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Purification and Properties of the Flavoenzyme D-Lactate Dehydrogenase from *Megasphaera elsdenii*†

Steven T. Olson and Vincent Massey*

ABSTRACT: A pyridine nucleotide independent D-lactate dehydrogenase has been purified to apparent homogeneity from the anaerobic bacterium *Megasphaera elsdenii*. The enzyme has a molecular weight of 105 000 by sedimentation equilibrium analysis with a subunit molecular weight of 55 000 by sodium dodecyl sulfate gel electrophoresis and is thus probably a dimer of identical subunits. It contains approximately 1 mol of FAD and 1 g-atom of Zn^{2+} per mol of protein subunit, and the flavin exhibits a fluorescence 1.7 times that of free FAD. An earlier purification [Brockman, H. L., & Wood, W. A. (1975) *J. Bacteriol. 124*, 1454–1461] results in substantial loss of the enzyme's zinc, which is required for catalytic activity. The new purification yields greater than 5 times the amount of enzyme previously isolated. The enzyme is specific for D-lactate, and no inhibition is observed with L-lactate. Surprisingly, the enzyme has a significant oxidase activity, which depends on the ionic strength. V_{max} values of 190 and 530 min^{-1} were obtained at a $\Gamma/2$ of 0.224 and 0.442, respectively. Except for this atypically high oxygen reactivity, D-lactate dehydrogenase resembles other flavoenzyme dehydrogenases in that the flavin does not react with sulfite, the tryptophan content is low, and a neutral blue semiquinone is formed upon photochemical reduction. The enzyme flavin is reduced either by dithionite, by oxalate plus catalytic 5-deazaflavin in the presence of light, or by D-lactate. Two electrons per flavin were consumed in a dithionite titration, implying no other oxidation–reduction active groups. From equilibrations of the enzyme with varying ratios of D-lactate and pyruvate, an E_{m7} of -0.219 ± 0.007 V at 20 °C was calculated for the flavin. The enzyme requires dithiothreitol for stability. Rapid inactivtion results when the enzyme is incubated with a substoichiometric level of Cu^{2+}. This inactivation can be reversed by dithiothreitol. It is proposed that the enzyme possesses a pair of cysteine residues capable of facile disulfide formation.

Baldwin & Milligan (1964) first reported the purification of a pyridine nucleotide independent lactate dehydrogenase from the anaerobic rumen bacterium *Megasphaera elsdenii* (formerly known as *Peptostreptococcus elsdenii*). Brockman later purified the enzyme to near homogeneity and found it to be a flavoprotein, specific for the D isomer of lactate. Its metabolic role is to couple the oxidation of D-lactate to the reduction of short-chain α,β-unsaturated acyl-CoA's, two other flavoproteins serving as intermediary electron carriers (Brockman, 1971; Brockman & Wood, 1975). These flavoproteins are an electron-transferring flavoprotein (ETF)[1]

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; DTT, dithiothreitol; DCIP, 2,6-dichloroindophenol; TLC, thin-layer chromatography; BSA, bovine plasma albumin; EFl_{ox}, $EFlH\cdot$, and EFl_{red}, oxidized, one-electron reduced, and two-electron reduced forms of D-lactate dehydrogenase, respectively; ETF_{ox} and ETF_{red}, the oxidized and reduced forms of electron transferring flavoprotein; BDH_{ox} and BDH_{red}, the oxidized and reduced forms of butyryl-CoA dehydrogenase.

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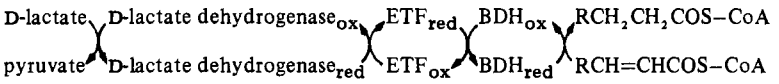
Scheme I



and a butyryl-CoA dehydrogenase (BDH), both of which have been purified to homogeneity and shown to be similar to the corresponding mammalian enzymes (Whitfield & Mayhew, 1974; Engel & Massey, 1971). The path of electron flow was deduced as shown in Scheme I.

The purified D-lactate dehydrogenase was inhibited by *o*-phenanthroline, the inhibition being reversible by addition of zinc (Brockman & Wood, 1975). It thus appeared to be similar to other pyridine nucleotide independent D-lactate dehydrogenases previously studied which also contained flavin and zinc (Gregolin & Singer, 1963; Nygaard, 1961; Cremona & Singer, 1966; Iwatsubo & Curdel, 1961).

We were initially interested in the enzyme after finding that a substrate analogue, α-hydroxybutynoate, covalently modified the flavin. This work is detailed in Olson et al. (1979) and Ghisla et al. (1979). Unfortunately, the published procedure for purification of the enzyme gave low yields and small amounts of enzyme, insufficient for structural characterization of the modified flavin. We subsequently discovered that zinc was removed from the enzyme during the purification. An improved purification was developed which now gives much greater quantities of enzyme.

Materials and Methods

Materials. FAD, NADH, NAD^+, sodium pyruvate, DL-lactate (Li salt), *Naja naja* venom phosphodiesterase, beef heart cytochrome *c* (Type V), and rabbit muscle L-lactate dehydrogenase were from Sigma Chemical Co. FAD was purified by DEAE-cellulose chromatography (Massey & Swoboda, 1963). Ultrapure guanidine hydrochloride was from Schwartz/Mann, D-lactate and L-lactate (both Li salts) and catalase (A grade) were from Calbiochem, and DCIP was from ICN. $NaDodSO_4$ and Chelex 100 (sodium form) were purchased from Bio-Rad, and acrylamide, bis(acrylamide), and Coomassie Blue were obtained from Eastman. Deoxyribonuclease (DNase) was from Worthington. Silica gel plates for TLC were from Brinkman Instruments, and Sephadex G-25 and Sephadex G-150 (medium grades) were from Pharmacia. DE-22 and CF1 cellulose came from Whatman, and ultrafiltration membranes and apparatus were from Amicon. Bovine plasma albumin was purchased from Armour Pharmaceuticals. Calcium phosphate gel was prepared by the method of Swingle & Tiselius (1951). Spectrographically pure zinc was from the Jarrell-Ash Co., Waltham, MA. Lumiflavin 3-acetate and 5-deazariboflavin were gifts from Dr. Peter Hemmerich. Superoxide dismutase (erythrocuprein) was isolated by the method of McCord & Fridovich (1969). All other chemicals were of the best grade commercially available.

Growth of Cells. Cells of *M. elsdenii*, strain LC1, were maintained and grown in iron-poor media as described by Mayhew & Massey (1969) with the following exceptions. Large-scale growth of cells was in a 200-L New Brunswick CF-250 Fermacell with the lactate concentration increased 1.5-fold. Cells were harvested in late log phase (cells grown for longer periods of time had lower quantities of enzyme), and the wet cell paste was stored at −20 °C.

Enzyme Assays. D-Lactate dehydrogenase couples the oxidation of D-lactate to the reduction of several artificial electron acceptors (Baldwin & Milligan, 1964; Brockman & Wood, 1975). A standard assay for the enzyme was adopted by using ferricyanide as the electron acceptor. The assay mixture consisted of 100 μmol of KP_i (KH_2PO_4/K_2HPO_4 = 0.4), 1 μmol of $K_3Fe(CN)_6$, 0.2 mg of BSA, and 200 μmol of lithium DL-lactate in a 1.0-mL volume (final pH 7.0). After incubation at 25 °C, the reaction was begun by addition of enzyme. Reduction of ferricyanide was measured by the loss in 420-nm absorption. One unit of activity is defined as the amount of enzyme giving an absorbance change of 1.0/min. An activity to flavin ratio (AFR) was defined as the ratio of units in this assay to absorbance at 454 nm. Inclusion of BSA was necessary for stability at low enzyme concentrations.

For a more sensitive assay, DCIP at 6×10^{-5} M was substituted for ferricyanide, and its reduction was monitored at 600 nm. Although a lower turnover number is observed with the DCIP assay, its 21-fold greater extinction (Loach, 1968) gives a greater rate of change in absorbance for the same amount of enzyme. If enzyme contained DTT, the assay mixture was preincubated without substrate, enzyme was added, and the nonenzymic oxidation of DTT by DCIP was followed to completion. Then the reaction was initiated with substrate.

Measurement of enzyme turnover using oxygen as an acceptor was made by either of two methods. First, by use of a coupled assay system, rates were measured with a stopped-flow apparatus. The stopped-flow apparatus is particularly useful for such measurements because of the ease of controlling O_2 concentration. For this method, the enzyme tonometer contained 2.20 μM D-lactate dehydrogenase, 0.2–0.3 mg/mL rabbit muscle L-lactate dehydrogenase, and 130–150 μM NADH in 0.1 M KP_i, pH 7.0, and was made anaerobic. Solutions of D-lactate in 0.1 M KP_i, pH 7.0, at varying concentrations were either air-equilibrated or purged with oxygen–nitrogen gas mixtures containing 10, 50, or 100% oxygen for several minutes. The reaction was followed by the decrease in A_{340} of NADH. A Corning CS7-54 filter was used to eliminate stray light and secondary scattering effects. Initial rates were unaffected by increasing the rabbit lactate dehydrogenase concentration, and concentrations of NADH as low as 40 μM were found to give the same initial rates as higher concentrations. This assured us that the rate of pyruvate production by D-lactate dehydrogenase was being measured. Rates were converted to turnover numbers by using ϵ_{340} = 6220 M^{-1} cm^{-1} for NADH (Horecker & Kornberg, 1948) and ϵ_{454} = 13 000 M^{-1} cm^{-1} at 3 °C for enzyme flavin determined as described under Results.

Alternatively, turnover numbers were measured with an oxygen electrode (Yellow Springs Instrument Co., Model 53). Assays were performed by preequilibrating 3.0 mL of 0.1 M KP_i, pH 7.0, 0.2 mg/mL BSA, and variable concentrations of D-lactate at 25 °C. The oxygen concentration was varied as described above by purging the solution for several minutes with different oxygen–nitrogen gas mixtures. Enzyme was then added with a Hamilton syringe to start the reaction. Excellent agreement was obtained between the two methods.

Protein Determination. Protein was determined by the micro-biuret method (Bailey, 1967) using BSA as the standard. The concentration of BSA was determined by using a value of 6.67 for $E_{279}^{1\%}$ (Janatova et al., 1968). Determination of the protein concentration of D-lactate dehydrogenase by amino acid analysis gave a value 94.1% of that obtained from the biuret analysis. Therefore, a factor of 0.941 was used to correct values obtained by the biuret method.

Flavin Constituent. For identification of the flavin coenzyme, the flavin was released from the enzyme by addition of Cl_3AcOH to 5% (w/v) final concentration. Cl_3AcOH was removed from the supernatant by extraction 3 times with a total of ~10 volumes of cold ether, and the pH was adjusted to between 6 and 7 with 0.1 M Na_2CO_3. The solution was taken to dryness under reduced pressure and then dissolved in a small volume of water for chromatography. Two solvent systems were used: 6% $Na_2HPO_4 \cdot 12H_2O$ in water and 1-butanol–acetic acid–water, 4:3:3, both run on silica gel plates (Kilgour et al., 1957; Fazekas & Kokai, 1971).

Analysis for Zinc. Zinc was analyzed on a Perkin-Elmer Model 306 atomic absorption spectrophotometer. Recommendations of Thiers (1957) were followed for preparing glassware free of contaminating metals. Standard zinc solutions were prepared by dissolving a weighed quantity of pure zinc metal in 0.1 N HCl and diluting to a known volume. For each analysis, several samples having the same protein concentration, but with varying amounts of added zinc, were analyzed to eliminate any effect of the protein. Correction was made for the small amount of zinc found in the enzyme buffer. For experiments with zinc-depleted enzyme, 1 M stock phosphate buffers were treated with Chelex 100 to remove trace quantities of Zn^{2+} before dilution with distilled, deionized water.

Electrophoresis. $NaDodSO_4$ gel electrophoresis was done according to Weber & Osborn (1969), except that staining was done for 30 min.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were performed in collaboration with Dr. Christopher Bull of The University of Michigan with a Spinco Model E ultracentrifuge using interference optics. The protein was dialyzed vs. 0.1 M KP_i, pH 6.0, plus 1 mM DTT for several days and then centrifuged at 18 000 rpm at 10.2 °C for 20 h at which time the interference pattern was photographed. After an additional 5 h, another photograph showed no change in the pattern, indicating equilibrium had been attained. Data were analyzed according to standard procedures (Chervenka, 1973).

Amino Acid Analysis. Amino acid analyses were performed by Dr. C. H. Williams, Jr., employing analytical methods similar to those previously described (Williams et al., 1967). Determination of tryptophan was done on separate samples either by magnetic circular dichroism (Holmquist & Vallee, 1973) using a Jasco J-41C spectropolarimeter equipped with a Jasco MCD-1B 15 000-G electromagnet or by comparing the protein fluorescence in 6 M guanidine hydrochloride to the fluorescence of known concentrations of tryptophan under the same conditions (Pajot, 1976).

Absorption and Fluorescence Measurements. Absorption spectra were measured with a Cary 17 or 118 recording spectrophotometer. Enzyme assays were done with a Gilford 2000 recording spectrophotometer. Fluorescence spectra were recorded with a ratio recording instrument build by Dr. David Ballou and Gordon Ford of this department, which is similar to one described earlier (Casola et al., 1966).

Anaerobic Experiments. Anaerobic spectral experiments were done using Thunberg-type cells equipped with two side arms. Anaerobiosis was achieved by at least 10 cycles of alternate evacuation using a water aspirator and filling with oxygen-free nitrogen [obtained by storage over Fieser's solution (Fieser, 1924)].

For dithionite titrations, a titration apparatus previously described (Foust et al., 1969) was used. Anaerobic conditions were achieved in this case by alternately evacuating with a vacuum pump and then admitting nitrogen freed of oxygen by passage over copper turnings at 450 °C.

Purification of D-*Lactate Dehydrogenase.* All operations were performed at 4 °C, and glass-distilled water was used to prepare all buffers. All buffers contained 1 mM DTT except where otherwise noted. In addition, buffers used for the initial cell suspension and DEAE-cellulose step contained 3×10^{-4} M EDTA. EDTA was omitted from all subsequent steps.

(*1*) *Extraction of Cells.* Frozen cells (300–500 gm) were thawed out overnight on ice and then suspended in a volume of 0.01 M KP_i, pH 6.0, approximately equal to the wet cell weight. DNase was added (~5 mg), and the mixture was passed 3 times through a Manton-Gaulin homogenizer (previously cooled with ice water) at 6000–9000 psi. The mixture was rapidly cooled by addition of ice after each passage. Cell debris was removed by centrifugation at 20000*g* for 30 min.

(*2*) *DEAE-Cellulose Chromotography.* The supernatant was applied to a ≃1-L DEAE-cellulose column (5 × 50 cm) equilibrated with 0.1 M KP_i, pH 6.0 (minus DTT). After loading, we washed the column with 4 L of 0.3 M KP_i, pH 6.0. The enzyme was then eluted with 0.6 M KP_i, pH 6.0. D-Lactate dehydrogenase elutes just before rubredoxin which is followed by butyryl-CoA dehydrogenase and finally flavodoxin. D-Lactate dehydrogenase is eluted in a sharp peak with a long tail.

The enzyme has no catalytic activity at this stage. Active fractions were located by supplementing the standard DCIP assay with $\sim 10^{-5}$ M $ZnCl_2$ and adding substrate last. The activity of the pooled enzyme-containing fractions was restored by addition of $ZnCl_2$ to 6×10^{-4} M final concentration. The recovery of activity is fast and requires no extended preincubation. Since addition of FAD gave significant increases in activity after dialysis (FAD reconstitution is slow at 4 °C; see Results), it was thought that some flavin loss had also occurred. Therefore, FAD was added equimolar with the estimated enzyme concentration. Additional DTT to 1 mM final concentration was also added as it was found that considerable loss of DTT had occurred at this stage.

The pooled fractions were dialyzed vs. 6 L of 10 mM K_2HPO_4 containing 0.5 mM DTT. The buffer was changed after about 8 h to 40 mM KP_i, pH 7.0, plus 0.5 mM DTT and dialyzed for at least 12 more h. During the dialysis a white precipitate formed which was removed by centrifugation. No activity is lost during dialysis, and the enzyme activity is not stimulated by Zn^{2+} or FAD at this stage.

(*3*) *Calcium Phosphate Gel–Cellulose Chromatography.* The calcium phosphate gel–cellulose column was prepared as described previously (Massey, 1960). After equilibrating with several liters of 0.05 M KP_i, pH 7.0 (minus DTT), the dialyzed enzyme was applied to the column and washed with about 500 mL of buffer, and then the protein was eluted with 0.1 M KP_i, pH 7.0. Fractions of 10 mL were collected. Green BDH precedes the yellow D-lactate dehydrogenase (also visible by its greenish yellow fluorescence). Fractions were assayed for activity in the standard ferricyanide assay and the visible absorption spectra taken. Typically, fractions with an activity to flavin ratio (AFR) of 60 or better were pooled. BDH is the major contaminating protein at this stage and is readily distinguished from D-lactate dehydrogenase by its unusual flavin absorption and a long wavelength band with a maximum at 710 nm (Engel & Massey, 1971).

(*4*) *Sephadex G-150 Gel Filtration.* The pooled fractions were concentrated to a volume of 10–20 mL by using an

Table I: Purification of D-Lactate Dehydrogenase

step	volume (mL)	total ferricyanide units	total protein (mg)	units/mg	AFR	yield (%)
crude[a]	585	1010[b]	19364	0.4[c]		
DEAE-cellulose	940	6148	1062	5.8		75[c]
calcium phosphate	183	4886	586	8.3	68	60
first Sephadex G-150	96	3821	nd[d]	–	101	47
second Sephadex G-150	92	3478	179	20.3[e]	108	42
third Sephadex G-150	98	2969	153	20.3[e]	107	36

[a] Starting material was 445 g, wet weight, of cell paste. [b] Variable inhibition of D-lactate dehydrogenase activity was found in the crude extract. [c] The recovery obtained when a second DEAE column was run in other purifications was taken as an estimate of the yield in the first step from which the true specific activity was calculated. [d] nd, not determined. [e] Protein concentration determined by the biuret method was corrected using a biuret coefficient of 0.941.

Amicon diaflo with a PM30 membrane. The enzyme was then applied to a 1200-mL column of Sephadex G-150 (3.7 × 120 cm) previously equilibrated with 0.1 M KP_i, pH 7.0, and eluted with the same buffer at a flow rate of about 0.4 mL/min. BDH elutes first, followed by D-lactate dehydrogenase. Fractions (5 mL) were assayed for activity in the standard ferricyanide assay, and their absorption spectra were recorded. Fractions with an AFR greater than 100 were pooled, concentrated as before on the diaflo, and reapplied to the column which was run as before. Fractions containing D-lactate dehydrogenase free of BDH were pooled, concentrated, and stored at −70 °C. The activity was stable to storage for at least 4 months at this temperature.

Although the protein is pure at this stage (see Results), variability in the AFR of isolated D-lactate dehydrogenase was observed. The range of AFR values was between 107 and 140, but most of the preparations exhibited values between 115 and 125. Enzyme with an AFR of at least 120 was used for experiments reported in this paper. A possible explanation for this variability is given under Discussion.

A summary of a typical purification is shown in Table I. In this particular case, a third gel filtration column was run, which gave no further improvement in specific activity. The ultraviolet to visible ratio (A_{274}/A_{454}) of the purified enzyme varied from 5.9 to 6.9. This could be accounted for by variable losses of a small amount of flavin during the purification (see Results). Final specific activities ranging between 20.3 and 24.5 units/mg were found, but, when normalized to an A_{274}/A_{454} ratio of 5.9 and an AFR of 140, the specific activity for fully active enzyme extrapolated to 27.7–28.3 units/mg.

Compared to the enzyme yield obtained by Brockman & Wood (1975), the present purification gives up to fivefold greater amounts of enzyme (based upon flavin content).

Results

Purity, Molecular Weight, and Subunit Composition. The purest preparations show only one protein band upon $NaDodSO_4$ gel electrophoresis in the presence of mercaptoethanol. The protein was also judged pure from the linear plot obtained from analysis of a high-speed sedimentation equilibrium experiment. The subunit molecular weight was determined by $NaDodSO_4$ gel electrophoresis using several standard proteins (Weber & Osborn, 1969). A value of 55 000 (range 54 000–56 000) was obtained from several experiments.

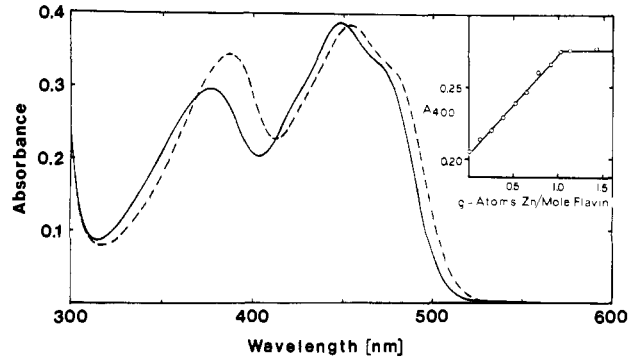


FIGURE 1: Absorption spectrum of zinc-depleted D-lactate dehydrogenase and the enzyme after titration with Zn^{2+}. Zinc-depleted enzyme was obtained by dialysis vs. 0.1 M EDTA in 0.1 M KP_i, pH 7.0, plus 1 mM DTT for 3 days. EDTA was removed by Sephadex G-25 gel filtration. (Trace amounts of zinc were removed from the buffer by Chelex 100.) Its spectrum (solid line) was recorded in 0.1 M KP_i, pH 7.0, plus 1 mM DTT at 10 °C. The inset shows the 400-nm absorption changes (corrected for dilution) upon titration of the zinc-depleted enzyme with 768 μM $ZnCl_2$. The dashed curve is the spectrum obtained (corrected for dilution) after addition of 1.42 equiv of $ZnCl_2$.

The molecular weight of the native protein was obtained from the sedimentation equilibrium experiment. From the partial specific volume of 0.742 calculated from the amino acid composition (Cohn & Edsall, 1943; McMeekin & Marshall, 1952) and the slope of the linear log fringe displacement vs. r^2 plot, a value of 105 000 was obtained. It is concluded, therefore, that D-lactate dehydrogenase is a dimer of two apparently identical subunits.

Identification of the Flavin Coenzyme. The visible absorption spectrum of the enzyme is characteristic of a flavoprotein (Figure 1). The flavin could be released quantitatively from the protein with 5% (w/v) Cl_3AcOH or by boiling.

The released flavin was confirmed to be FAD, first, from its identical migration with authentic FAD on TLC in two solvent systems[2] (see Materials and Methods) and, second, from an approximately 10-fold fluorescence enhancement upon adding snake venom phosphodiesterase (Bessey et al., 1949).

Analysis of the flavin content of several preparations of homogeneous enzyme gave a range of 0.84–0.96 mol/54 000 g of protein. Small losses of flavin were further indicated by the amino acid analysis. Therefore, the native enzyme contains one FAD per subunit or two FAD per dimer.

Absorption and Fluorescence Spectra. The absorption spectrum (Figure 1) shows maxima at 454, 386 and 274 nm with minima at 412 and 316 nm. Compared to free FAD, the visible peaks are red-shifted with a resolution of the long wavelength maximum to give a shoulder at about 470 nm. No absorption appears beyond 540 nm.

An extinction coefficient for protein-bound FAD was determined from the change in extinction of the flavin on release from the enzyme, by using ϵ_{450} = 11 300 M^{-1} cm^{-1} for free FAD (Whitby, 1953). An average value of 13 000 M^{-1} cm^{-1} was found at 3 °C. The extinction coefficient was observed to decrease by 270 M^{-1} cm^{-1} at 25 °C.

The flavin of D-lactate dehydrogenase is fluorescent with an emission maximum at 525 nm. The fluorescence intensity

[2] A few minor yellow spots having greenish yellow fluorescence were also detected in flavin released from the enzyme. These were shown to be chemically unique forms of flavin bound to D-lactate dehydrogenase from their reaction with the suicide substrate, α-hydroxybutynoate [see Olson et al. (1979)].

is 1.7 times that of free FAD at 3 °C (on a molar basis) and decreases by about 35% at 25 °C.

Requirement for Zinc. Brockman has reported that D-lactate dehydrogenase is inhibited by *o*-phenanthroline and can be reactivated by adding Zn^{2+} or Co^{2+}, zinc being the most effective. In analogy to other similar flavoprotein D-lactate dehydrogenases shown to contain zinc, it was concluded that the enzyme possesses an essential zinc cofactor (Brockman & Wood, 1975).

When enzyme isolated by slight modifications of Brockman's procedure was analyzed for zinc by atomic absorption spectrophotometry, substoichiometric quantities were found. One typical preparation was found to contain 0.22 g-atoms of Zn^{2+} per 54 000 g of protein, casting doubt on a possible role for zinc. Subsequently, it was found that purification of the enzyme with buffers including 3×10^{-4} M EDTA resulted in a complete loss of D-lactate dehydrogenase activity from the DEAE-cellulose column. When 10^{-5} M Zn^{2+} was added to assays, however, enzyme activity reappeared in those fractions where it was expected. Surprisingly, enzyme fractions showed activities several-fold higher than had ever been observed. This suggested that previous DEAE columns run without EDTA in the buffers had removed much of the enzyme's zinc. Indeed, greater than a twofold activity stimulation by zinc was observed in pooled enzyme fractions from a DEAE column run without EDTA in the buffers. After reconstitution with zinc and further purification to homogeneity, the enzyme was found to contain nearly 1 equiv of zinc per protein subunit. In several preparations the zinc content was found to vary from 0.98 to 1.15 g-atoms of zinc per mol of protein subunit.

To confirm the essential role of zinc, we attempted to remove it from purified enzyme by dialysis vs. EDTA. It was found that 3 days of dialysis vs. 200 volumes of 0.1 M EDTA in 0.1 M KP_i, pH 7.0, plus 1 mM DTT, with a buffer change every day, reduced the enzymic activity to 1% the initial value. Analysis of the resulting enzyme (after removal of EDTA by gel filtration with Sephadex G-25) showed a reduction in zinc content from 1.1 to 0.11 g-atoms per mol of flavin. Thus, loss of zinc is accompanied by loss of catalytic activity.

The spectrum of the zinc-depleted enzyme indicated that negligible flavin loss had occurred, but the visible absorption peaks of the flavin coenzyme had shifted to 449 and 377 nm with a significant decrease in the extinction of the shorter wavelength maximum (Figure 1). Addition of a stoichiometric amount of zinc caused an immediate change of the spectrum to that typical of the native enzyme (Figure 1), with nearly complete recovery of activity (94%). After passage once more through a Sephadex G-25 column to remove unbound zinc, the protein showed the expected stoichiometric binding (1.08 g-atoms/mol of FAD).

Another sample of the zinc-depleted enzyme was titrated with Zn^{2+} (Figure 1). A plot of the absorbance changes at several wavelengths vs. added zinc was linear up to the final stoichiometric end point, indicating a very tight binding ($K_d < 10^{-8}$ M) of the metal. The end point occurs after 1.03 g-atoms of Zn^{2+} has been added per mol of flavin.

Preparation of Deflavo-D-lactate Dehydrogenase. Flavin can be removed from the enzyme by dialysis vs. 2 M KBr buffered at either pH 5.5 or 8.5 (Massey & Curti, 1966). At pH 5.5, approximately 70% of the zinc is also removed while at pH 8.5 only about 15% loss of zinc is found (as judged from the difference between activity reconstituted with FAD plus zinc and FAD alone). The rate of reconstitution is time dependent, exhibiting first-order kinetics, and is independent of flavin concentration. The half-time for reconstitution measured at 12 °C was about 6 min. This is reminiscent of the behavior of the apoenzyme of D-amino-acid oxidase, where it was shown that reconstitution proceeds by an initial rapid binding of flavin, followed by a slow conformational change (Massey & Curti, 1966).

Table II: Amino Acid Composition of D-Lactate Dehydrogenase

amino acid	mol of residue per 54 000 g of protein[a]
aspartic acid	52.7
threonine[b]	23.8
serine[b]	19.6
glutamic acid	60.0
proline	19.0
glycine	40.7
alanine	35.9
valine[c]	57.6
methionine	18.3
isoleucine[c]	31.5
leucine	38.5
tyrosine	17.3
phenylalanine	11.2
lysine	37.8
histidine	7.3
arginine	12.2
half-cystine[d]	8.6
tryptophan[e]	2.9

[a] Each value represents the average of values obtained after 24, 48, and 72 h of hydrolysis, unless specified otherwise. [b] Extrapolated to zero time of hydrolysis. [c] Value obtained after 72 h of hydrolysis. [d] Determined on a separate sample by dimethyl sulfoxide oxidation (Spencer & Wold, 1969). [e] A value of 2.3 mol of residue per 54 000 g of protein was obtained from the magnetic circular dichroism spectrum and a value of 3.4 mol of residue per 54 000 g of protein was obtained by fluorescence (see Materials and Methods).

Amino Acid Analysis. The amino acid composition of the enzyme is shown in Table II. The results are expressed as residues per 54 000 molecular weight subunit (an average of the native and subunit molecular weights). Calculation of a minimum molecular weight based upon flavin content gave a value of 63 000. If 54 000 is the correct subunit molecular weight, 14% of the flavin should have been lost in this particular preparation. This was confirmed by flavin analysis (0.87 mol/54 000 g of protein) and from an ~10% stimulation of the catalytic activity upon adding FAD. Notable is the low tryptophan content which appears to be typical of flavoprotein dehydrogenases (Williams, 1976).

Specificity for D-Lactate and Lack of Inhibition by L-Lactate. It was confirmed that L-lactate is not a substrate for the enzyme. A study of stoichiometry showed the oxidation of only 0.34% of added L-lactate, which is presumably due to contaminating D-lactate.

The L isomer must bind extremely weakly, if at all, since no competitive inhibition could be observed. The presence of L-lactate was actually found to increase measured turnover rates in either the ferricyanide or oxygen electrode assay. Substitution of an equivalent concentration of KCl gave about the same enhancement of rate, implying this to be an ionic strength effect. Especially significant was that, even at 480 mM L-lactate or KCl with 20 mM D-lactate, the observed rates in a ferricyanide assay (1 mM ferricyanide and 0.1 M KP_i, pH 6.7, 25 °C) were the same, both being 1.4 times the rate with D-lactate alone.

Steady-State Kinetic Analysis. In addition to ferricyanide, 2,6-dichloroindophenol and oxygen are also good electron acceptors, ETF being the natural acceptor of reducing equivalents from the flavin (Brockman & Wood, 1975). In the case of oxygen, the product was identified as H_2O_2 by

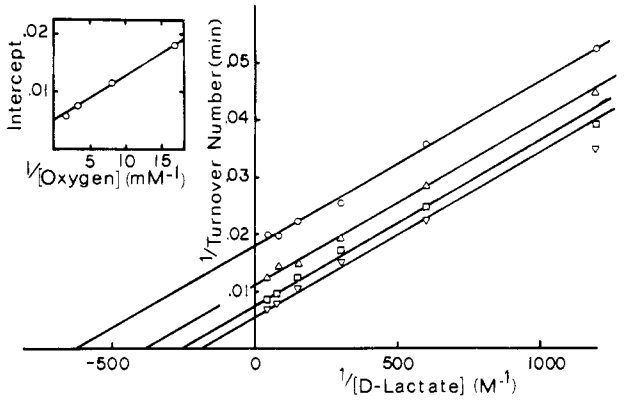


FIGURE 2: Lineweaver–Burk plots of steady-state turnover rates of D-lactate dehydrogenase at varying D-lactate and oxygen concentrations. Rates were measured at 25 °C in 0.1 M KP_i, pH 7.0, by using 1.10 μM D-lactate dehydrogenase. Pyruvate production was followed by coupling with rabbit muscle L-lactate dehydrogenase and NADH (see Materials and Methods for further details). Data were obtained at oxygen concentrations of 59.5 (○), 125 (△), 300 (□), and 595 μM (▽). Each point represents the average of at least three determinations. The inset shows a secondary plot of the intercepts vs. reciprocal oxygen concentration.

Table III: Kinetic Constants Obtained from a Steady-State Analysis of D-Lactate Dehydrogenase at Two Ionic Strengths

	Γ/2 = 0.224	Γ/2 = 0.442
K_{O_2}	1.5×10^{-4} M	6.9×10^{-4} M
$K_{D\text{-lactate}}$	5.4×10^{-3} M	1.5×10^{-2} M
V_{max}	190 min^{-1}	530 min^{-1}

observing a burst of oxygen upon addition of catalase to an enzyme reaction monitored by the oxygen electrode, followed by a decrease of the oxygen consumption rate to half its value before the catalase was added.

Steady-state analyses were done using oxygen as the acceptor at two different ionic strengths. The lower ionic strength study was done in 0.1 M KP_i, pH 7.0; the higher ionic strength study was conducted in the same buffer, but the ionic strength was increased by 0.2 by addition of L-lactate.[3]

Figure 2 shows the results obtained from the low ionic strength study. Lineweaver–Burk plots varying D-lactate at a constant oxygen concentration were linear over the entire concentration range, and the lines obtained at different oxygen concentrations were parallel. A secondary plot of the intercepts vs. reciprocal oxygen concentration (Figure 2 inset) was linear, the intercept giving a V_{max} of 190 min^{-1}. The kinetic constants obtained by analysis of the data are tabulated in Table III.

At the higher ionic strength, a set of parallel lines was also obtained when Lineweaver–Burk plots were made (data not shown). The kinetic constants are given in Table III. Comparison of the two sets of data shows that ionic strength increases V_{max} and K_m for both substrates. In fact, the enzyme becomes quite a good oxidase at the higher ionic strength with a V_{max} of 530 min^{-1}, representing a 2.8-fold rate enhancement. Also notable is that the K_m for oxygen increases 4.6 times while the K_m for D-lactate is raised only 2.8 times.

Some steady-state data were reported by Brockman & Wood (1975) using both ferricyanide and ETF as acceptors. Whereas nonlinear Lineweaver–Burk plots were observed when D-lactate was varied by using ferricyanide as the acceptor, such plots were linear when ETF was used as the acceptor.

[3] The amount of L-lactate added was adjusted so that the total concentration of D-lactate plus L-lactate was held constant at 0.2 M.

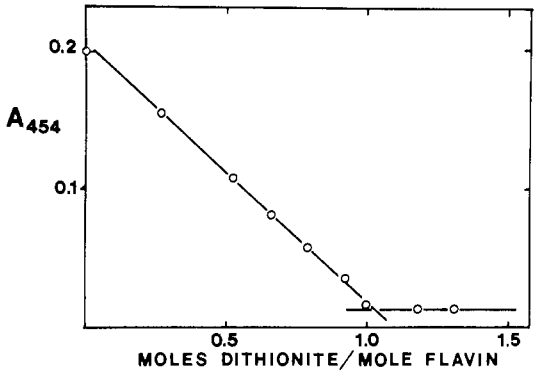


FIGURE 3: Anaerobic reduction of D-lactate dehydrogenase by sodium dithionite. Enzyme (15.3 μM) in 0.1 M KP_i, pH 7.0, plus 0.1 mM DTT was made anaerobic and titrated with 4.08 mM dithionite (standardized by titration with lumiflavin 3-acetate) at 3 °C. The absorbance at 454 nm (corrected for dilution) is plotted as a function of added dithionite.

Furthermore, with ferricyanide, an extremely large concentration of D-lactate was required to reach half of V_{max} (3.3 M) whereas ETF gave a more normal apparent K_m for D-lactate of 2.6×10^{-3} M (0.25 M KP_i, pH 7.0, 37 °C). These workers concluded that the enzyme exhibited negative cooperativity with ferricyanide.

In agreement with these results, we find that, when the D-lactate concentration was varied in the ferricyanide assay, biphasic Lineweaver–Burk plots of the type expected for negative cooperativity (Koshland, 1970) were obtained. Very high apparent K_m values for D-lactate were also observed with ferricyanide as the acceptor if, at D-lactate concentrations above 50 mM, no compensation for changes in the pH of the phosphate buffer or increases in the ionic strength was made. When the latter two variables were held constant, apparent K_m values on the order of 10^{-2} M were observed, although the biphasicity persisted.

It should be noted that since ferricyanide is an obligatory one-electron acceptor, the oxidative half-reaction with ferricyanide must occur in two steps with the flavin radical participating as an intermediate. With ETF and oxygen, however, direct two-electron transfers may be involved.

That a two-electron transfer is the favored path with oxygen as an acceptor was shown by turnover experiments in which cytochrome *c* in the presence and absence of superoxide dismutase was used to detect superoxide production. In the presence of a saturating level of cytochrome *c* in air-equilibrated buffer, D-lactate dehydrogenase was found to reduce cytochrome *c* at a rate equivalent to 19% of its rate of oxygen consumption. This rate was inhibited 85–87% by superoxide dismutase, demonstrating this to be due to superoxide production. Therefore, only 9.5% of the total peroxide formed in turnover arises by dismutation of superoxide.

Further experiments are clearly needed to determine whether the kinetic patterns observed are indeed correlated with the ability of the electron acceptor to take up one or two electrons.

Dithionite Reduction. The enzyme is rapidly reduced by dithionite. In a quantitative titration with a standardized dithionite solution, the enzyme spectrum was converted to the characteristic featureless spectrum of two-electron reduced flavin. An isosbestic point occurred at 342 nm and little, if any, intermediate semiquinone was detectable. In Figure 3, the 454-nm absorption is plotted vs. added dithionite. After reaction of a small amount of residual oxygen, the 454-nm absorption decreases linearly with added dithionite. The end of the titration is evident from the cessation of spectral changes

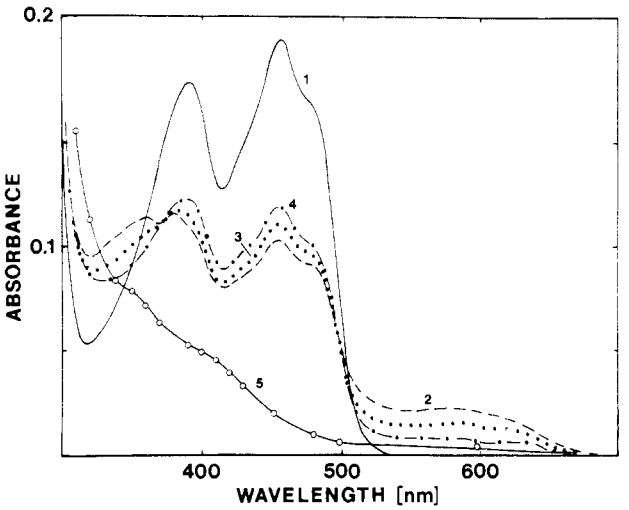


FIGURE 4: Photochemical reduction of D-lactate dehydrogenase by oxalate and catalytic 5-deazaflavin at 3 °C. Enzyme (15.3 μM), sodium oxalate (8.3 mM), and 5-deazariboflavin (0.8 μM) in 0.1 M KP_i, pH 7.0, were made anaerobic, and the spectrum was recorded (curve 1). The enzyme was irradiated for a 15-s interval which caused the 454-nm absorption to decrease by about half. Spectra were then recorded 2 (curve 2), 12 (curve 3), and 81 min (curve 4) after the light irradiation at a speed of 60 nm/min. Curve 5 was obtained from another experiment in which 39 μM enzyme was taken to full reduction by 105-s light irradiation (same light intensity and temperature) and normalized to the same enzyme concentration.

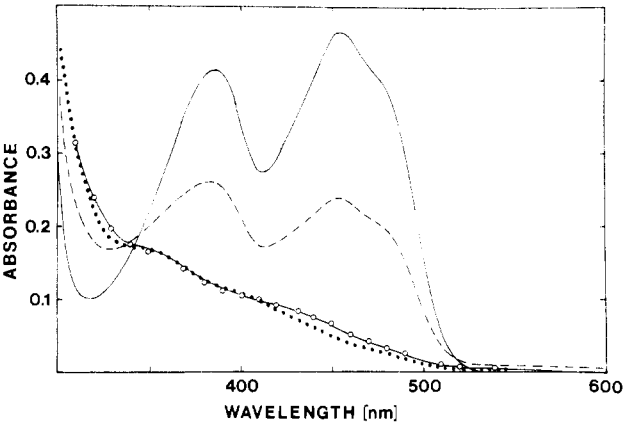


FIGURE 5: Anaerobic reduction of D-lactate dehydrogenase by D-lactate. Enzyme (36.5 μM) in 0.1 M KP_i, pH 7.0, plus 0.2 mM DTT was made anaerobic, and the spectrum was recorded at 20 °C (—). A final concentration of 450 μM D-lactate was mixed in, and, after changes in the 454-nm absorption had stopped (~50 min), a spectrum was recorded (- - -) (92% of the 454-nm bleaching occurred within 1 min after mixing in the D-lactate). Hydroxylamine (8.9 mM) was then mixed in. After 6 h, a spectrum was reached which did not change over the next 10 h (···). (O) shows the spectrum obtained in another experiment (normalized to the same enzyme concentration) in which 42 mM D-lactate was mixed with 14.3 μM enzyme anaerobically. Although 95% of the 454-nm bleaching occurred within 1 min after mixing, the remainder required ~2 h to complete.

above 350 nm and the appearance of the characteristic absorbance of unreacted dithionite (λ_{max} 314 nm). After full reduction, the characteristic fluorescence of the oxidized flavin disappears. When the solution is then exposed to air, complete return of the original spectrum is observed. Using an extinction coefficient of 13 000 M^{-1} cm^{-1} at 3 °C and the experimentally determined dithionite concentration, we calculated that 1.00 mol of dithionite was consumed per mol of flavin. Thus, flavin is the only redox active species present in the isolated enzyme.

Photochemical Reduction. The enzyme is also reduced by EDTA in the presence of light and catalytic amounts of 5-deazaflavin as has been shown for other flavoproteins (Massey & Hemmerich, 1977, 1978). However, EDTA produces complications by removing some of the enzyme's zinc. Oxalate was found to be much better suited as a reductant, being a much weaker metal chelator (Bard, 1966) and giving only carbon dioxide as the oxidation product (Halliwell, 1972). In contrast to the dithionite reduction, the light reduction method results in the appearance of a long wavelength absorbing intermediate. This is clearly shown in the experiment of Figure 4 where enzyme was irradiated with light under anaerobic conditions to give about 50% loss in the 454-nm absorption. (The initial oxidized spectrum shows perturbations due to the binding of oxalate.) The spectrum revealed a long wavelength absorbing band beyond 500 nm, characteristic of a blue neutral semiquinone (Massey & Palmer, 1966). Subsequently, this band decayed with concomitant increases in the 454-nm band. Further light irradiation produced a fully reduced flavin spectrum indistinguishable from that obtained by dithionite reduction. Again, the original oxidized flavin spectrum was fully recovered upon admission of air. In other experiments in which several short light pulses were used to take the enzyme to complete reduction, such a burst in radical absorption, followed by its decay, was observed after each interval of light irradiation.

These results show that, in the case of D-lactate dehydrogenase, a radical is produced, but then it slowly decays. This decay is most likely due to a significant rate of disproportionation:

$$2EFlH\cdot \rightleftarrows EFl_{ox} + EFl_{red}$$

Thus, the appearance of radical would reflect a kinetic rather than a thermodynamic stabilization of the one-electron reduced species.

With dithionite reduction, little detectable semiquinone was seen. Since dithionite is a one-electron reductant (Mayhew & Massey, 1973; Lambeth & Palmer, 1973), the rate of reduction of oxidized enzyme to radical must be comparable to that of reduction of radical to fully reduced enzyme.

The kinetic stabilization of the semiquinoid species during photochemical reduction was found to depend on pH. When carried out at pH 8.5, no intermediate radical was observed. To check for thermodynamic stabilization at this pH, photoreduction was carried out to 50% reduction, and then the spectrum was monitored for appearance of radical via comproportionation of oxidized and reduced enzyme. No detectable radical was observed over a period of 2 days.

Substrate Reduction. D-Lactate dehydrogenase can also be reduced anaerobically by its substrate, D-lactate. To obtain complete reduction, however, we found that a large excess (~40 mM) of D-lactate is required (Figure 5). At lower concentrations of D-lactate, incomplete reduction is observed even though the substrate concentration is in excess of that of the enzyme. For example, 39 and 60% reduction of ~15 μM enzyme was observed with a 6.4- and 30-fold excess of D-lactate, respectively. This behavior suggested the flavin potential was unfavorable for reduction by low levels of D-lactate, resulting in a redox equilibrium between flavin and substrate. That this was indeed the case was shown by the complete reduction of the enzyme on the subsequent addition of carbonyl trapping agents such as hydroxylamine (Figure 5).

The existence of an equilibrium between flavin and substrate provided a means of determining the oxidation–reduction potential of the enzyme flavin. Enzyme was equilibrated anaerobically at 20 °C and pH 7.0 with varying ratios of D-lactate to pyruvate. From the spectrum, the amount of

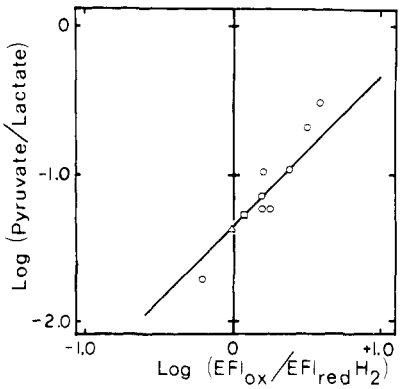


FIGURE 6: Analysis of anaerobic equilibrations of D-lactate dehydrogenase with varying ratios of D-lactate to pyruvate by the Nernst equation. All experiments were performed in 0.1 M KP_i, pH 7.0, plus 0.1–0.2 mM DTT at 20 °C. The log of the pyruvate to D-lactate ratio is plotted as a function of the log of the ratio of oxidized to reduced enzyme. Circles represent data obtained at enzyme concentrations between 14 and 15 μM. The triangle and square are data points obtained by using enzyme concentrations of 36 and 6.5 μM, respectively.

oxidized and reduced enzyme was obtained, and an equilibrium constant could be calculated for this temperature and pH:

$$K_{eq} = \frac{[\text{D-lactate}]_{eq}[EFl_{ox}]_{eq}}{[\text{pyruvate}]_{eq}[EFl_{red}]_{eq}}$$

Using the Nernst equation, we could calculate the separation between the potential of flavin and that of substrate (relative to the standard hydrogen electrode) for the two electron transfer:

$$\Delta E_{m7} = 0.0291 \log K_{eq}$$

Rearrangement of the above equation gives

$$\log \frac{[\text{pyruvate}]_{eq}}{[\text{D-lactate}]_{eq}} = \frac{-\Delta E_{m7}}{0.0291} + \log \frac{[EFl_{ox}]_{eq}}{[EFl_{red}]_{eq}}$$

Thus, a plot of log ($[\text{pyruvate}]_{eq}/[\text{D-lactate}]_{eq}$) vs. log ($[EFl_{ox}]_{eq}/[EFl_{red}]_{eq}$) should be a straight line with unit slope and a y-axis intercept of $-\Delta E_{m7}/0.0291$. The data obtained from several experiments were plotted in this manner and are shown in Figure 6. The circles represent experiments conducted at enzyme concentrations between 14 and 15 μM. An average potential separation of +0.039 V was determined from these experiments. The line drawn through the points is the theoretical line for a ΔE_{m7} = +0.039 V.[4]

Since the potential can be shifted by a differential binding affinity of substrate and product, concentrations of D-lactate and pyruvate no greater than 1 and 0.1 mM, respectively, were used. (Preliminary stopped-flow experiments studying the reductive half-reaction indicate a K_d for D-lactate on the order of 10^{-2} M.) That binding is not affecting the determined potential is supported by the experiments performed at different enzyme concentrations. The results of two such experiments, performed at 6.5 (square) and 36 μM (triangle), are shown in Figure 6. Both gave values within 1 mV of the Δ 0.039-V average.

[4] It should be pointed out that the slope of Figure 6 gives no information about the number of electrons involved; since the n value is contained in the intercept term, the slope must always be 1 if the data follow the Nernst equation. The deviation from unit slope obvious in Figure 6 is presumably due to errors inherent to small contamination with O_2 which, at low pyruvate concentrations, can affect markedly the calculated [pyruvate]/[lactate] ratio.

Table IV: Inhibition of D-Lactate Dehydrogenase by Cu^{2+} [a]

mol of Cu^{2+} per mol of FAD	time after adding Cu^{2+} (min)	% initial act.
10	13.5	6.2
	390	7.4
2.0	1	6.9
	4.5	7.7
1.0	1	34
	3	20
	20	9.3
0.5	90	22
	180	12
	500	5.8

[a] To 9–45 μM enzyme was added $CuSO_4$ at the levels indicated, and the mixture was incubated at 0 °C. Activity was measured before Cu^{2+} addition and at varying intervals afterward by the standard ferricyanide assay.

The flavin potential is readily obtained given the substrate potential. Literature values of E_{m7} varying from −0.176 to −0.193 V for the lactate/pyruvate couple at 25 °C are given by Clark (1960). A determination of this potential by equilibrating L-lactate with NAD^+ by using rabbit muscle L-lactate dehydrogenase as the catalyst gave a value of −0.186 V at 25 °C. The ΔE_{m7} at 25 and 20 °C for this latter system was found to be 0.140 V, and from the temperature dependence of the NAD^+/NADH couple given by Clark (1960), the potential of the lactate/pyruvate couple at 20 °C was calculated to be −0.180 V. From the +0.039-V average potential separation determined above between flavin and substrate, a potential of −0.219 ± 0.007 V is calculated for the flavin at 20 °C, pH 7.0.

Reduction of the enzyme by D-lactate is predominantly rapid. When reduced with ~40 mM D-lactate, 95% of the 454-nm absorption bleaching occurs within seconds after mixing in the substrate even at 3 °C. The remaining 5% takes a couple of hours to complete. As discussed earlier, removal of zinc from the enzyme causes a loss in its catalytic activity. It was thus of interest to see whether D-lactate could reduce the flavin of zinc-depleted enzyme. When a large excess of D-lactate (~40 mM) was mixed with the anaerobic metal-depleted enzyme at 3 °C, a slow bleaching of the 450-nm absorption took place. After 3 h, about 50% reduction had occurred while 80–90% reduction was reached by 20 h.

Sulfite Reactivity. D-Lactate dehydrogenase did not undergo any spectral changes upon addition of sulfite at concentrations as high as 0.13 M. Sulfite does react with most flavoprotein oxidases to give an adduct at the N(5) position of the isoalloxazine nucleus with a spectrum resembling that of 1,5-dihydroflavin. The lack of sulfite reaction with D-lactate dehydrogenase is consistent with the previous correlation between sulfite reactivity and stabilization by the protein of the red flavin anionic semiquinone (Massey et al., 1969).

Lability of Enzyme Thiols to Oxidation. We confirmed a report that DTT prevented activity losses from occurring during purification of D-lactate dehydrogenase (Brockman & Wood, 1975). When DTT, present during the purification, was removed by passage through a Sephadex G-25 column, activity was gradually lost on storage at 4 °C. For example, 84, 65, and 28% residual activity was found after 2, 5, and 13 days, respectively, of storage at 4 °C while enzyme containing 1 mM DTT was stable over this time. Such activity losses could be partially restored by incubation with DTT.

These results suggested that the enzyme might possess one or more thiol residues which are sensitive to oxidation by contaminating trace metals, e.g., cupric ions (Casola et al.,

1966). Indeed, rapid inactivation of the enzyme was observed even with a substoichiometric level of Cu^{2+} (Table IV). A residual activity of 6–8% persisted even with prolonged incubation with excess Cu^{2+} and after Sephadex G-25 gel filtration to remove the copper. Additon of 2 mM DTT to the copper-treated enzyme and incubation at 0 °C gave about 50% recovery of lost activity after 2 h and complete restoration of native activity after 1 day. The ability to reactivate the copper-treated enzyme with DTT was gradually lost with storage at 0 °C. After 1, 2, and 3 days of storage, 92, 68, and 58% recovery, respectively, of the native activity was found upon incubation with 2 mM DTT for 1 day at 0 °C.

Discussion

A pyridine nucleotide independent lactate dehydrogenase catalyzing specifically the oxidation of D-lactate to pyruvate has been purified to homogeneity and shown to contain approximately 1 mol each of FAD and zinc per mol of protein subunit. The native enzyme is a dimer with a molecular weight of about 105 000 and has apparently identical subunits.

This flavoenzyme shares several properties with other flavin-dependent D-α-hydroxyacid dehydrogenases studied previously. Those best characterized include two enzymes from yeast: a D-α-hydroxyacid dehydrogenase present under anaerobic growth conditions (Iwatsubo & Curdel, 1961; Cremona & Singer, 1966; Slonimski & Tysarowski, 1958) and a D-lactate–cytochrome *c* reductase appearing under aerobic growth conditions (Gregolin & Singer, 1963; Nygaard, 1961). Both are FAD-containing flavoenzymes, the former containing 2–3 zinc atoms per flavin, and the latter also containing an essential metal believed to be zinc. A D-α-hydroxyacid dehydrogenase from rabbit kidney mitochondria has also been shown to contain FAD and an essential metal (Tubbs, 1960, 1962; Tubbs & Greville, 1959; Cammack, 1969). The membrane-bound D-lactate dehydrogenase from *Escherichia coli* which couples the oxidation of D-lactate to the transport of a wide variety of metabolites is also an FAD-containing flavoenzyme but differs in that it oxidizes both D and L isomers of lactate (Kohn & Kaback, 1973; Futai, 1973). No requirement for metal has been reported for this enzyme. Several other incompletely characterized pyridine nucleotide independent D-lactate dehydrogenases have been reported and are thought to contain flavin (Snoswell, 1966).

It seems clear from the present work that Brockman's purification of this enzyme resulted in substantial losses of zinc. This is evident from the similarity of his reported spectrum (λ_{max} 450 nm, 378) to that of zinc-depleted enzyme (Figure 1). Furthermore, with his enzyme preparation, only about 50% reduction of the flavin occurred in 10 min with 0.4 M D-lactate (Brockman & Wood, 1975), compared to the nearly quantitative reduction obtained over the same time with ~40 mM D-lactate with the present enzyme.

The isolation of smaller amounts of enzyme by the Brockman procedure must be at least partially attributable to the removal of zinc by DEAE-cellulose. Brockman used a gradient elution from 0.3 to 0.8 M KP_i in the DEAE-cellulose chromatography step (Brockman & Wood, 1975). When this elution procedure was used by us, a small peak of activity eluting between 0.45 and 0.6 M KP_i was observed. When assays were supplemented with zinc, however, the activity peak increased several-fold and was spread out over nearly the entire gradient. Therefore, it is believed that in earlier work much of the enzyme was discarded at this stage. The levels of enzyme estimated to be present in the bacterium are about equivalent to the amount of ETF present (Whitfield & Mayhew, 1974).

The role of zinc in this enzyme is unknown, but its absolute requirement for catalytic activity has been shown in this work. In contrast to the yeast D-lactate dehydrogenases which were inhibited by metal chelators via binding and not actual metal removal (Ghiretti-Magaldi et al., 1961), EDTA inhibits the *M. elsdenii* enzyme by removing the zinc. For the yeast D-α-hydroxyacid dehydrogenase, it has been shown that no reduction of flavin by D-lactate occurs with the EDTA-treated, dialyzed enzyme but that rapid flavin reduction occurs upon addition of zinc (Ghiretti-Magaldi et al., 1961). With the *M. elsdenii* enzyme, zinc-depleted enzyme is reduced by D-lactate, but extremely slowly. This suggests that zinc is required for rapid reduction of the flavin. Whether zinc plays a structural role or is more intimately involved in catalysis as has been found with the zinc-containing alcohol dehydrogenases remains to be determined (Shore & Santiago, 1975).

A steady-state analysis of the enzyme using oxygen as an acceptor revealed an unusually high turnover number for a dehydrogenase. Typically, reduced flavoprotein dehydrogenases react with oxygen at rates of about 1 min^{-1} (Massey et al., 1969). The rate of reaction of reduced D-lactate dehydrogenase with oxygen must be at least as fast as V_{max}. This implies minimal rates of 190 or 530 min^{-1}, depending upon the ionic strength. Surprisingly, these rates lie in the neighborhood of reactivity of glycollate oxidase, reported to react with oxygen at a rate of 620 min^{-1} (Dickenson, 1965). This might argue that oxygen could be a physiologically relevant acceptor in normal turnover. This is improbable, however, since the organism is a strict anaerobe. Whereas dehydrogenases found in aerobic organisms may have had to evolve ways of depressing oxygen reactivity to prevent short circuiting of their electron-transfer reactions, in the case of the *M. elsdenii* enzyme, such an evolutionary change was unnecessary. Thus, it is probable that the high oxygen reactivity for this enzyme is fortuitous.

A minimal mechanism for D-lactate dehydrogenase with oxygen as the acceptor must involve both a reductive half-reaction in which the flavin gets reduced by substrate and an oxidative half-reaction in which oxygen reoxidizes the flavin to complete the catalytic cycle. The set of parallel Lineweaver–Burk plots (Figure 2) is typical of a ping-pong mechanism, in which the product, pyruvate, would be required to dissociate from the reduced enzyme prior to the reaction with O_2. However, in the case of two flavoprotein oxidases, D-amino-acid oxidase (Massey & Gibson, 1964; Porter et al., 1977) and L-lactate oxidase (Lockridge et al., 1972), even though the steady-state analysis yielded parallel Lineweaver–Burk plots, it was shown by stopped-flow studies that O_2 reoxidized the reduced enzyme while the product was still bound. Similar stopped-flow studies are planned for D-lactate dehydrogenase.

The enzyme appears to be quite sensitive to the oxidation of some cysteine residue(s) which affect the catalytic activity. A substoichiometric amount of cupric ions rapidly reduces the catalytic activity to a level of about 7% of the native activity. The lost activity is regained by incubation with DTT. Similarly, enzyme incubated without DTT slowly loses activity which can be partially restored by DTT. Since the inactivation occurs over a much longer period of time in the latter case, a greater lability to denaturation of the inactivated enzyme might preclude full reactivation by DTT. The copper-treated enzyme was, in fact, found to gradually lose its ability to convert back to the native activity level upon storage at 0 °C. These findings are reminiscent of another flavoenzyme, pig heart lipoamide dehydrogenase, which is also inactivated by

Cu^{2+} to give an enzyme with only 5–10% of the native activity. This was shown to be a result of a catalyzed oxidation of two neighboring cysteine residues on the protein to produce a disulfide bond. Cysteine could reverse this process (Casola et al., 1966; Thorpe & Williams, 1975). Thus, a logical hypothesis consistent with these data is that D-lactate dehydrogenase possesses a pair of neighboring cysteine residues which can readily oxidize to form a disulfide bond, thereby decreasing the enzyme's catalytic capacity. Such a hypothesis could provide a reasonable explanation for the variable AFR of the isolated enzyme. Typically, enzyme with an AFR of 115–125 is isolated, but a range of 107–140 has been observed over the course of several preparations. This variability might arise from different levels of the disulfide form of the enzyme in the preparations which must have undergone subsequent changes such that DTT will no longer restore its native state. This disulfide form of the enzyme could be present before cell breakage or else have formed during DEAE-cellulose chromatography due to the substantial loss of DTT that occurs in this step (see Materials and Methods). Work is now in progress to correlate the thiol content of the enzyme with its AFR value.

Acknowledgments

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Suicide Inactivation of the Flavoenzyme D-Lactate Dehydrogenase by α-Hydroxybutynoate†

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ABSTRACT: The acetylenic α-hydroxy acid 2-hydroxy-3-butynoate (αHB) is a substrate and an irreversible inactivator of the FAD-containing flavoenzyme D-lactate dehydrogenase from *Megasphaera elsdenii*. On the average, the enzyme undergoes five catalytic turnovers with αHB in air at pH 7.0 before being inactivated. Irreversible inactivation is due to the conversion of the flavin to a pink adduct with visible absorption peaks at 522, 382, and 330 nm and weak fluorescence with an emission maximum at 635 nm. The adduct is stable and can be released from the enzyme and purified. It retains a structure analogous to FAD since it binds to the FAD-specific apo-D-amino acid oxidase. It can be further converted to an FMN analogue with phosphodiesterase which binds to the FMN-specific apoflavodoxin. Experiments were conducted to test whether inactivation was initiated by an αHB allene carbanion or the dehydrogenation product of αHB. Kinetic studies proved inconclusive in that a rapid equilibrium between an oxidized enzyme–allene carbanion pair and reduced enzyme–keto acid pair would make these two species kinetically equivalent. The olefinic substrate 2-hydroxy-3-butenoate, however, produced no flavin adduct. Since the keto acid derived from the oxidation of this α-hydroxy acid is expected to be as reactive as 2-keto-3-butynoate, it is concluded that an allene carbanion produced by abstraction of the α-hydrogen of αHB is the reactive species which covalently adds to the flavin.

Acetylenic suicide substrates were first used by Bloch and his co-workers, who found that β-hydroxydecanoylthioester dehydrase was irreversibly inactivated by a β-acetylenic substrate analogue (Bloch, 1971). This inactivation was shown to result from an enzyme-catalyzed rearrangement of the acetylenic function to a reactive allene, which then alkylated an active-site histidine. Since the enzyme had caused its own destruction, the acetylenic substrate was appropriately called a suicide substrate.

Since then, several other enzymes have been demonstrated to be irreversibly inactivated by acetylenic suicide substrates in a like manner (Walsh, 1977). Such studies have provided indirect evidence for carbanionic intermediates in the normal enzyme-catalyzed reactions. Thus, the formation of a carbanion adjacent to an acetylenic function would be expected to promote the rearrangement to a reactive allene which can then serve as an active-site alkylating agent.

In the case of several flavin enzymes which catalyze dehydrogenation reactions, acetylenic substrates were also found to cause irreversible inactivation. Instead of alkylating the protein, however, these suicide inhibitors were found to covalently attach to the flavin coenzyme. A few cases were found in which alkylation of the protein by the acetylenic suicide substrate occurred (Walsh, 1977, 1978).

Two different types of covalent suicide inhibitor–flavin adducts have been described. With monoamine oxidase, covalent addition at position N(5) occurred (Maycock et al., 1976), and with the L-specific lactate oxidase, cyclic addition to N(5) and C(4a) of the flavin nucleus was found (Walsh et al., 1972; Ghisla et al., 1976; Schonbrunn et al., 1976). Since these positions of the flavin coenzyme are electrophilic in the oxidized state, an attack of an electrophilic allene is considered unlikely. Either an allene carbanion or the oxidized acetylenic substrate is considered a potential inactivating species (Walsh, 1977, 1978). Studies of these suicide reactions have thus focused on discriminating between these two possible inactivation pathways with the intent of discerning the involvement of carbanions in these flavoenzymes.

The flavoenzyme D-lactate dehydrogenase from the anaerobic bacterium *Megasphaera elsdenii* (formerly known as *Peptostreptococcus elsdenii*) catalyzes the oxidation of D-lactate to pyruvate, an electron transferring flavoprotein (ETF) serving as the natural electron acceptor (Brockman, 1971; Brockman & Wood, 1975). The enzyme has been purified to homogeneity and shown to be specific for the D isomer of lactate and to contain 1 equiv each of FAD and zinc per mol of protein subunit (Olson & Massey, 1979).

The production of a stereochemically precise N(5)–C(4a) cyclic flavin adduct upon reaction of α-hydroxybutynoate (αHB)[1] with the L-specific lactate oxidase (Walsh et al., 1972; Schonbrunn et al., 1976) prompted the investigation of the reaction of αHB with the D-specific lactate dehydrogenase.

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[1] Abbreviations used: $ApAD^+$, 3-acetylpyridine adenine dinucleotide, oxidized form; DCIP, 2,6-dichloroindophenol; Cl_3AcOH, trichloroacetic acid; DTT, dithiothreitol; TLC, thin-layer chromatography; ETF, electron transfer flavoprotein; LDH, lactate dehydrogenase (pyridine nucleotide dependent); DAAO, D-amino acid oxidase; αHB, 2-hydroxy-3-butynoate; αKB, 2-keto-3-butynoate.

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