Metal Binding to D-Lactate Dehydrogenase†

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ABSTRACT: The kinetic and spectral properties of native and cobalt-substituted D-lactate dehydrogenase have been compared. Optical and MCD spectra are consistent with high-spin tetrahedral cobalt. The cobalt enzyme is strongly inhibited by D-lactate when oxygen is the electron acceptor. This is probably due to the formation of an abortive complex of reduced enzyme and lactate. Like the native zinc enzyme, the cobalt-substituted enzyme is reduced by substrate in a triphasic fashion involving a transient charge-transfer complex. The kinetics of the cobalt enzyme suggest that the metal is involved in substrate binding and reduction. A second metal-binding site is also described. Evidence is presented to suggest that the flavin cofactor and an essential thiol are involved in this site.

Zinc is one of the more widely found transition metals in enzyme systems, and its various roles have been subject to extensive investigation [for reviews, see Friedberg (1974) and Coleman (1971)]. The D-lactate dehydrogenases from various bacterial and mammalian sources are the only flavoproteins yet reported to contain zinc (Stachiewicz et al., 1961; Ghiretti-Magaldi et al., 1961; Cremona, 1964). Olson & Massey (1979) have purified the enzyme from the anaerobe *Megasphera elsdenii* and selectively removed the catalytic zinc by dialysis against ethylenediaminetetraacetic acid (EDTA). D-Lactate dehydrogenase is a dimer of 55 000 subunit molecular weight with one noncovalently bound FAD and one catalytically essential zinc ion per protomer. After removal of the Zn^{2+}, all the enzyme activity is lost, but it is recoverable by addition of 1 equiv of $ZnCl_2$.

In this study we report how the properties of D-lactate dehydrogenase are altered when zinc-depleted enzyme is reconstituted with cobalt. This is an approach to studying zinc-containing enzymes which has been used to great effect by other workers. The unique value of cobalt lies in that it is paramagnetic and has distinctive optical and magnetic circular dichroism (MCD) properties (e.g., Holmquist & Vallee, 1978; Fee, 1973; Lingskog & Nyman, 1964; Latt & Vallee, 1971).

Brockman & Wood (1975) had previously reported that D-lactate dehydrogenase from *M. elsdenii* may be reactivated by cobalt after incubation with metal chelating agents. Similar reactivation had also previously been seen with the D-lactate dehydrogenase from yeast (Curdel & Labeyrie, 1961; Stachiewicz et al., 1961).

We also give evidence for more than one metal-binding site in *M. elsdenii* D-lactate dehydrogenase.

Experimental Procedures

Materials

All solutions were prepared in glass-distilled water which had been previously deionized by passage through an I.W.T. ion exchanger, Illinois Water Treatment Co., Rockford, IL.

Glassware used was left to soak for at least 12 h in 10% nitric acid and then rinsed thoroughly in distilled deionized water before use.

Enzyme. D-Lactate dehydrogenase was prepared and assayed with potassium ferricyanide as previously described by Olson & Massey (1979).

Metal Salts. All metal salts were of the highest grade commercially available. $MnCl_2$, $MgCl_2$, and $CoCl_2$ were from M.C.B., $NiCl_2$ and $CdCl_2$ were from Aldrich Chemical Co., $ZnCl_2$ was from Mallinckrodt, and $AgNO_3$ was from Allied Chemicals while $CuCl_2$ was a product of the General Chemical Company of New York.

Methods

Zinc-Free Enzyme. Enzyme free from zinc was prepared by a modification of the method of Olson & Massey (1979). The enzyme was dialyzed against 0.1 M EDTA at pH 6 in 0.1 M potassium phosphate buffer plus 1 mM dithiothreitol (DTT), rather than at pH 7. This greatly facilitated the loss of zinc from the enzyme.

Absorption and Fluorescence Measurements. Absorption spectra were taken with Cary 17, 118, or 219 recording spectrophotometers. Fluorescence spectra were obtained with a ratio-recording instrument similar to one described earlier (Casola et al., 1966).

Anaerobic Experiments. These were performed as described in Olson & Massey (1979). When flavin semiquinone was formed by using 5-deazariboflavin (Massey & Hemmerich, 1978), either 10 mM oxalate or 0.1 M glycine was used as the electron donor.

Magnetic Circular Dichroism. Magnetic circular dichroism measurements were performed with a Jasco J-41E instrument. All spectra were recorded at 9 °C in 0.1 M potassium phosphate buffer, pH 7.

Results

Cobalt-Substituted D-Lactate Dehydrogenase. (1) Spectral Studies. When Co^{2+} is added to the zinc-free enzyme, similar spectral changes are found to those seen on reconstituting the enzyme with zinc (Olson & Massey, 1979; Morpeth & Massey, 1982a), as shown in Figure 1. A plot of the absorbance change at 400 nm vs. added Co^{2+} showed a sharp end point at 1 atom of Co^{2+}/equiv of enzyme-bound flavin. The zinc-free enzyme spectrum had maxima at 447 and 373 nm. When reconstituted with zinc, the enzyme spectrum showed maxima at 454 and 386 nm, identical with those of the native enzyme. The cobalt enzyme had a similar spectrum with maxima at 452 and 384 nm plus a new peak with a maximum at 550 nm. This long wavelength peak is characteristic of high-spin cobalt and may be clearly seen in Figure

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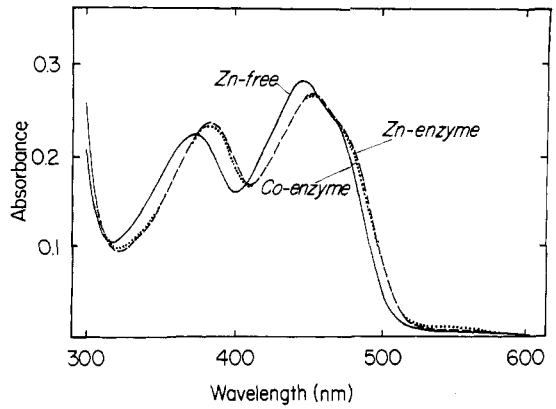

FIGURE 1: Absorption spectra of zinc-depleted D-lactate dehydrogenase and the enzyme after titration with Zn^{2+} or Co^{2+}. The spectra were recorded in 0.1 M KP_i, pH 7, at 25 °C. (—) Zinc-free enzyme; (---) after addition of 1.08 equiv of $ZnCl_2$; (···) after addition of 1.14 equiv of $CoCl_2$ instead of $ZnCl_2$.

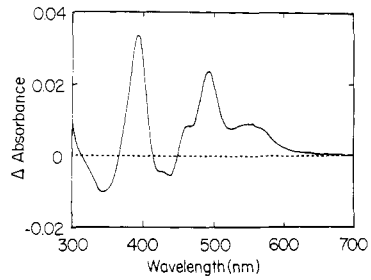

FIGURE 2: Difference spectrum between zinc-depleted enzyme reconstituted with Zn^{2+} vs. the same enzyme reconstituted with Co^{2+}. Solutions of zinc-free D-lactate dehydrogenase (59 μM) in dual compartment silica cells were titrated with $CoCl_2$ and $ZnCl_2$ to give maximal difference spectra in 0.1 M potassium phosphate buffer, pH 7, at 4 °C.

2, which shows a difference spectrum of zinc-free enzyme remade with zinc in the reference cuvette and cobalt in the sample cuvette. The molar extinction coefficient of the cobalt peak is 180 M^{-1} cm^{-1}, which is in the range expected for tetrahedral Co^{2+}, 150–600 M^{-1} cm^{-1} (Cotton & Soderberg, 1962; Coleman, 1971).

D-Lactate dehydrogenase is a fluorescent flavoprotein (Olson & Massey, 1979). Emission and excitation spectra of the zinc-free enzyme and enzyme reconstituted with zinc or cobalt are shown in Figure 3. The small peak at 325 nm in the excitation spectra is due to the formation of a covalent adduct between FAD and the enzyme on exposure to light (F. F. Morpeth and V. Massey, unpublished observations). Addition of metal to the enzyme resulted in a loss of flavin fluorescence, with cobalt having a greater effect than zinc. Further addition of cobalt or zinc to the respective enzyme did not cause any more loss of fluorescence. There was no change in the fluorescence of the aromatic amino acid residues of the enzyme on addition of zinc or cobalt (exciting at 280 nm and emitting at 325 nm). Thus the quenching of the flavin fluorescence is probably due directly to the heavy metal ions. This suggests that the cobalt and zinc probably bind in close proximity to the flavin coenzyme. We do not know the mechanism of quenching; it is unlikely to be due to direct coordination with the oxidized flavin, which shows only weak complexes with metal ions (Hemmerich & Lauterwein, 1973).

(*2*) *Stability.* Native D-lactate dehydrogenase is not a particularly stable enzyme. It has a labile essential thiol and a tendency to lose FAD irreversibly (Olson & Massey, 1979, 1980). However, substantial stability may be conferred upon the zinc-containing enzyme by the addition of 1 mM DTT

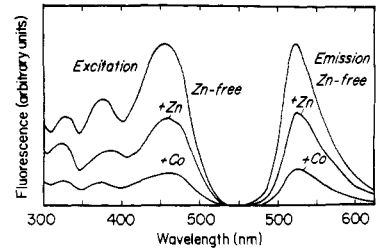

FIGURE 3: Fluorescence emission spectra (exciting at 455 nm) of Zn^{2+}-depleted, Zn^{2+}-reconstituted, and Co^{2+}-reconstituted D-lactate dehydrogenase. Fluorescence excitation spectra (emitting at 525 nm) of Zn^{2+}-depleted, Zn^{2+}-reconstituted, and Co^{2+}-reconstituted D-lactate dehydrogenase. All spectra were recorded at 25 °C in 0.1 M potassium phosphate buffer, pH 7. The concentration of D-lactate dehydrogenase in all cases was 22 μM.

(Olson & Massey, 1979). Unfortunately if the cobalt enzyme is stored in the presence of DTT, the substituted D-lactate dehydrogenase rapidly loses all its activity, and cobalt is removed from the enzyme and forms a highly absorbing orange complex. Storage under nitrogen does not stabilize the cobalt-substituted D-lactate dehydrogenase. Thus the zinc-free enzyme was routinely separated from DTT and EDTA and then reconstituted with cobalt immediately before use.

(*3*) *Reduction by Photochemistry, Dithionite, or D-Lactate.* The cobalt enzyme is very similar to the native zinc enzyme in that on reduction with D-lactate or sodium dithionite, the flavin is reduced and no semiquinone is formed. When the cobalt enzyme was reduced by irradiation with 0.1 M glycine as an electron donor, in the presence of catalytic amounts of 5-deazaflavin (Massey & Hemmerich, 1978), substoichiometric amounts of the blue semiquinone formed. Like the native enzyme this semiquinone was kinetically rather than thermodynamically stabilized (Olson & Massey, 1979). The semiquinone formed with the cobalt enzyme had a very similar absorption spectrum to that seen with the native zinc enzyme (Olson & Massey, 1979).

(*4*) *Magnetic Circular Dichroism.* A magnetocircular dichroic difference spectrum of cobalt-substituted minus zinc-depleted D-lactate dehydrogenase has two negative bands. One at 555 nm, $\Delta\xi = 0.21$ M^{-1} cm^{-1} τ^{-1}, with a shoulder at 490 nm corresponds to tetrahedral cobalt (Holmquist & Vallee, 1978). The second at 360 nm, $\Delta\xi = 0.74$ M^{-1} cm^{-1} τ^{-1}, presumably reflects a change in the flavin spectrum on adding cobalt to the enzyme.

(*5*) *Steady-State Kinetic Analysis of the Cobalt Enzyme.* The steady-state kinetics of native D-lactate dehydrogenase have been investigated in some detail (Morpeth & Massey, 1982b). When ferricyanide is an electron acceptor at pH 8, linear double-reciprocal plots are seen. At pH values less than 8, a series of parallel, concave-down curves are seen. With oxygen as acceptor the double-reciprocal plots are linear at pH values above 6.

Primary plots varying D-lactate or potassium ferricyanide at 25 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23, show some substrate activation with the cobalt-substituted enzyme. The series of roughly parallel curves were comparable to the steady-state pattern seen at pH 6 with the native enzyme. At 4 °C the substrate activation was much less pronounced, and the primary plots converge. This suggests that a simple ping-pong kinetic mechanism is not in operation. Rather, potassium ferricyanide appears to be reacting with a reduced enzyme–pyruvate complex, as is the case for the native enzyme (Morpeth & Massey, 1982a–c). Steady-state parameters obtained from the linear portions of the double-reciprocal plots and expressed in the notation of Dalziel (1957)

Table I: Kinetic Coefficients Describing the Oxidation of D-Lactate by Oxygen and Potassium Ferricyanide Catalyzed by Native and Cobalt-Substituted D-Lactate Dehydrogenase[a]

		ϕ_0 (min) $\times 10^3$	ϕ_1 (mM min) $\times 10^2$	ϕ_2 (mM min) $\times 10^3$	ϕ_{12} (mM^2 min)	ϕ_1/ϕ_0 (mM)[b]	ϕ_2/ϕ_0 (mM)[b]
			Electron Acceptor Oxygen				
native enzyme:	25 °C	4.5	2.9	1.3		6.4	0.278
cobalt enzyme:	25 °C	5.7	0.178	1.8			0.315
			Potassium Ferricyanide				
native enzyme:	25 °C	1.2	1.9	0.13		15.8	0.11
	4 °C	2.7	7.1	1.78	1.6	26.3	0.66
cobalt enzyme:	25 °C	1.3	0.055	0.37		0.042	0.28
	4 °C	5.6	0.415	5.6	0.00326	2.4	1

[a] The kinetic coefficients shown are those in the reciprocal rate equation $e/v = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2] + \phi_{12}/([S_1][S_2])$ where e is the concentration of active sites based on the flavin concentration, S_1 is D-lactate, and S_2 is potassium ferricyanide or oxygen. [b] ϕ_1/ϕ_0 is the Michaelis constant for D-lactate, and ϕ_2/ϕ_0 is the Michaelis constant for oxygen and potassium ferricyanide. All data were obtained in potassium phosphate buffer, pH 8, ionic strength = 0.23.

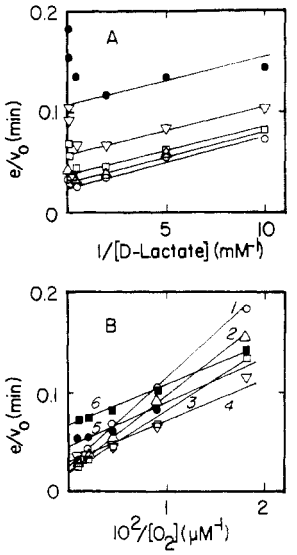

FIGURE 4: Oxygen kinetics of cobalt-substituted D-lactate dehydrogenase. (A) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 8, 25 °C, with the reciprocal of the D-lactate concentration at constant oxygen concentrations. The oxygen concentrations were (○) 560, (△) 280, (□) 118, (▽) 56, and (●) 28 μM. (B) Primary plots showing the variations of the reciprocal of the specific initial rate at pH 8, 25 °C, with the reciprocal of the oxygen concentration at several constant D-lactate concentrations. The D-lactate concentrations were (○) 20, (△) 10, (□) 2, (▽) 0.5, (●) 0.2, and (■) 0.1 mM. For clarity in panel B the curves are numbered 1–6 in order of decreasing D-lactate concentration. The experiment was carried out in potassium phosphate buffer, ionic strength = 0.23.

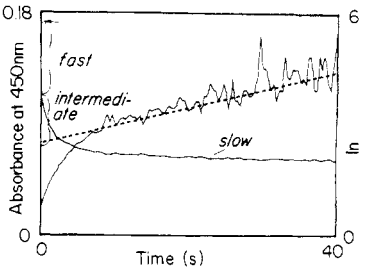

FIGURE 5: Change in absorbance at 450 nm with time when anaerobic oxidized cobalt-substituted D-lactate dehydrogenase (13.3 μM) is mixed with an equal volume of D-lactate (10 mM) in potassium phosphate buffer, pH 8, ionic strength = 0.23, at 4 °C. The reaction was followed in a stopped-flow spectrophotometer equipped with a 2-cm path-length cell. Curve 1 shows the bleaching of the flavin absorbance at 450 nm, and curve 2 is the natural logarithm of curve 1.

are given in Table I. The data in Table I for the cobalt enzyme are corrected to an AFR of 130 for comparative purposes. However, the AFR of the cobalt enzyme as made was usually in the range 50–80.

The substrate activation seen with the native zinc enzyme at low pH has been shown to be due to negative interactions within the protein (Morpeth & Massey, 1982b,c). This could also be the case with the cobalt-substituted enzyme, with the complex behavior manifesting itself at higher pH values.

When oxygen is the electron acceptor for D-lactate oxidation at 25 °C and pH 8, in potassium phosphate buffer, ionic strength = 0.23, the primary plots shown in Figure 4 are seen. Figure 4A shows that at concentrations of D-lactate greater than 0.5 mM significant substrate inhibition occurs. The inhibition is much greater at lower oxygen concentrations. This suggests that D-lactate and oxygen are competing for reduced enzyme. The abortive complex between reduced enzyme and D-lactate may also react with oxygen, but at a much reduced rate. Figure 4B shows the alternative primary plots varying the oxygen concentration. This complex pattern of curves has been shown by Segal (1975) to be entirely consistent with the above conclusion.

Native enzyme also shows some substrate inhibition by D-lactate. At high ionic strength and low oxygen concentration (28 μM), D-lactate concentrations greater than 40 mM elicit substrate inhibition (data not shown).

Rapid-Reaction Studies on the Cobalt Enzyme. (1) Reductive Half-Reaction. As with native D-lactate dehydrogenase (Morpeth & Massey, 1982c), the reductive half-reaction is a complex triphasic process. Figure 5 (curve 1) shows a trace at 450 nm when anaerobic cobalt-substituted D-lactate dehydrogenase is mixed with 10 mM D-lactate in a stopped-flow spectrophotometer at 4 °C. The natural logarithm plot (curve 2 in Figure 5) clearly shows the triphasic nature of the reaction. The slowest phase (k_{slow}) is independent of substrate concentration between 2 and 50 mM D-lactate with a rate of 0.06 ± 0.015 s^{-1}. This phase accounts for no more than 10% of the total absorbance change at any wavelength. The slowest phase seen with the native enzyme under these conditions has a similar rate constant, 0.07 ± 0.015 s^{-1}, and also accounts for about 10% of the total reduction (Morpeth & Massey, 1982c). However, the intermediate phase of reduction seen with the cobalt-substituted enzyme, which accounts for 30–40% of the total absorbance change, is also saturated in the D-lactate concentration range used. The rate constant for this phase is 0.7 ± 0.1 s^{-1}. This is strikingly different from the native enzyme where the intermediate phase accounts for 70–80% of flavin reduction, and its rate is substrate concentration dependent.

The cobalt enzyme fast phase of reduction accounts for 50–60% of the flavin bleaching, and it is substrate concentration dependent. This is again unlike the native enzyme in

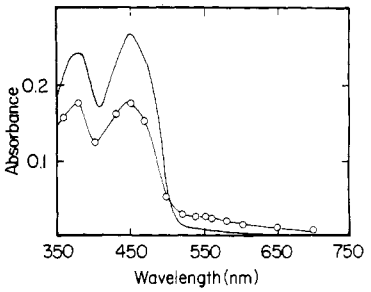

FIGURE 6: Transient species seen during the reduction of cobalt-substituted D-lactate dehydrogenase by 50 mM D-lactate at 4 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23. The solid line is the oxidized enzyme and the broken line the transient charge-transfer complex after 0.1 s.

Scheme I

$$E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{red}P$$

which the fast phase accounts for 10–15% of the absorbance change at a substrate concentration independent rate of 11 ± 3 s^{-1}. With both the native enzyme and the cobalt-substituted enzyme there is a transient increase in long wavelength absorbance (510–700 nm). In both cases, this absorbance appears with the same rate constant as the fast bleaching of the flavin peak. The amount of long wavelength absorbance formed was independent of the D-lactate concentration with the cobalt-substituted enzyme while its rate of formation was not. This is exactly opposite to the native enzyme, where the rate of formation of the long wavelength absorbance is substrate independent while the amount formed is substrate dependent. The rate of loss of long wavelength absorbance with both the native and cobalt-substituted enzymes is equal to the intermediate phase of bleaching of the flavin peak.

A spectrum of the long wavelength absorbing intermediate seen after 0.1 s on reduction of the cobalt-substituted D-lactate dehydrogenase with D-lactate is shown in Figure 6. This spectrum is very similar to that seen with the native enzyme and no well-defined long wavelength peak is seen. The molar extinction coefficient at 540 nm of the long wavelength intermediate of the cobalt-substituted D-lactate dehydrogenase is 1220 M^{-1} cm^{-1}, compared with that of the native enzyme under identical conditions of 900 M^{-1} cm^{-1}.

When the substrate dependence of k_{fast} is analyzed as described by Strickland et al. (1975), both the plot of k_{fast} vs. D-lactate and the corresponding double-reciprocal plot are curved. This suggests that a mechanism of the type shown in Scheme I is in operation and that k_4 is significant.

From the direct plot k_4 can be estimated as 26 s^{-1}, and from the corrected double-reciprocal plot of $1/(k_{fast} - k_4)$ vs. 1/[D-lactate], k_3 = 77 s^{-1} and K_d^{app} = 20 mM.

(2) Reoxidation of Reduced Enzyme by Pyruvate. The reductive half-reaction of D-lactate dehydrogenase is freely reversible (Olson & Massey, 1979; Morpeth & Massey, 1982c). The reaction product, pyruvate, will reoxidize reduced native D-lactate dehydrogenase in a monophasic reaction at pH 8 (Morpeth & Massey, 1982c).

When reduced cobalt-substituted enzyme is mixed anaerobically at 4 °C with varying concentrations of pyruvate (0.5–100 mM), a biphasic rise in flavin absorbance is seen. The rate of the slow phase is independent of pyruvate concentration and accounts for 20% of the absorbance change with a rate constant of 0.038 ± 0.002 s^{-1}. When the variation of the faster phase with pyruvate concentration was analyzed according to Strickland et al. (1975), the data fit a two-step

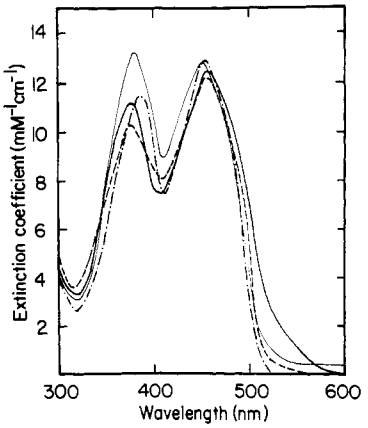

FIGURE 7: Spectra of native D-lactate dehydrogenase free and complexed to Cd^{2+}, Cu^{2+}, and Ag^{+}. The native D-lactate dehydrogenase (-·-) was separated from 1 mM DTT normally present in the buffer by passage through a Sephadex G-25 column. The Cd^{2+} complex (—) (dark) was prepared by titrating in 1.12 equiv of $CdCl_2$, and the Ag^{+} complex by titration of 2.07 equiv of $AgNO_3$ (---), and the spectrum of the Cu^{2+} (—) complex was taken immediately after the addition of 1 equiv of $CuCl_2$. All spectra were taken in 0.1 M potassium phosphate buffer, pH 7, at 4 °C.

scheme similar to Scheme I. From the direct plot and a corrected double-reciprocal plot, values for the kinetic constants were determined (the primes on the rate constants are to distinguish them from rate constants involved in lactate oxidation): k_3' = 6.1 s^{-1}, k_4' = 1.3 s^{-1}, and K_d^{app} = 8.3 mM.

A very small transient increase in long wavelength absorption (510–700 nm) was seen when reduced cobalt-substituted D-lactate dehydrogenase was mixed with concentrations of pyruvate greater than 100 mM. Thus the charge-transfer band produced must have a very high K_d^{app}, in contrast to the native enzyme where a similar transient long wavelength absorbance is saturated at 1 mM pyruvate (Morpeth & Massey, 1982c).

(3) Reoxidation of Reduced Enzyme by Oxygen. When reduced cobalt-substituted D-lactate dehydrogenase was mixed at 25 °C with 0.1 M potassium phosphate buffer, pH 7, equilibrated with various concentrations of oxygen, a monophasic rise of the oxidized flavin peak was seen. As with the native enzyme, there was no evidence for a C(4a) hydroperoxy intermediate (Morpeth & Massey, 1982c; Entsch et al., 1976). The reaction between reduced cobalt-substituted enzyme and oxygen was simple second order and had a rate constant of 9.75 × 10^3 M^{-1} s^{-1}. Under identical conditions, reduced native D-lactate dehydrogenase reacts with oxygen at 15 × 10^3 M^{-1} s^{-1} (Morpeth & Massey, 1982c).

Other Metals Binding to the Zinc Site. Several other metal ions were also added to the zinc-depleted enzyme to see if they could substitute for zinc in the enzyme. Stoichiometric amounts or large excesses (at least 10-fold) of either Mg^{2+} or Mn^{2+} did not cause any activity to return or any spectral change when added to the zinc-depleted enzyme. If zinc was added subsequently, full activity reappeared, and the expected spectral change occurred. The transition metal Cd^{2+} did not restore activity, but it did cause an unusual spectral change and bound tightly with a stoichiometry of two Cd^{2+} per flavin. The Cd^{2+} enzyme had no catalytic activity.

The presence of two high-affinity binding sites for Cd^{2+} on zinc-depleted D-lactate dehydrogenase led us to examine Cd^{2+} binding to native D-lactate dehydrogenase.

Secondary Metal-Binding Site. Native enzyme containing its full complement of zinc was titrated in 0.1 M potassium phosphate buffer, pH 7, 4 °C, with 1 equiv of $CdCl_2$ which gave the spectral change shown in Figure 7. This is very

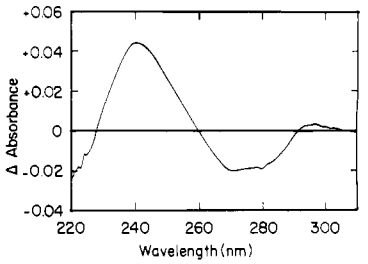

FIGURE 8: Ultraviolet difference spectrum between native enzyme and the Cd^{2+}–D-lactate dehydrogenase complex. Solutions of DTT-free D-lactate dehydrogenase (9.8 μM) in dual compartment silica cells were titrated with $CdCl_2$ to give a maximal difference spectrum (1.24 equiv of $CdCl_2$ per FAD) at 4 °C in 0.1 M potassium phosphate buffer, pH 7.

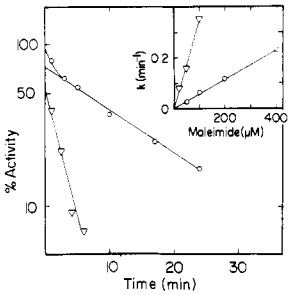

FIGURE 9: Kinetics of inactivation of native D-lactate dehydrogenase by *N*-ethylmaleimide and by *N*-benzylmaleimide. The plots show the loss of activity when 4 μM enzyme (free of DTT) in 0.1 M potassium phosphate buffer, pH 7, was reacted with 100 μM *N*-ethylmaleimide (○) and 100 μM *N*-benzylmaleimide (▽). The insert shows the apparent second-order rate constant for inactivation by *N*-ethylmaleimide (○) and *N*-benzylmaleimide (▽). The pseudo-first-order rate constants from experiments such as those shown in the panel are plotted against the maleimide concentration, with the slope giving the apparent second-order rate constant.

similar to the spectral change seen when 2 equiv of Cd^{2+} is added to the zinc-free enzyme. The addition of Cd^{2+} to the native enzyme also quenches the flavin fluorescence by about 30%. In the standard assay, enzyme complexed with Cd^{2+} displays nonlinear progress curves. After an initial lag phase, the enzyme exhibits its full native activity. The lag phase could represent the breakdown of the cadmium–enzyme complex.

Spectral perturbation of the flavin in the presence of metal ions has also been seen with another flavoprotein, lipoamide dehydrogenase (Veeger & Massey, 1962; Casola et al., 1966; Thorpe & Williams, 1976). These spectral changes were shown to be due to the catalytic oxidation by Cu^{2+} of vicinal thiols leading to the insertion of a second disulfide bridge into the enzyme and not to copper bound in the vicinity of the flavin.

When Cu^{2+} was added to D-lactate dehydrogenase in 0.1 M potassium phosphate buffer, pH 7, at 4 °C, the spectral change shown in Figure 7 occurred. There is an increase in absorbance in the range 370–400 nm, a slight decrease from 420 to 470 nm, and a long wavelength end absorption with no well-resolved bands, extending to 800 nm.

The copper–enzyme complex was very unstable and rapidly broke down for reasons discussed below. However, the same initial increase in absorption at 380 nm was seen with 1 or 2 equiv of $CuCl_2$, suggesting that there was only one binding site.

The Cu^{2+}–native enzyme complex also yields nonlinear standard assay progress curves. However, in this case full native activity was not seen after the lag phase. This is due presumably to copper oxidizing an essential sulfhydryl in the enzyme (Olson & Massey, 1980).

Other transition metals were also examined with the native enzyme for their ability to cause spectral perturbations of the flavin. Large excesses of Zn^{2+}, Co^{2+}, Ni^{2+}, and Hg^{2+} (10–20-fold) had no effect. However, Ag^{+} bound tightly, producing the spectral changes shown in Figure 7 with a stoichiometry of 2 and again quenching the fluorescence of the flavin by about 30%. On assaying the $(Ag^{+})_2$–enzyme complex, a lag was again seen. As with Cu^{2+}, at the end of the lag the rate was less than that with the native enzyme, again suggesting some thiol oxidation.

The spectral changes seen in D-lactate dehydrogenase on addition of Ag^{+}, Cu^{2+}, and Cd^{2+} are very similar to those reported in model studies for flavoquinone chelates (Hemmerich & Lauterwein, 1972). These chelates involve ligation of the transition metal to the N(5) and C(4) positions of the flavin isoalloxazine ring, and they are usually very unstable under aqueous conditions. This raises the possibility that in D-lactate dehydrogenase the flavin cofactor is in a hydrophobic environment.

Thus, the metal ions in the secondary metal-binding site appear to be bound directly to the flavin. In addition, these metal ions appear to be liganded to thiols. Evidence to support this suggestion comes from the UV absorbance spectrum of the native enzyme–cadmium complex (Figure 8). The new peak which appears at 240 nm is characteristic of Cd^{2+}–thiol compounds (Kagi & Vallee, 1961; Vasak et al., 1981), and its molar extinction coefficient is 5.3×10^3 M^{-1} cm^{-1}. This should be compared to extinction coefficients of 14×10^3 M^{-1} cm^{-1} for Cd^{2+} bound to four thiols in metallothionein (Kagi & Vallee, 1961) and 10.2×10^3 M^{-1} cm^{-1} for Cd^{2+} bound to an average of three thiols in horse liver alcohol dehydrogenase (Drum & Vallee, 1970). Thus it appears that Cd^{2+} is bound to thiols in D-lactate dehydrogenase, possibly two thiols. Further it appears that one of the thiols involved in binding the transition metal ions to the secondary metal binding site is the essential thiol described by Olson & Massey (1980). This conclusion is based on a comparison of percentage activity remaining and the percentage of enzyme–Cu^{2+} complex remaining with time. The time courses of these two processes are very similar. Thus it is possible that the breakdown of the Cu^{2+} complex represents formation of a disulfide bridge between the essential thiol and another thiol, preventing the Cu^{2+} from binding next to the flavin.

These results suggest that the N(5) position of the flavin and the essential thiol of D-lactate dehydrogenase are in reasonably close proximity.

We suggested above that the stability of these flavoquinone–metal chelates could be due to a hydrophobic binding pocket for the metal. Anderson and co-workers (Heitz et al., 1968; Anderson & Vasini, 1970) have developed the techniques of using *N*-alkylmaleimides with different side chains to probe the polarity of the environment of enzyme sulfhydryl groups. In Figure 9 the kinetics of inactivation of D-lactate dehydrogenase by *N*-ethylmaleimide and *N*-benzylmaleimide are shown at 4 °C in 0.1 M potassium phosphate buffer, pH 7. With both maleimide derivatives, the kinetics are markedly biphasic. This type of behavior is seen on inactivation of D-lactate dehydrogenase by a wide range of sulfhydryl reagents (Schopfer et al., 1981; F. F. Morpeth, unpublished observations). However, the inactivation obviously proceeds at a much greater rate with *N*-benzylmaleimide. The apparent second-order rate constants for the slow phases of inactivation were calculated to be 9.5 M^{-1} s^{-1} for *N*-ethylmaleimide and 55 M^{-1} s^{-1} for *N*-benzylmaleimide. Maleimide derivatives have been shown to react in model studies with cysteine and glutathione

at rates which are essentially independent of the side chain (Heitz et al., 1968). Thus the more rapid rates of inactivation by the more hydrophobic derivative probably reflect a rate-determining interaction with a nonpolar region of the enzyme in the vicinity of the essential thiol. This supports the idea that the essential sulfhydryl is in a hydrophobic environment near the N(5) position of the flavin.

Discussion

Cobalt-Substituted Enzyme. Like with many other zinc metalloenzymes, the cobalt analogue of D-lactate dehydrogenase is catalytically active. MCD and optical studies indicate that the catalytic metal ion is bound as a tetrahedral complex. No other metal ions tested with the exception of Cd^{2+} bound tightly to the zinc site of the enzyme, and the Cd^{2+} enzyme was catalytically inactive.

The cobalt enzyme shows many interesting differences in both its steady-state and rapid reaction behavior from the native enzyme (Morpeth & Massey, 1982b,c). Perhaps the most striking change in catalytic properties is the very strong excess-substrate inhibition seen when oxygen is the electron acceptor (Figure 4). This is probably due to the enzyme being trapped in an abortive E_{red}–lactate complex at high concentrations of D-lactate.

From the linear portions of the double-reciprocal plots, estimates of the Dalziel kinetic coefficients could be found (Table I). These show that on changing from the native zinc to cobalt-substituted D-lactate dehydrogenase, the parameter ϕ_1 is most affected, decreasing some 30-fold. An estimate of ϕ_1 could not be made from the rapid reaction data since the reductive phase of flavin bleaching was saturated at low D-lactate concentrations. The above observations and the high affinity for lactate of the reduced enzyme, as judged by the ready formation of E_{red}–lactate, suggest that the metal is involved in substrate binding and reduction. This is especially interesting in view of the finding of Goulden (1960) that the 1:1 Co^{2+} chelate with lactate is much more stable than the Zn^{2+} chelate.

The reductive half-reaction of the cobalt-substituted D-lactate dehydrogenase, like that of the native enzyme, is a complex triphasic process involving the transient formation of a long wavelength intermediate, which is probably a charge-transfer complex (Morpeth & Massey, 1982c). The steady-state results at 4 °C (for enzyme AFR = 50) suggest a V_{max} of 68 min^{-1}, which is in reasonable agreement with the estimate of k_3 = 42 min^{-1} for the intermediate phase of flavin reduction. Thus the conversion of E_{ox}–lactate to E_{red}–pyruvate is probably rate limiting.

If, as suggested above, the zinc in D-lactate dehydrogenase binds lactate directly, then it would have two effects which would be consistent with a transient carbanion being involved in catalysis. First, it would polarize the α carbon–hydrogen bond, making it significantly more acidic. Second, the positive charge of the Zn^{2+} would tend to stabilize any carbanion formed. The second metal-binding site in D-lactate dehydrogenase we envisage to contain two thiols binding the metal, where one is the essential thiol of Olson & Massey (1980). The very rapid oxidation of D-lactate dehydrogenase by Cu^{2+} is probably partly due to the excellent coordination environment provided for the metal by the binding site.

In summary, we believe that we may make the following statements concerning the active site of D-lactate dehydrogenase. First, the three essential catalytic elements so far identified, FAD, zinc, and an essential thiol, are all in reasonable proximity. Thus, they are presumably all in the active site. Second, it seems that the active site is mainly hydrophobic in native.

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