Redox Potentials of the Flavoprotein Lactate Oxidase†

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ABSTRACT: The redox potentials for both electron transfers for the enzyme lactate oxidase have been measured at pH 7.0 in 0.01 M imidazole buffer at 25 °C. Methylviologen is the electrochemically generated reducing agent capable of transferring both electrons to the enzyme in this spectroelectrochemical experiment. The $E^{0\prime}$ values are as follows: for $EFl_{ox} + e^- = EFl^{-}\cdot$, $E^{0\prime}_1 = -0.067 \pm 0.006$ V; for $EFl^{-}\cdot + e^- + H^+ = EFl_{red}H^-$, $E^{0\prime}_2 = -0.231 \pm 0.004$ V. All potentials are reported vs. the standard hydrogen electrode (SHE). Both electron transfers are reversible. Consistent with the 164-mV potential separation, 95% of the enzyme anion radical is thermodynamically stabilized at half-reduction in all experiments.

The flavoenzyme lactate oxidase catalyzes the oxidative decarboxylation of lactate to the products CO_2, acetate, and water. The overall reaction mechanism outlined below has been proposed by Massey and Lockridge (Lockridge et al., 1972). The overall catalytic reaction occurs in two separate two-electron steps. First, two electrons are transferred from the bound substrate (lactate) to the enzyme to form the two-electron-reduced enzyme–pyruvate complex. The two-electron-reduced enzyme–pyruvate complex then transfers two electrons to oxygen to form H_2O_2. The H_2O_2 remains bound in the active site along with pyruvate. The bound intermediates then further react to give products acetate, CO_2, and H_2O.

Electron transfer between lactate oxidase and normal substrates appears to involve two electrons. Three mechanisms have been proposed for this transfer: a free radical mechanism, a hydride transfer mechanism, and a covalent mechanism (carbanion mechanism) (Ghisla & Massey, 1977). Considering the free radical mechanism, no radical intermediates have been found with normal substrates. In contrast, free enzyme reduced by the deazaflavin–glycine light method in the absence of normal substrates is known to form the anion radical (Choong & Massey, 1980). When pyruvate is mixed with anion radical, a tightly bound ($K_d = 1.36 \times 10^{-5}$) complex species is formed. This anion radical–pyruvate complex is stable to oxygen reoxidation, while the anion radical and the two-electron-reduced enzyme–pyruvate complex are both very reactive with O_2. These observations indicate the anion radical is not in the normal catalytic path.

The redox potentials of the individual electron transfers of lactate oxidase have not yet been measured. Previously, no good reducing agent for this enzyme existed. Lactate oxidase binds a wide variety of anions, including most of the commonly used buffers (Lockridge et al., 1972). Since most chemical reducing agents are anions, they are bound to the enzyme. This causes changes in both the spectral properties and redox properties of the enzyme (Massey et al., 1969). As a result, indirect evidence from reductive reactions and complexation reactions have been used to predict possible properties of electron transfer. Three methods and their contradictory predictions for redox behavior are given below.

(1) Standardized solutions of $Na_2S_2O_4$ are typically used for reductive titrations of enzyme. However, $Na_2S_2O_4$ cannot be used to reduce lactate oxidase because SO_3^{2-}, the oxidation product of $Na_2S_2O_4$, complexes lactate oxidase very tightly; the dissociation constant is 3.7×10^{-6} M (Massey et al., 1969). Thus, the binding of enzyme by SO_3^{2-} is almost quantitative.

Although SO_3^{2-} binding prevents the $Na_2S_2O_4$ reduction of lactate oxidase, the SO_3^{2-} binding constant was used to estimate the redox potential of the enzyme. Müller and Massey discovered that there is a linear correlation between the log of the SO_3^{2-} binding constant and redox potentials for free modified flavins of varying redox potential (Müller & Massey, 1969). Lactate oxidase has one of the highest SO_3^{2-} binding constants of any flavoprotein. According to the criteria of Müller and Massey, lactate oxidase is predicted to have a redox potential of +0.040 V vs. SHE[1] at pH 7.0. This is the most positive value predicted thus far for a flavoprotein.

(2) Recently Massey and Hemmerich characterized a deazariboflavin-mediated light reduction method for flavoproteins (Hemmerich & Massey, 1978). The postulated reducing agent is deazariboflavin radical which has a redox potential value of −1.00 V vs. SHE. When this deazaflavin-catalyzed photoreduction is used, the enzyme undergoes a one-electron reduction, stabilizing 100% anion radical (Choong & Massey, 1980). The second electron cannot be transferred by using this reducing system. This behavior implies that the redox potential of the two-electron transfers of lactate oxidase are widely separated in potential, the second electron being transferred at a potential more negative than −1.00 V.

(3) Finally, structural information has been obtained about the active site of the flavoprotein oxidases. Some recent experiments, in which modified flavins are used, strongly suggest (Massey & Hemmerich, 1980) that flavoprotein oxidases have a protonated base near the N-1, O-2 position of the flavin. By an inductive effect, the positively charged group should make the first electron transfer to the flavoprotein occur at a more positive value (Massey & Hemmerich, 1980). The effect of the positively charged group is the stabilization of the anion radical.

We have measured the redox potentials for lactate oxidase. The measurements were performed by using the previously developed spectroelectrochemical methodology (Stankovich, 1980). Methylviologen was selected to be the electrochemically

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[1] Abbreviations: EFl_{ox}, oxidized lactate oxidase; $EFl^{-}\cdot$, anion radical; $EFl_{red}H^-$, two-electron-reduced lactate oxidase unprotonated; MV, methylviologen with no oxidation state specified; MV^{2+}, oxidized methylviologen; $MV^{+}\cdot$, one-electron-reduced methylviologen; PMSF, phenazine methosulfate; PYC, pyocyanine; IDS, indigodisulfonate; AQS, 2-anthraquinonesulfonic acid; DHNQ, 2-hydroxy-1,4-naphthoquinone; 8ClFl, 8-chlororiboflavin; 1DFl, 1-deazariboflavin; PHE, phenosafranine; EDTA, ethylenediaminetetraacetic acid; SHE, standard hydrogen electrode

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reduced titrant. One-electron-reduced methylviologen is positively charged (Clark, 1960); therefore, there is no anionic reducing agent in this system. We chose experimental conditions in which changes for binding of enzyme and mediators would be minimized; e.g., positively charged redox indicators were used where possible.

Experimental Procedures

Materials

Lactate oxidase [L-lactate:oxygen 2-oxidoreductase (decarboxylating), EC 1.13.12.4] isolated from *Mycobacterium smegmatis* by the method of Sullivan (1968) was the generous gift of V. Massey. All work was done in 0.01 M imidazole hydrochloride buffer, pH 7.0, at 25 °C. Indigodisulfonate redox indicator, purified, was the gift of Dr. F. Guengerich, Vanderbilt University. Pyocyanine was synthesized via the photochemical decomposition of phenazine methosulfate (McIlwain, 1937). Redox indicator 8-chlororiboflavin was the generous gift of Dr. J. P. Lambooy, University of Maryland; 1-deazariboflavin was the gift of Dr. D. Graham, Merck Sharpe & Dohme; 5-deazariboflavin was the generous gift of Dr. V. Massey, University of Michigan.

Methods

Three types of experiments were performed on lactate oxidase. In all experiments the basic spectroelectrochemical cell, the spectral and electrochemical equipment (Stankovich, 1980), and the methodology of the photochemical experimental (Choong & Massey, 1980) were the same as described previously. However, the electrode construction was improved in the following ways: (a) The glass frits and agar in the reference and auxiliary electrodes of the original design were replaced by porous Vycor or "thirsty glass" (Dow Corning, Toledo, OH). Thirsty glass was the gift of Dr. R. Ramette, Carleton College, Northfield, MN. (b) The $Na_2S_2O_4$ oxygen scrubbing solution originally used for storing the reference and auxiliary electrodes was replaced by methylviologen maintained in the reduced form by the 5-deazariboflavin-mediated glycine light reaction (Choong & Massey, 1980).

Coulometric Titration. The coulometric titration was done as described previously (Stankovich, 1980). In this experiment only MV, enzyme, and buffer were present. The total concentration of enzyme was calculated from the initial absorbance of EFl_{ox} at 450 nm and the published molar absorptivity value of EFl_{ox} at 450 nm (Lockridge et al., 1972). MV^{2+} does not absorb in the visible region. Since the concentration and volume were known, the number of coulombs required to transfer 1 equiv of charge could be calculated from Faradays number. The number of reducing equivalents transferred was determined by integration of current. Enzyme spectra were recorded after incremental additions of reducing equivalents. The absorbances at 530, 450, and 370 nm were plotted vs. number of reducing equivalents added.

This type of plot will be linear with distinct breaks occurring at $n = 1$ and $n = 2$ if the enzyme electron transfers occur in sequential single electron steps. If this occurs, the molar absorptivity at any wavelength of both one-electron- and two-electron-reduced species can be calculated as well as the percentage of one-electron-reduced species observed. Current efficiency can also be calculated, being the percentage of the total current transferred which is transferred to the species of interest.

From this reductive titration we learned that the first electron transfer to lactate oxidase is 95% complete before the second electron starts to transfer (see Results). Therefore, during the first part of the titration, the equilibrium is between only two enzyme species, EFl_{ox} and $EFl^{-}\cdot$; during the second electron transfer, the equilibrium is between only $EFl^{-}\cdot$ and EFl_{red} H^{-}. This behavior allowed simplification of the calculation of concentrations of species present at any point in the titration. At any point in the titration, the amount of radical can be calculated from the A_{530} by using the molar absorptivity (Choong & Massey, 1980). The ratio of EFl_{ox} to $EFl^{-}\cdot$ can be calculated from the total concentration of enzyme and molar absorptivity. Likewise, if the second electron transfer is being studied, the two species in equilibrium are $EFl^{-}\cdot$ and EFl_{red} H^{-}.

Potentiometric Titration. Lactate oxidase was reduced in a solution containing a mixture of nearly optically transparent redox indicator dyes (including MV) covering a potential range of +0.080 to −0.500 V. From this type of experiment, estimates of both redox potentials and spectral properties of the enzyme can be obtained (Stankovich, 1980; Meckstroth et al., 1981; Hendler et al., 1977). The solution was 2 μM each in PYC, PMSF, and AQS, 100 μM in MV, and 25 μM in lactate oxidase, and the potentials measured for the two electron transfers were approximately −0.050 and −0.250 V vs. SHE at pH 6.7.[2] MV was omitted in a later experiment because it is reported to complex AQS (Nakahara & Wang, 1963). AQS was needed to control the electrode potential at −0.225 V, close to the potential of the second electron transfer. This experiment was used as a control experiment to test the enzyme potential and behavior in the absence of MV.

Potentiometric titrations of enzyme were also done with a single redox indicator present in addition to the MV. The purpose of this type of experiment is to rigorously measure the redox potential of each electron transfer of the enzyme by using a Nernst plot. The experiments were performed in such a way that if an enzyme complexes with a particular dye, it would be reflected by deviations from the expected redox potential for both dye and enzyme. The following are the basic strategies used in experimental design. (1) Anionic redox indicators were avoided whenever possible. (2) The standard redox potentials of the dyes alone were measured. Then, the potential of dye and the potential of enzyme were measured simultaneously in another experiment. If the dye potential shifted in the presence of enzyme, binding was occurring. (3) The ratio of dye to enzyme was low (ranging from 1/3 to 1/10). In this way, the effect of complexation would be minimized on the redox potential of the enzyme, while the complexation effect would maximize the dye potential shift. (4) The redox potential of each electron transfer of the enzyme was measured independently in two separate experiments by using two different redox indicators. (5) The redox indicators chosen in these experiments have redox potentials near those of the enzyme, as estimated by the preliminary experiment. (6) The spectral properties of the indicators are distinct from those of the enzyme, so the spectral properties of each can be measured separately.

In control experiments, the electron transfer mediator MV and the redox indicators (IDS, 8ClFl, 1DFl, and PHE) were titrated individually. The concentrations of redox indicators in these control experiments were typically 10 μM, that is, 10-fold higher than mediator concentration in the presence of enzyme.

The first electron transfer to the enzyme was measured with IDS and 8ClFl. IDS has a relatively long wavelength absorbance maximum at 603 nm. From the absorbance measurement at 603 nm, it was possible to calculate and correct

[2] Unpublished results.

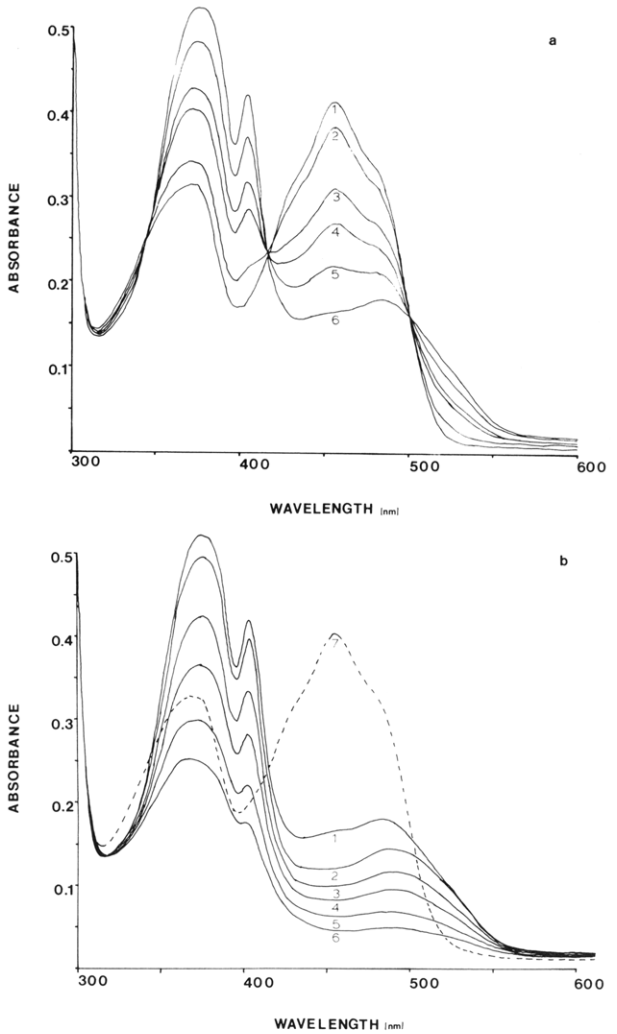

FIGURE 1: (a) Controlled potential titration of L-lactate oxidase (LOD). LOD, 37.3 μM, and 100 μM MV^{2+} in 4.20 mL of 0.01 M imidazole, pH 7.0. (1) Oxidized enzyme, (2) n = 0.15 electrons/mol of FMN, (3) n = 0.48, (4) n = 0.65, (5) n = 0.85, and (6) n = 1.04. (b) (1) n = 1.04 electrons/mol of FMN, (2) n = 1.31, (3) n = 1.49, (4) n = 1.64, (5) n = 1.81, (6) n = 1.94, and (7) reoxidized enzyme.

for the dye absorbance at any wavelength. 8ClFl does not absorb at 530 nm. At this wavelength $EFl^{-}\cdot$ absorbs alone. Therefore, the concentration of $EFl^{-}\cdot$ can be calculated directly from A_{530}. No redox potential value for 8ClFl in the presence of enzyme could be made, for the concentrations of oxidized and reduced 8ClFl could not be measured.

PHE and 1DFl were used to determine the potential of the second electron transfer. PHE has an absorbance maximum at 520 nm and an isosbestic point at 405 nm; 1DFl has an absorbance maximum at 535 nm and an isosbestic point at 392 nm. Concentrations of $EF1^{-}\cdot$ and $EFl_{red}\ H^{-}$ were calculated by using the absorbance observed at the isosbestic points for the dyes. These enzyme concentrations were used to correct the absorbance at the maxima for the dyes so that Nernst plots for the dyes could be made.

The $E^{0\prime}$ of MV was measured in the presence of enzyme. MV^{2+} does not absorb in the visible region. $MV^{+}\cdot$ absorbs at long wavelengths where no enzyme species absorb. The molar absorptivity at 602 nm is known (Szentrimay et al., 1977). The ratio of $MV^{2+}/MV^{+}\cdot$ was calculated from total concentration and the absorbance at 602 nm.

Photochemical Experiment. The fourth type of experiment did not involve electrochemical reduction of the enzyme. Instead, reduction was accomplished with the 5-deazariboflavin-catalyzed glycine light reduction (Choong & Massey, 1980). MV was added to this system to test its effect on the mechanism of electron transfer.

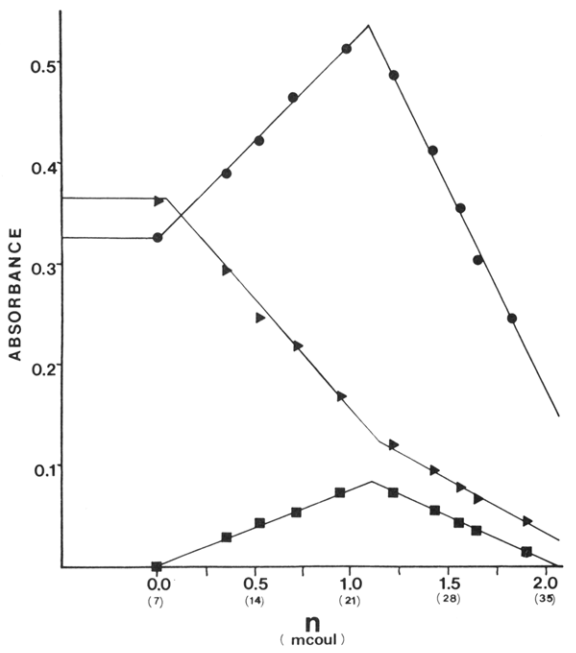

FIGURE 2: Reduction titration of L-lactate oxidase. Plot of absorbance at 370 (●), 450 (▲), and 530 (■) nm vs. number of reducing equivalents. Actual millicoulombs added in parentheses. Same experiment as Figure 1. Absorbance values at 530 nm corrected to show absorbance due to $EFl^{-}\cdot$ only.

Results

Coulometric Titration. Lactate oxidase (37.3 μM) was reduced with MV (100 μM) as electron transfer mediator. Parts a and b of Figure 1 show the spectral changes that occurred upon reduction of lactate oxidase. Figure 1a shows the spectral changes that occurred as the first electron was transferred to the enzyme. The spectrum obtained upon completion of the first electron transfer (spectrum 6 of Figure 1a and spectrum 1 of Figure 1b) is the characteristic spectrum of the anion radical (Choong & Massey, 1980). The isosbestic points at 501, 416, and 343 nm held during the first electron transfer. This indicates that between n = 0 and n = 1, there is an equilibrium between EFl_{ox} and $EFl^{-}\cdot$.

The spectra in Figure 1b were recorded as the second electron was transferred to lactate oxidase. The isosbestic points which held as the reduction proceeded from n = 0 to n = 1 were broken as the reduction proceeded from n = 1 to n = 2. A new isosbestic point at 318 nm was established and maintained in this part of the reduction. This indicated that a new equilibrium between between $EFl^{-}\cdot$ and $EFl_{red}\ H^{-}$ was established. The final dotted line in Figure 1b shows that upon admission of oxygen, the oxidized enzyme was obtained. The absorbance at 450 nm is 97% of the initial absorbance.

The greatest differences in molar absorptivities of EFl_{ox}, $EFl^{-}\cdot$, and $EFl_{red}\ H^{-}$ occur at 530, 450, and 370 nm.[3] Figure 2 is a graph of A_{530}, A_{450}, and A_{370} as a function of the number of reducing equivalents added per FMN. Note that there is an initial lag as 7 mC of reducing equivalents added to the system was consumed by oxygen. No spectral changes occurred until the oxygen was consumed (3–6 μM oxygen). The

[3] Molar absorptivities ($mM^{-1}\ cm^{-1}$) used in this paper are, for 530, 450, and 370 nm, respectively, the following EFl_{ox}, 0.3, 10.6, 9.0 (Choong & Massey, 1980); $EFl^{-}\cdot$, 2.1, 3.6, 14.0 (Choong & Massey, 1980); $EFl_{red}\ H^{-}$, 0.3, 0.6, 5.8 (this paper).

Table I: Coulometric Titration Data

	expt		
	1	2	3
concentration of enzyme (μM FMN)	37.3	26.8	16.1
concentration of MV^{2+} (μM)	100	100	100
first electron transfer			
Q calculated (n = 1) (mC)	15.1	10.9	6.5
Q observed	15.9	13.8	9.0
n measured	1.05	1.27	1.38
second electron transfer			
Q calculated (n = 2) (mC)	15.1	10.9	6.5
Q observed	15.4	11.8	7.1
n measured	1.02	1.08	1.09
A_{530} predicted	0.0840	0.0549	0.0365
A_{530} observed	0.0783	0.0499	0.0339
% (A_{530} observed/ A_{530} predicted)	93	91	93
Q, initial oxygen reduction (mC)	7.0	3.0	6.9

A_{530} and A_{370} maxima occurred at exactly the same value n = 1.05. The inflection of the A_{450} curve differed from the others by 4%. The second electron transfer is complete at n = 1.02. The first electron is transferred with 95% current efficiency and the second with 98% efficiency. (The molar absorptivity values for $EFl_{red}H^-$ are taken from the end point of this experiment.) Table I summarizes the coulometric reduction experiments on lactate oxidase. The previously described experiment is experiment 1.

A convenient wavelength to monitor $EFl^{-}\cdot$ production is 530 nm since EFl_{ox} and $EFl_{red}H^-$ absorb very little at that wavelength. The intersection of the A_{530} lines in Figure 3 yields an absorbance value for 100% radical formation. This value agrees with the absorbance value calculated from known molar absorptivity of $EFl^{-}\cdot$ and measured concentration. The measured experimental value (Figure 2) for maximum radical absorbance is 95% of the calculated value. This kind of behavior indicates that enzyme radical is thermodynamically stabilized. That is, the two electron transfers to this enzyme are widely separated in energy. The first electron is 95% transferred before the second starts to transfer.

Deazariboflavin-Catalyzed Glycine Light Reduction Reaction. The deazaflavin–glycine light reaction (Choong & Massey, 1980) was used to reduce 12.1 μM lactate oxidase in an anaerobic spectrophotometric cuvette. (No electrodes were used). The conditions were identical with those described except that MV was stored in the side arm of the cuvette. Lactate oxidase was irradiated for 53 min. The photochemical reduction produced quantitative anion radical, and the reduction of enzyme did not progress past the first electron transfer. MV stored in the side arm was then mixed anaerobically with the $EFl^{-}\cdot$. The spectrophotometric cell now containing 10 μM MV and 10.9 μM lactate oxidase enzyme radical was further irradiated. The enzyme radical was further reduced to the two-electron-reduced enzyme in 40 min.

Potentiometric Titration. The possibility of binding between MV and enzyme was tested by comparing the redox potential of MV alone with the redox potential in the presence of a 3-fold excess of enzyme. The $E^{0\prime}$ for MV alone in, pH 7.0, imidazole buffer was shifted 40 mV positive to −406 mV. This shift in imidazole buffer was observed with the other redox indicators. However, the $E^{0\prime}$ of MV (10–30 μM) in the presence of lactate oxidase (30 μM) was −406 mV, unchanged, in the presence of enzyme. The slope of the Nernst plot of MV is 60 mV, in the presence and in the absence of enzyme. From this evidence, we concluded that MV does not bind

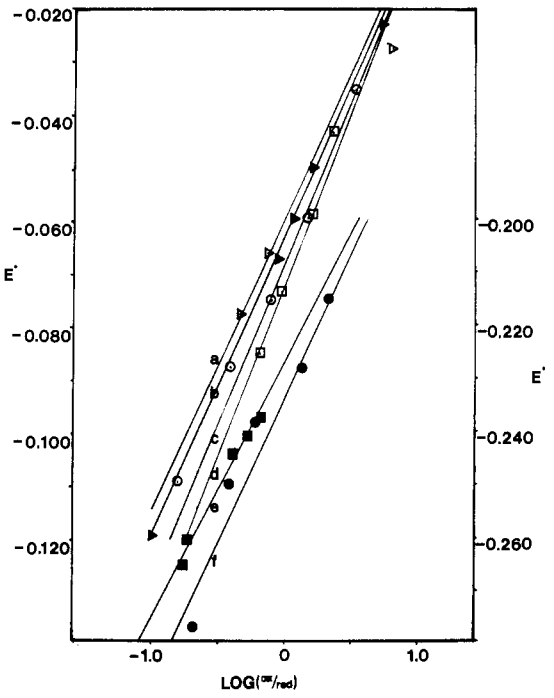

FIGURE 3: Nernst plots for the two electron transfers to L-lactate oxidase. The y axis on the left is used for lines a–d, while the y axis on the right is used for lines e and f. Experimental conditions were the following: (a) first electron oxidative direction, 27.1 μM LOD, 4 μM PMSF, 4 μM PYC, 4 μM DHNQ, and 100 μM MV^{2+}; (b) first electron reductive direction, 27.1 μM LOD, 4 μM PMSF, 4 μM PYC, 4 μM DHNQ, and 100 μM MV^{2+}; (c) first electron, 21.5 μM LOD, 4 μM PMSF, 4 μM PYC, and 4 μM AQS. (d) first electron, 31.1 μM LOD, 2.4 μM IDS, and 12.7 μM MV^{2+}; (e) second electron, 14.4 μM LOD, 1.1 μM 1DFl, and 30.6 μM MV^{2+}; (f) second electron, 23.2 μM LOD, 0.9 μM PHE, and 30.5 μM MV^{2+}.

lactate oxidase. This is reasonable because lactate oxidase usually binds negatively charged species (Lockridge & Massey, 1972). MV is positively charged in both oxidized and reduced states.

The redox potential of the first electron transfer to lactate oxidase was measured in three individual experiments with IDS, 8ClFl, and a mixture of optically transparent mediators excluding MV. The average $E^{0\prime}$ value is

$$EFl_{ox} + e^- = EFl^{-}\cdot \qquad E^{0\prime}_1 = -0.067 \pm 0.006 \text{ V vs. SHE} \tag{1}$$

The average value of the slope of the Nernst plots for the first electron transfer is +0.061 V.

The redox potential of IDS was the same in the presence and absence of enzyme; therefore, no binding occurred according to our criteria. The redox potential of the enzyme in the presence of a mixture of optically transparent mediators omitting MV is the same as the potential measured in the presence of MV. This confirms previous work showing that there was no binding of MV.

The redox potential of the second electron transfer was determined by using two independent potentiometric experiments in which PHE and 1DFl were used as redox indicators. The potential for the second electron transfer is

$$EFl^{-}\cdot + e^- + H^+ = EFl_{red}H^- \qquad E^{0\prime}_2 = -0.231 \pm 0.004 \text{ V vs. SHE} \tag{2}$$

The average value for the slope of the Nernst plots is +0.052 V.

PHE was found to bind to the reduced enzyme by observation of a shift in redox potential for the dye and by the non-Nernstian slope initially calculated. Correction of the data

Table II: Redox Potential Data

no.	redox mediator	mediator concn (μM)	$E^{0\prime}$, mediator alone (V)	$E^{0\prime}$, mediator with enzyme (V)	enzyme concn (μM)	$E^{0\prime}$, enzyme (V)	slope (V)	no. of points
				First Electron Transfer				
1	IDS	2.4	−0.088	−0.085	31.1	−0.072	+0.065	4
2	IDS[a]	2.4	−0.076	−0.074	11.1	−0.065	+0.069	6
3	8ClFl	4.5	−0.084	−0.085	17.8	−0.078	+0.059	3
4	PMSF	4						
	PYC	4			21.5	−0.069	+0.061	5
	AQS	4						
5	PMSF	4						
	PYC	4			27.1	−0.062[b]	+0.057	5
	DHNQ	4				−0.059[c]	+0.055	3
				Second Electron Transfer				
6	1DFl	1.1	−0.230	−0.233	14.4	−0.227	+0.049	5
7	PHE	0.9	−0.212	−0.226	23.2	−0.234	+0.055	5
8	PMSF	4						
	PYC	4			27.1	−0.220[b]		
	DHNQ	20				−0.205[c]		
	PHE	1						

[a] Gift of F. Guengerich. [b] Reductive direction. [c] Oxidative direction.

for binding between reduced PHE and reduced enzyme gave a Nernst plot with $E^{0\prime} = -0.234$ V and a slope of +0.055 V. The reason for the nonideal slope in the presence of 1DFl is not known. Conditions and results of the potentiometric titration are given in Table II. Figure 3 contains representative Nernst plots for both electrons.

The assignments of electron and proton transfer were made on the basis of the spectral data available for each oxidation state of the enzyme. The spectrum at one-electron reduction is characteristic of the spectrum of anion radical, not of the neutral radical species (Massey & Palmer, 1966). The enzyme spectrum at the completion of the second electron transfer is characteristic of the spectrum of the anion $EFl_{red}\ H^-$ (Ghisla et al., 1974). The spectral properties of the two-electron-reduced form of lactate oxidase have been reported by others to indicate that the enzyme stabilizes the anion two-electron-reduced form (Ghisla et al., 1974).

Reversible Potentiometric Titration. The reversibility of the individual electron transfers was tested in the following manner: The first electron was transferred to lactate oxidase in the presence of 4 μM PMSF, 4 μM PYC, 4 μM DHNQ, and 100 μM MV. Potentials and spectra were recorded. Then the lactate oxidase was reoxidized by controlling the potential of the working electrode at a positive value (+0.150 V). This oxidized PMSF, which in turn reoxidized the enzyme. Figure 3 shows the Nernst plots for the forward and reverse first electron transfer. The first electron is reversible according to this criteria, with $E^{0\prime}_1$ differing by only 2 mV in either direction. This electron transfer is equally rapid in both directions (1 h/point for transfer of charge and potential equilibration).

A similar experiment was performed for the second electron transfer. PHE (1 μM) was added to the previously described mixture of dyes in order to poise the potential of the second electron transfer. The concentration of DHNQ was increased to 20 μM.

The $EFl_{red}\ H^-$ was then reoxidized by controlling the potential of the working electrode at −0.100 V in order to transfer the −0.230 V electron only. At this potential value DHNQ should have acted as an electron acceptor. The production of $EFl^{-\cdot}$ was quantitative and very slow (3–5 h/point). The potential of the working electrode was then changed to +0.150 V as in the first electron transfer case. Under these conditions the reoxidation was more rapid, but the path of reoxidation was different. The rapid oxidation produced mostly oxidized form of the enzyme EFl_{ox}, but relatively little radical. The mixture of oxidized and reduced enzyme then slowly dismuted (2–3 h) to form the radical. However, the final equilibrium positions of the system and potentials were identical with those on the reduction path.

The spectra from reduction and reoxidation parts of the reaction were superimposable, and the potentials associated with these spectra were within 15 mV for the second electron and 1 mV for the first electron. The 95% radical was generated upon reoxidation. Both spectral and electrochemical properties of the enzyme were identical in either direction. Therefore, both electron transfers were reversible. However, the Nernst plot is not given for the second electron oxidation because of difficulty in interpreting potential values for the second electron transfer.

The potential measured at the gold indicator electrode during reoxidation in the presence of DHNQ is not consistent with other experimental observations. A possible explanation for this inconsistent electrode behavior is that the reduced DHNQ redox indicator appears to be adsorbing to the gold electrode. Thus, the electrode is being poised by the adsorbed DHNQ. This interferes with the ability of the gold electrode to accurately sense the potential of the enzyme solution.[4]

This adsorption can account for the puzzling experimental

[4] Cyclic voltammetric experiments were performed on 160 μM 2-hydroxy-1,4-naphthoquinone (DHNQ) at pH 7.0 in 0.1 M phosphate buffer by using both gold and mercury working electrodes. At the hanging mercury drop electrode, DHNQ gave a well-shaped reversible cyclic voltammogram with a peak potential separation of 30 mV. There was a small postwave, indicating a small amount of adsorption of the reduced species. Thus, DHNQ exhibited almost ideal behavior on mercury. In contrast, at the gold electrode DHNQ exhibited very small flattened waves. The peak potentials in the reductive and oxidative direction was separated by 400 mV. This behavior is so ill-defined it is impossible to model it accurately. A possible explanation for the observed behavior is that there is some species adsorbing to the electrode surface, preventing diffusion-controlled electron transfer. It is well-known that adsorption of a monolayer of species to the electrode surface may partially block that electrode to further reactions. Adsorption of material to the surface would account for the flattened shape of the reduction wave, for the rate of the reaction depends on diffusion through a surface layer. The resistance due to the adsorbed layer could account for the large separation in the peak potentials. We have investigated the behavior of 33 other redox indicators. None of the others show this marked difference in behavior with electrode materials.

observations in the presence of high concentrations of (20 μM) DHNQ. (1) The potential value of the first electron agrees with previously measured values even when the oxidation is in the sequence $EFl_{red}H^-$ to EFl_{ox} to $EFl^{-}\cdot$, because at these relatively positive potential values the DHNQ is in the nonadsorbing oxidized form. (2) The redox potentials of all species that have redox potentials negative of $E^{0\prime} = -0.112$ V of DHNQ (i.e., the second electron of lactate oxidase, PHE, and MV) were all shifted positive by 120 mV. At negative potential values, the equilibrium DHNQ species is the reduced form and is adsorbed at the electrode. The Nernst plot slope of 30 mV observed is consistent with adsorbed dye, since it is a two-electron-transferring agent. This positive 120-mV shift was observed only in this experiment which contains the DHNQ (Table II). When the measured value of the potential of the second electron was corrected for the observed potential shift of +120 mV, the redox potentials of the second electron transfer yield midpoint values that agree within 15 mV with the previously measured values.

Discussion

We have performed both coulometric and potentiometric titrations of lactate oxidase at a single pH value using electrochemically reduced methylviologen as the reductive titrant. These experiments provide the first titration and first measurement of redox potentials of this enzyme. The $E^{0\prime}_1$ measured for the first electron is −0.067 V with a slope of +0.061 V. The $E^{0\prime}_2$ measured for the second electron was −0.231 V with a slope of +0.052 V. The experiments presented here show that lactate oxidase can be titrated in two distinct electron transfers with formation of 95% of the anion radical, that the potential values of the two transfers are indeed widely separated in potential, and that the potential values measured and percent radical observed are the same in either the reductive or oxidative directions. The experimentally measured potential difference of 164 mV predicts 93% formation of radical.

Methylviologen was able to transfer the second electron in the reductive titration of the enzyme, both by electrochemical methods and with glycine light. In the absence of methylviologen, the transfer of the second electron to lactate oxidase is virtually impossible (Choong & Massey, 1980). Therefore, there is a kinetic barrier to the transfer of the second electron which the positively charged MV^{2+} can overcome. In our reductive reactions both electrons were transferred relatively easily. Our data indicate that methylviologen is acting only to transfer electrons. It is not altering the enzyme electron transfer properties.

By using two kinds of experiments, we have shown that MV does not bind lactate oxidase. First, the redox potential of MV is identical in the presence and absence of a 3-fold excess of enzyme. Second, the redox potential of the first electron transfer of the enzyme is the same whether MV or a two-electron-transferring dye is used. Since no binding of MV to enzyme occurs, the coulometric titration yields accurate spectral properties and electron transfer properties for the enzyme. Additional data which argue against MV influencing the course of the reduction are from a reductive experiment performed in the absence of MV. The 95% anion radical was generated by using the two-electron reductant AQS. If $EFl^{-}\cdot$ were kinetically stabilized by the $MV^{+}\cdot$ reducing agent, the presence of other mediators capable of two-electron transfer should enable thermodynamic equilibration to occur, thus lowering the amount of radical observed while shifting the redox potential. This behavior does not occur.

The thermodynamic stability of 95% anion radical fits well with the predictions of Massey and Hemmerich (Massey & Hemmerich, 1980). They stated this entire class of enzymes thermodynamically stabilizes anion radical. Their prediction was based on the observations that when equimolar amounts of oxidized and two-electron-reduced D-amino acid oxidase or L-amino acid oxidase were mixed anaerobically, anion radical was generated (Massey & Palmer, 1966; Massey & Curti, 1967). Our data are also consistent with the presence of a protonated base near the N-1 nitrogen of flavin. This base is postulated to stabilize anion radical (Massey & Hemmerich, 1980).

Since the $E^{0\prime}_2$ for the second electron transfer is −0.231 V, the deazaflavin–glycine light reaction should have been capable of transferring the second electron easily. This would suggest that the barrier to the second electron transfer in the 5-deazaflavin-catalyzed glycine light reaction is kinetic, not thermodynamic. Methylviologen was able to overcome that kinetic barrier.

Our data do not fit with the redox potentials predicted from the SO_3^{2-} binding constants. Lactate oxidase has a SO_3^{2-} binding constant of 3.7×10^{-6} M (Massey et al., 1969), which would predict a midpoint potential value of +0.048 V vs. SHE. The predictive value of the SO_3^{2-} binding is not valid for other flavoproteins whose redox potentials have been measured. For example, the $E^{0\prime}$ for glucose oxidase (extrapolated to pH 7.0 is about −0.180 V) is considerably more negative than the $E^{0\prime}$ value predicted from SO_3^{2-} binding (−0.035 V; Stankovich et al., 1978; Swodoba & Massey, 1966). In addition, the measured $E^{0\prime}$ value of D-amino acid oxidase of −0.004 V vs. SHE is considerably more positive than the value of −0.080 V predicted from SO_3^{2-} binding studies (Brunori et al., 1971). Therefore as a predictive tool for redox potentials of flavoprotein oxidases, the SO_3^{2-} binding constant is of questionable value.

Although our data agree with previously reported behavior of lactate oxidase, the redox properties reported here do not immediately cast light on the catalytic mechanism of this enzyme. However, from this work we now know the energy levels of the electrons of the free enzyme. By comparison of these potentials to the redox potentials of lactate oxidase bound to substrates or inhibitors, it may be possible to measure the effect of binding and charge-transfer complexes on the energy levels of lactate oxidase.

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References

Brunori, M., Rotilio, G., Antonini, E., Curti, B., Branzoli, U., & Massey, V. (1971) *J. Biol. Chem. 246*, 3140–3144.

Choong, Y. S., & Massey, V. (1980) *J. Biol. Chem. 255*, 8672–8677.

Clark, Wm. (1960) *Redox Potentials of Organic Compounds*, Williams and Wilkins, New York.

Ghisla, S., & Massey, V. (1977) *J. Biol. Chem. 252*, 6729–6735.

Ghisla, S., Massey, V., Lhoste, J. M., & Mayhew, S. G. (1974) *Biochemistry 13*, 589–597.

Hemmerich, P., & Massey, V. (1978) *Biochemistry 17*, 8–15.

Hendler, R. W., Songco, D., & Clem, T. R. (1977) *Anal. Chem. 49*, 1908–1913.

Lockridge, O., Massey, V., & Sullivan, P. (1972) *J. Biol. Chem. 247*, 8097–8106.

Massey, V., & Palmer, G. (1966) *Biochemistry 5*, 3181–3189.

Massey, V., & Curti, B. (1967) *J. Biol. Chem. 242*, 1259–1264.
Massey, V., & Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246–256.
Massey, V., Muller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., & Foust, G. P. (1969) *J. Biol. Chem. 244*, 3999–4006.
McIlwain, H. (1937) *J. Chem. Soc. 2*, 1704–1711.
Meckstroth, M., Norris, B., & Heineman, W. R. (1981) *Bioenergetics 8*, 63–70.
Müller, F., & Massey, V. (1969) *J. Biol. Chem. 244*, 4007–4016.
Nakahara, A., & Wang, J. H. (1963) *J. Phys. Chem. 67*, 496–498.
Stankovich, M. (1980) *Anal. Biochem. 109*, 295–308.
Stankovich, M., Schopfer, L., & Massey, V. (1978) *J. Biol. Chem. 253*, 4971–4979.
Sullivan, P. A. (1968) *Biochem. J. 110*, 363–370.
Swoboda, B. P., & Massey, V. (1966) *J. Biol. Chem. 241*, 3409–3416.
Szentrimay, R., Yeh, R. P., & Kuwana, T. (1977) in *Electrochemical Studies of Biological Systems* (Sawyer, D. T., Ed.) ACS Symp. Ser. No. 38, pp 142–169, American Chemical Society, Washington, DC.

Influence of pN_2 and pD_2 on HD Formation by Various Nitrogenases†

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ABSTRACT: Formation of HD from D_2 has been demonstrated with nitrogenase preparations from *Azotobacter vinelandii*, *Clostridium pasteurianum*, *Klebsiella pneumoniae*, and *Azospirillum* sp. We conclude that the formation of HD from D_2 is a general property of nitrogenases. However, the nitrogenases differ in their K_i values for D_2 (N_2 fixation) and in their rates of catalyzing HD formation; among the nitrogenases tested, *C. pasteurianum* nitrogenase had the lowest activity for formation of HD. When contaminating N_2 was removed from the atmospheres above reaction mixtures, less than 1% of the total electron flux in the system was directed to HD formation; hence, we doubt that N_2-independent HD formation is significant. A working hypothesis is suggested that operates without invoking an N_2-independent reaction for forming HD.

The formation of HD from D_2 by N_2-fixing organisms was discovered in soybean nodules by Hoch et al. (1960). They found this reaction was inhibited by CO and N_2O but enhanced by N_2. Their suggestion that HD was formed from an enzyme-bound diimide moiety may have constituted the first experimental evidence for diimide as an intermediate in biological N_2 fixation.

These observations were expanded by other investigators. Formation of HD from D_2 was observed by Bergersen (1963) in soybean nodules, Dixon (1967) in pea nodules, and Kelly (1968) in nodules of *Medicago lupulina* and *Alnus glutinosa*. Jackson et al. (1968) studied HD formation by crude nitrogenase preparations from *Azotobacter vinelandii* (Av)[1] and confirmed the requirement for N_2 and the inhibition by CO. The facts that MgATP (McNary & Burris, 1962) and a reductant such as ferredoxin (Mortenson, 1964) were required for N_2 fixation had been established, and Jackson et al. (1968) reported that they also were required for formation of HD. Turner & Bergersen (1969), with cell-free extracts from soybean nodule bacteroids, confirmed the work of Jackson et al. (1968). They also reported that the apparent $K_m(N_2)$ for HD formation was much smaller than that for NH_3 production. Kelly (1968, 1969), using crude extracts from *A. vinelandii* and partially purified nitrogenases from *Azotobacter chroococcum* (Ac) and other free-living nitrogen fixers, contrary to others found no enhancement of HD formation by N_2. Vandecasteele & Burris (1970) reported that a small amount of HD was formed by partially purified nitrogenase preparations from *Clostridium pasteurianum* (Cp).

The Kettering research group has studied HD formation in more detail with the Av nitrogenase complex (Bulen, 1976) and later with purified Av nitrogenase (Newton et al., 1976, 1977; Stiefel, 1977; Stiefel et al., 1980; Burgess et al., 1980, 1981; Wherland et al., 1981). They pointed out that HD formation via diimide, as formulated by Hoch et al. (1960), implies electron transfer rather than simple exchange and that one electron is used for each molecule of HD formed. They also suggested that H_2 inhibition and HD formation are two manifestations of the same molecular process. The group also took the position that HD formation has two pathways: an N_2-dependent pathway that is inhibited completely by 1% CO, and an N_2-independent pathway that is inhibited only partially by 1% CO. They reported that in the absence of added N_2 the percentage of total electrons flowing through the N_2-independent pathway is independent of the Av2/Av1 (Av2 = dinitrogenase reductase and Av1 = dinitrogenase) ratio

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[1] Abbreviations: Av, *Azotobacter vinelandii*; Ac, *Azotobacter chroococcum*; Cp, *Clostridium pasteurianum*; Av1, *Azotobacter vinelandii* dinitrogenase; Av2, *Azotobacter vinelandii* dinitrogenase reductase (the same convention is applied to the other nitrogenase components, i.e., Cp1, Kp1, Al1, Cp2, Kp2, Al2, etc.); Kp, *Klebsiella pneumoniae*; Al, *Azospirillum* sp. (apparently *A. lipoferum*); AE, activating enzyme; Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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