport systems in which these enzymes function.

The kinetic and physical properties of the D-lactate dehydrogenase of *P. elsdenii* are consistent with its role in electron transport. Furthermore, the unusual kinetics of the substrate binding and the possible existence of effectors of enzyme activity suggest that the control of this enzyme is important in the control of lactate metabolism and, hence, cell growth of this anaerobic bacterium.

ACKNOWLEDGMENTS

This research was supported by a grant from the U.S. Atomic Energy Commission and by a traineeship (H.B.) from the National Institutes of Health. We are grateful to the A. E. Staley Manufacturing Co. of Decatur, Ill., for the corn steep liquor.

LITERATURE CITED

1. Brockman, H. L., and W. A. Wood. Electron-transferring flavoprotein of *Peptostreptococcus elsdenii* that functions in the reduction of acrylyl-coenzyme A. *J. Bacteriol.* **124**:1447-1453.
2. Cremona, T., and T. P. Singer. 1964. The lactic dehydrogenases of yeast. V. Chemical properties and function of the zinc component of D-lactic cytochrome reductase. *J. Biol. Chem.* **239**:1466, 1473.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum problems. *Ann. N. Y. Acad. Sci.* **121**:404-427.
4. Dunne, C. P., J. A., Gerlt, K. W. Rabinowitz, and W. A. Wood. 1973. The mechanism of action of 5'-adenylic

- acid-activated threonine dehydrase. Characterization of kinetic effect of adenosine monophosphate. *J. Biol. Chem.* **248**:8189-8199.
5. Elsdon, S. R., B. E. Volcani, F. M. C. Gilchrist, and D. Lewis. 1956. Properties of a fatty acid-forming organism isolated from the rumen of sheep. *J. Bacteriol.* **72**:681-689.
 6. Gutierrez, J., R. E. Davis, I. L. Lindahl, and E. J. Warwick. 1959. Bacterial changes in the rumen during the onset of feed-lot bloat in cattle and characteristics of *Peptostreptococcus elsdonii* n. sp. *Appl. Microbiol.* **7**:16-22.
 7. Kilgour, G. L., S. P. Felton, and F. M. Huennekens. 1957. Paper chromatography of flavins and flavin nucleotides. *J. Am. Chem. Soc.* **79**:2254-2256.
 8. Koshland, D. E., Jr., G. Nemethy, and D. Filmer. 1966. Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* **5**:365-385.
 9. Levitzki, A., and D. E. Koshland, Jr. 1969. Negative cooperativity in regulatory enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **62**:1121-1128.
 10. Molinari, R., and F. J. S. Lara. 1960. The lactic dehydrogenase of *Propionibacterium pentosaceum*. *Biochem. J.* **75**:57-65.
 11. Nygaard, A. P. 1961. Reversible transformation of the acceptor specificity of yeast D-lactic cytochrome *c* reductase. *Acta Chem. Scand.* **15**:1627-1628.
 12. Symons, R. H., and L. A. Burgoyne. 1966. L-lactate (cytochrome) dehydrogenase (crystalline, yeast), p. 314-321. *In* W. A. Wood (ed.), *Methods in enzymology*, vol. 9. Academic Press Inc., New York.
 13. Warburg, O., and W. Christian. 1942. Isolation and crystallization of enolase. *Biochem. Z.* **310**:384-421.
 14. Wittenberger, C. L., and A. S. Haaf. 1966. Isolation of NAD-dependent and NAD-independent lactate dehydrogenases from extracts of *Butyribacterium rettgeri*. *Biochim. Biophys. Acta* **122**:393-405.