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Oxidation–Reduction Properties of Glycolate Oxidase†

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ABSTRACT: This is the first report of the redox potentials of glycolate oxidase. The pH dependence of the redox behavior as well as the effects of activators and inhibitors was studied. At pH 7.1 in 10 mM imidazole–chloride, $E_1^{\circ\prime}$ ($EF1_{ox}/EF1^{-}\cdot$) is -0.033 ± 0.010 V and $E_2^{\circ\prime}$ ($EF1^{-}\cdot/EF1_{red}H^{-}$) is -0.017 ± 0.017 V vs. the standard hydrogen electrode at 10 °C. A maximum of 29% flavin mononucleotide (FMN) anion radical is stabilized at half-reduction at pH 7.1 and 10 °C. Both redox couples of glycolate oxidase are pH-dependent from pH 7 to pH 9, and the FMN anion radical is stabilized in this range. The redox potentials of glycolate oxidase are shifted markedly positive of those of unbound FMN, consistent with the enzyme's function. The midpoint potential of glycolate oxidase is more positive than that of the glyoxalate/glycolate couple, and two-electron reduction of glycolate oxidase is thermodynamically favorable. The redox behavior of glycolate oxidase markedly contrasts that of other flavoprotein oxidases. For most flavoprotein oxidases, $E_1^{\circ\prime}$ is independent of pH from pH 7 to pH 9 and is much more positive than $E_2^{\circ\prime}$, which is pH-dependent. We present a mechanism that suggests a structural basis for the positive shifts and pH dependence of both $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ of glycolate oxidase.

Glycolate oxidase (glycolate:oxygen oxidoreductase, EC 1.1.3.1) catalyzes oxidation of α-hydroxy acids by molecular oxygen to produce α-keto acids and hydrogen peroxide. Previous studies of the isolated enzyme from pig liver have explored its physicochemical and steady-state kinetic properties (Dickinson & Massey, 1963; Schuman & Massey, 1971a,b). Particular attention was paid to the spectral and kinetic effects of mono- and dianions, e.g., chloride or oxalate ions. These anions are inhibitors except for phosphate and arsenate, which are activators (Schuman & Massey, 1971b).

A consistent picture of the redox behavior and structure of free and inhibitor-bound flavoprotein oxidases has emerged from electrochemical studies conducted in this laboratory (Stankovich & Fox, 1983, 1984; Van den Berghe-Snorek & Stankovich, 1985). Briefly, E_m[1] for flavin in flavoprotein oxidases is shifted positive relative to E_m for uncomplexed flavin. The positive shift is more pronounced for $E_1^{\circ\prime}$ than for $E_2^{\circ\prime}$. In addition, $E_1^{\circ\prime}$ for L-amino acid oxidase or D-amino acid oxidase is pH-independent from pH 7 to pH 9 but $E_2^{\circ\prime}$ is pH-dependent.

We wondered whether or not glycolate oxidase fit this pattern and whether or not the redox properties of glycolate oxidase were changed by binding of activators and inhibitors. Previously, redox titration of native glycolate oxidase had not

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[1] Abbreviations: FMN, flavin mononucleotide; $EF1_{ox}$, glycolate oxidase with oxidized FMN cofactor; $EF1^{-}\cdot$, glycolate oxidase with the FMN cofactor in the anion radical form; $EF1_{red}H^{-}$, glycolate oxidase with reduced FMN cofactor; E_m, midpoint potential; $E_1^{\circ\prime}$, formal potential for the redox couple oxidized flavin/flavin radical; $E_2^{\circ\prime}$, formal potential for the redox couple flavin radical/reduced flavin; AMPD, 2-amino-2-methyl-1,3-propanediol; CD, circular dichroism; 8CR, 8-chlororiboflavin; EDTA, ethylenediaminetetraacetate; IDS, indigo disulfonate; $MV^{+}\cdot$, methyl viologen radical; Pyo, pyocyanine; PSF, phenosafranine; SHE, standard hydrogen electrode; TEA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane.

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been performed because the most common reducing agents for flavoproteins cannot be used with glycolate oxidases. Glycolate oxidase binds the bisulfite that is produced during dithionite titration; the binding constant is the largest reported for the flavoprotein oxidases (Jorns & Hersh, 1976). Nor can EDTA, the most commonly employed ultimate electron donor for photochemical reduction, be used with glycolate oxidase because glyoxalate, a substrate and product of glycolate oxidase action, can be produced during the photochemical reaction (Enns & Burgess, 1965).

It has been suggested that E_m for native glycolate oxidase should be more positive than −0.170 V, which is the E_m of glycolate oxidase in which FMN is replaced by 5-deaza-FMN (Jorns & Hersh, 1976). E_m for the glyoxalate/glycolate couple is −0.086 V at pH 7 (Clark, 1960), and this fact would also suggest that E_m for glycolate oxidase should be significantly more positive than that of unbound FMN (−0.205 V). If this were not true, electron transfer from glycolate to glycolate oxidase would be very unfavorable.

Materials and Methods

Ammonium sulfate (special enzyme grade) was from Schwarz/Mann. Hyflo Super-Cel was from Johns-Manville. Methyl viologen was from BDH Chemicals. Phenazine methosulfate (99%) was from Aldrich. Pyo was obtained by photooxidation of phenazine methosulfate. IDS and 8CR were gifts from respectively Dr. Fred Guengerich, Vanderbilt University, and Dr. John P. Lambooy, University of Maryland. Lactate oxidase was a gift from Dr. Vincent Massey, University of Michigan.

Glycolate oxidase was isolated from pig liver according to the method of Schuman and Massey (1971a) except the eluate from DEAE-cellulose was treated with saturating ammonium sulfate and stored at −20 °C. The three preparations used here had ratios of absorbance at 277 nm to absorbance at 452.5 nm of 8.2 or less and specific activities of 1.1–1.2 as compared to specific activities of 1.2–1.4 obtained by Schuman and Massey (1971a). Autogenous reduction (Schuman & Massey, 1971a) was not observed during preparation of glycolate oxidase or during the electrochemical experiments. Jorns (1975) reported that 13–20% of the flavin content of pig liver glycolate oxidase was in the form of 6-hydroxy-FMN. In contrast, from the absorbance at 600 nm and a molar absorptivity of 3450 M^{-1} cm^{-1} for 6-hydroxy-FMN (Mayhew et al., 1974), it may be estimated that 6-hydroxy-FMN accounted for less than 10% of the flavin content of the glycolate oxidase preparations used here.

In preparation for each experiment, a sample of glycolate oxidase was thawed and dialyzed against 0.1 M phosphate, pH 7.0–7.1, with or without 0.3 mM EDTA. The glycolate oxidase concentration ws determined in this buffer by using a molar absorptivity of 11 700 M^{-1} cm^{-1} (Schuman & Massey, 1971a) at 452.5 nm. Phosphate buffer was then replaced by means of further dialysis. Dialyzed samples were filtered (Millipore, 0.45 μm) and mixed with methyl viologen and indicator dyes (potentiometric experiments) in the spectroelectrochemical cell. The pH was checked at the end of the experiment, and this value was taken to be the pH of the experiment. The spectroelectrochemical apparatus and procedures used here have been described (Stankovich, 1980; Stankovich & Fox, 1983).

One of the objectives of coulometric titrations was to measure the molar absorptivities of the red flavin radical form of glycolate oxidase. Large quantities of $EF1^{-}\cdot$ were kinetically stabilized during coulometric titrations in the absence of poising dyes. Therefore, coulometric experiments were conducted as rapidly as possible in order to minimize slow decay of $EF1^{-}\cdot$ toward equilibrium by dismutation. After addition of charge sufficient to remove residual oxygen, charge was added in aliquots of 0.5 mC. Eight to thirteen minutes was required per data point until $MV^{+}\cdot$ was observed. Recording of subsequent spectra was delayed until the absorbance due to $MV^{+}\cdot$ was insignificant. At pH 8.2, this delay was less than 25 min. At pH 9, the time per data point increased to as much as 2.5 h near the end of the titration.

For each data point in a spectrocoulometric titration, concentrations of $EF1_{ox}$, $EF1^{-}\cdot$, and $EF1_{red}H^{-}$ were calculated from the equations

$$\epsilon_0[EF1_{ox}] + \epsilon_1[EF1^{-}\cdot] + \epsilon_2[EF1_{red}H^{-}] = A \quad (1)$$

$$[EF1_{ox}] + [EF1^{-}\cdot] + [EF1_{red}H^{-}] = E_t \quad (2)$$

where A is absorbance, E_t is the total concentration of enzyme, and ϵ_0, ϵ_1, and ϵ_2 are the molar absorptivities of, respectively, $EF1_{ox}$, $EF1^{-}\cdot$, and $EF1_{red}H^{-}$. Equation 1 was evaluated at 375 and 450 nm. The path length was 1.00 cm. E_t was determined as described above from the absorption spectrum of glycolate oxidase in 0.1 M phosphate at pH 7. It was assumed that negligible loss of glycolate oxidase occurred during the manipulations described above for changing to new experimental conditions. Absorption spectra from each experiment yielded the values for ϵ_0 and ϵ_2 that are presented in Table IV of the supplementary material (see paragraph at end of paper regarding supplementary material). For ϵ_1, 18 000 M^{-1} cm^{-1} and 3700 M^{-1} cm^{-1} were used at 375 and 450 nm, respectively (Table I).

Potentiometric titrations were routinely conducted in the reductive direction. In some cases, an oxidative titration was also performed after completion of the reductive titration. Potentials are reported vs. SHE and are not corrected for temperature. All experiments were run at 10 °C.

Indicator dyes were titrated individually in each buffer at each pH value. These reduction potentials were compared to those obtained in the presence of glycolate oxidase. If E_m of the indicator dye was unchanged in the presence of glycolate oxidase, this was taken as evidence that the dye was not binding to glycolate oxidase. Absorbance values of enzyme–dye mixtures were corrected for each indicator dye either from the method described previously (Stankovich, 1980) or from the absorbance of the isolated dye at the same potential. Corrections were then made for residual absorbance at 800 nm, if necessary, either as a flat base-line correction or as a correction for turbidity. The correction for turbidity was based upon the spectrum of turbid bovine serum albumin.

E_m for glycolate oxidase was calculated by two methods. First, absorbance values at 375 and 450 nm were used as described above to determine the concentrations of $EF1_{ox}$, $EF1^{-}\cdot$, and $EF1_{red}H^{-}$. E_m was then obtained from a linear least-squares analysis of potential vs. log ($[EF1_{ox}]/[EF1_{red}H^{-}]$). $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ were calculated from the equations

$$E_1^{\circ\prime} - E_2^{\circ\prime} = 0.11236 \log [2M/(1 - M)] \quad (3)$$

$$E_1^{\circ\prime} + E_2^{\circ\prime} = 2E_m \quad (4)$$

where M is the maximum concentration of $EF1^{-}$ calculated from absorbance data. Note that the subscripting is opposite that used by Clark (1960). Linear regression analysis of potential vs. log ($[EF1_{ox}]/[EF1^{-}\cdot]$) or log ($[EF1^{-}\cdot]/[EF1_{red}H^{-}]$) was also performed. These results are not reported although they were usually in reasonable agreement with values for $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ obtained from E_m and M.

In the second method of calculation of E_m, the data were fitted by means of a computer program (provided by Dr. Gary

Table I: Summary of Coulometric Titrations of Glycolate Oxidase

	pH				
	7.1	8.2	9.1	9.2	9.0
buffer[a]	4	4	6	11	10
enzyme concn (μM FMN)	12	10	12	8.4	9.0
initial oxygen (mC)	3.8	6.0	3.9	2.1	2.9
equiv/mol of FMN					
first phase	1.3	1.2	1.2	1.1	1.1
total	2.2	2.5	2.1	2.1	2.5
ϵ_1[b] at 375 nm (M^{-1} cm^{-1})					
A vs. *Q*	14 000	13 000	17 000	17 000	18 000
A vs. *A*	15 000	16 000	16 000	18 000	18 000
ϵ_1 at 450 nm (M^{-1} cm^{-1})					
A vs. *Q*	3 200	2 800	3 500	3 600	4 200
A vs. *A*	3 500	4 000	3 500	3 700	4 000
max % radical[c]	53	53	79	81	87

[a] Buffers are listed in Table IV of the supplementary material. [b] Molar absorptivities were obtained from plots of absorbance vs. charge (*A* vs. *Q*) or absorbance at 375 nm vs. abosrbance at 450 nm (*A* vs. *A*). [c] The maximum percent radical that was observed during each titration was calculated as described under Materials and Methods.

Williamson, Emory University) to an equation derived by Michaelis (Clark, 1960):

$$E_h = E_m + \frac{RT}{2F} \ln \frac{1+\mu}{1-\mu} + \frac{RT}{2F} \ln \frac{[1+g(1-\mu^2)]^{1/2}+\mu}{[1+g(1-\mu^2)]^{1/2}-\mu} \quad (5)$$

where E_h is the experimentally measured redox potential, $g = (4/K) - 1$, $K = [EF1^{-}\cdot]^2/([EF1_{ox}][EF1_{red}H^{-}])$, and μ is the degree of oxidation that is scaled from −1 (100% reduced) to 1 (100% oxidized). Redox potentials for the separate electron transfers were calculated from E_m and K by use of eq 4 and

$$E_1^{\circ\prime} - E_2^{\circ\prime} = 0.05618 \log K \quad (6)$$

Circular dichroism measurements were made with a Jasco spectropolarimeter with *d*-10-camphorsulfonic acid as standard. Measurements were made at ambient temperature with the enzyme solutions in quartz cuvettes of 1-cm path length.

Results

The results presented here are discussed in terms of two reactions:

$$EF1_{ox} + e^{-} + H^{+} \rightleftarrows EF1^{-}\cdot \quad (7)$$

$$EF1^{-}\cdot + e^{-} + H^{+} \rightleftarrows EF1_{red}H^{-} \quad (8)$$

These two reactions are referred to as the first and second electron transfers in reduction with corresponding potentials, $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$, respectively.

Coulometric Titrations. The results of spectrocoulometric titration of glycolate oxidase are summarized in Table I. Electrochemical reduction with methyl viologen as mediator of electron transfer produced the red flavoprotein radical (Massey & Palmer, 1966) from pH 7 to pH 9 as illustrated in Figure 1. $EF1^{-}\cdot$ increased to a maximum upon addition of about 1 equiv of charge beyond that required to remove residual oxygen. A second equivalent of charge completed the conversion to $EF1_{red}H^{-}$. During the first phase of the titration, absorbance increased at 375, 400, and 520 nm while absorbance decreased at 450 nm. Isosbestic points were present at 498, 409, and 347 nm. During the second phase of the titration, absorbance due to $EF1^{-}\cdot$ was lost and an isosbestic point appeared at 332 nm.

Absorbance data from coulometric experiments were plotted at wavelengths where spectral differences were greatest vs. the reducing equivalents added per FMN (inset to Figure 1). The

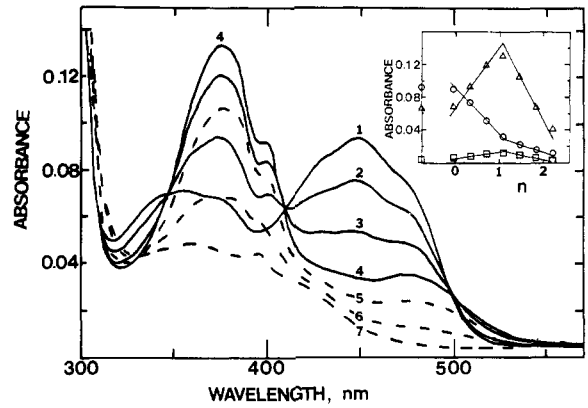


FIGURE 1: Absorption spectra from reductive coulometric titration of glycolate oxidase (8.4 μM as FMN) in 0.26 M TEA-HCl adjusted to pH 9.2 with NaOH. Methyl viologen (0.12 mM) was mediator of electron transport. After 2 mC was added to remove residual oxygen, glycolate oxidase was titrated with aliquots of 0.5 mC. Spectrum 2 corresponds to addition of a total of 3 mC. Succeeding spectra are shown at intervals of 1 mC. Solid lines show radical increasing from zero at the start of the titration (spectrum 1) to a maximum of 81% of the total FMN at 1.1 equiv added per mole of FMN (spectrum 4). Dashed lines show the decrease in radical that occurred between 1.1 and 2.1 equiv added per mole of FMN. (Inset) Absorbance vs. equivalents of charge at 375 (triangles), 450 (circles), and 520 nm (squares).

intersections of the linear portions of these plots represent absorbances due to 100% formation of $EF1^{-}\cdot$ and also the amount of charge required for completion of the first phase of the titration. Extrapolation of these lines to the initial and final absorbance values determined the values that were used for respectively charge taken by initial oxygen and total charge required for the titration.

Molar absorptivities of $EF1^{-}\cdot$ were obtained from plots of absorbance vs. charge (inset to Figure 1) or from plots of absorbance at 375 nm vs. absorbance at 450 nm. Results from the two types of plot are in reasonable agreement. Since a large amount of $EF1^{-}\cdot$ is obtained at a pH of 9 or higher (80 ± 8%), the extrapolation methods should yield reasonable values for the molar absorptivities of $EF1^{-}\cdot$.

In the absence of poising dyes, transfer of electrons to $EF1^{-}\cdot$ became more difficult as the pH was increased. This fact is indicated by the higher percentage of $EF1^{-}\cdot$ observed at pH 9 (Table I) and by the stage of the titration at which absorbance due to $MV^{+}\cdot$ began to appear in the spectra. At pH 7.1, $MV^{+}\cdot$ did not appear until the end of the coulometric titration. At pH 8 and 9, absorbance due to $MV^{+}\cdot$ appeared in the spectra after the titrations were approximately three-fourths and one-half completed, respectively.

As shown by potentiometric titrations described below, the percentages of $EF1^{-}\cdot$ given in Table I are not equilibrium values. Less than 40% of glycolate oxidase was present as $EF1^{-}\cdot$ during potentiometric titrations whereas 50–90% conversion to $EF1^{-}\cdot$ was obtained during coulometric titrations. The high percentages of $EF1^{-}\cdot$ observed during coulometric titrations must be due to kinetic barriers that are overcome in the presence of indicator dyes. Similar effects have been reported for glucose oxidase (Stankovich et al., 1978).

Potentiometric Titrations. In the majority of cases, no indicator dye had an E_m that matched both $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ of glycolate oxidase. For this reason, two dyes were often used. The two dyes, Pyo and IDS or Pyo and 8CR, bracketed the potentials of glycolate oxidase.

The pH dependence values of E_m for the poising dyes were 84–98% of expected values, indicating a small systematic error. For Pyo the value was −0.051 V/unit pH; for IDS or 8CR,

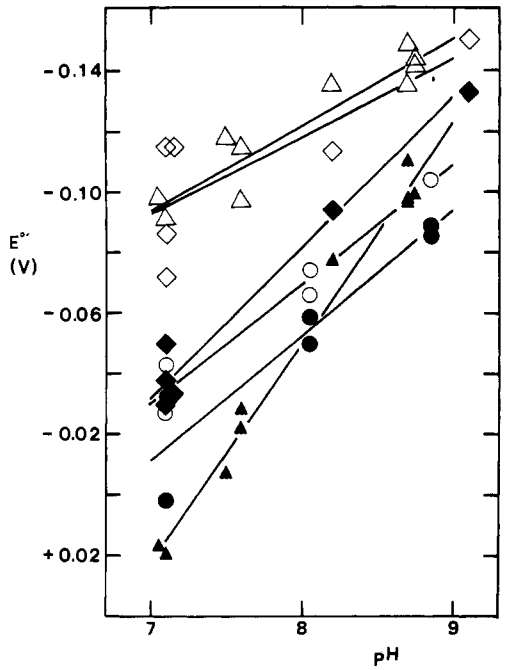


FIGURE 2: Potential vs. pH plots for glycolate oxidase. $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ values are represented by open symbols and filled symbols, respectively. The lines are derived from least-squares analysis of the data from experiments conducted in the presence of 5 mM Cl^- (○, ●), 0.1 M Cl^- (△, ▲), or 0.1 M phosphate (◇, ◆).

the value was −0.026 V/unit pH. E_m of Pyo (−0.012 V) in 0.1 M phosphate at pH 7.1 was similar to that reported by Szentrimay et al. (1977) but differed from other values in the literature (Clark, 1960; Prince et al., 1981; Cammack et al., 1976). Detailed results of potentiometric titrations of indicator dyes are available in Table II of the supplementary material.

Pyo and IDS absorb at 687 and 610 nm, respectively, where glycolate oxidase absorbance is negligible. Therefore, reduction of the indicator dye could be monitored during titration of glycolate oxidase as long as interference from turbidity was negligible. E_m for Pyo or IDS in the presence of glycolate oxidase was usually within 0.007 V of that of the dye in the absence of enzyme. This agreement indicates no strong binding of Pyo or IDS by glycolate oxidase. The absence of binding of 8CR to glycolate oxidase could not be confirmed in a similar way since there is no wavelength at which 8CR absorbs and glycolate oxidase does not absorb. However, at pH 7.1 in 0.1 M imidazole–chloride, similar results were obtained in the presence of Pyo as the only indicator dye or in the presence of both Pyo and 8CR.

The results of potentiometric titrations of uncomplexed, inhibited, or activated glycolate oxidase are summarized in Figure 2 and in Table III in the supplementary material. In the pH range 7–9, $E_1^{\circ\prime}$ values for glycolate oxidase are very similar in 0.1 M chloride (inhibitor) or 0.1 M phosphate (activator) and are 0.035–0.065 V more negative than those obtained in 5 mM chloride (uncomplexed glycolate oxidase). At pH 7.1, $E_1^{\circ\prime}$ of glycolate oxidase in 0.1 M phosphate and 0.1 M chloride is −0.096 ± 0.017 V. Specific ion effects on $E_2^{\circ\prime}$ are more pronounced. At pH 7.1, $E_2^{\circ\prime}$ is −0.038 ± 0.010 V in 0.1 M phosphate, 0.019 ± 0.002 V in 0.1 M chloride, and −0.017 ± 0.017 V in 5 mM chloride. Both $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ are pH-dependent; values ranged between 0.7 and 1.3 protons per electron.

Oxidative and reductive titrations of glycolate oxidase showed average differences of 0.002 V for E_m and 0.008 V for $E^{\circ\prime}$ values. These differences are within the range observed for potentiometric titrations of flavoprotein oxidases (Stankovich & Fox, 1983, 1984). A notable exception is the experiment at pH 7.1 in 5 mM chloride where the differences between oxidative and reductive titrations are 0.019 V and 0.034 for $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$, respectively. A certain amount of hysteresis is expected due to the approach to equilibrium from opposite directions; however, the changes in $E^{\circ\prime}$ at pH 7.1 in 5 mM chloride are much larger than usual. The combination of low ionic strength and neutral pH may slow equilibration of electrons between the three redox states of glycolate oxidase or between glycolate oxidase and the poising dyes. Slow equilibration may involve binding of the dyes to glycolate oxidase. Binding of IDS to glycolate oxidase would be consistent with the −0.013 V shift of E_m for IDS in the presence of glycolate oxidase at pH 7.1 in 5 mM chloride.

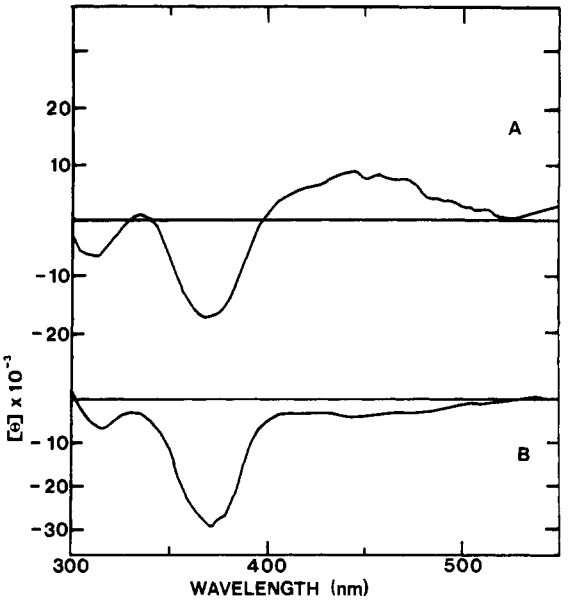


FIGURE 3: Circular dichroism spectra of 29.2 μM glycolate oxidase (A) and 22.5 μM lactate oxidase (B) in 10 mM imidazole–4 mM Cl^-, pH 7.0. [θ] is in units of deg cm^2 $dmol^{-1}$.

The effect of the competitive inhibitor oxalate upon the redox behavior of glycolate oxidase was examined in 0.10 M inidazole, 0.099 M chloride, and 0.01 M oxalate at pH 7.6 and 10 °C. Oxalate was assumed to have no effect upon the redox behavior of the poising dyes that were used in this experiment, Pyo and 8CR. E_m of glycolate oxidase in the presence of oxalate was −0.085 V, and a maximum of 2% $EF1^{-\cdot}$ was calculated to be present during the experiment. Calculations based upon such a low percentage of $EF1^{-\cdot}$ are subject to large errors, but the values obtained were $E_1^{\circ\prime}$ = −0.16 V and $E_2^{\circ\prime}$ = −0.01 V. These results for glycolate oxidase are qualitatively similar to those obtained for D-amino acid oxidase in the presence of the competitive inhibitor benzoate (Van der Berghe-Snorek & Stankovich, 1985). For both enzymes, the competitive inhibitor causes a decrease in radical formation and a large negative shift in $E_1^{\circ\prime}$.

Circular Dichroism. What structural feature, unique to glycolate oxidase, causes the redox properties to be different from those of the other flavoprotein oxidases that have been studied? Is that feature revealed by CD? The CD spectrum of glycolate oxidase in the visible region of the spectrum appeared to be somewhat unusual compared to those of other flavoprotein oxidases (Edmondson & Tollin, 1971; Merrill et al., 1979). The spectrum is shown in Figure 3 along with that of lactate oxidase. Glycolate oxidase exhibits positive circular dichroism, which extends from 400 to 500 nm. The maximum molar ellipticity in this region was 8000 deg cm^2 $dmol^{-1}$. Between 300 and 400 nm, glycolate oxidase inhibits two

negative circular dichroism peaks centered at 367 (−18 000 deg cm^2 dmol^{-1}) and 304 nm (−5500 deg cm^2 dmol^{-1}).

The CD spectrum of lactate oxidase resembles that of glycolate oxidase between 300 and 400 nm. There is a prominent negative CD peak centered at 370 nm (−28 000 deg cm^2 dmol^{-1}) and a weaker peak centered at 315 nm (−5500 deg cm^2 dmol^{-1}). From 400 to 500 nm, lactate oxidase exhibited a broad negative CD (−4000 deg cm^2 dmol^{-1}), in contrast to the positive CD shown by glycolate oxidase in this region. The CD peaks of lactate oxidase in the visible region are of opposite sign to those of the FAD-containing oxidases (Edmondson & Tollin, 1971). This is consistent with the results obtained by Edmondson and Tollin (1971) for FMN- and FAD-containing dehydrogenases. The CD spectrum of pyridoxamine oxidase (Merrill et al., 1979), another FMN-containing oxidase, resembles that of lactate oxidase in having negative circular dichroism throughout the visible region, although the relative strengths of the bands above and below 400 nm are reversed. Therefore, glycolate oxidase is the only flavoprotein oxidase that exhibits a different sign for the CD bands that correspond to the two major flavin electronic transitions in the visible spectrum. In this respect, at least, glycolate oxidase resembles a dehydrogenase.

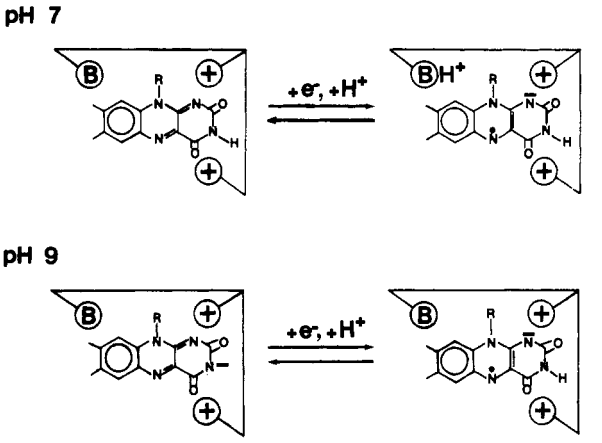
Discussion

The redox potentials of glycolate oxidase are shifted markedly positive of those of unbound FMN. E_m for glycolate oxidase at pH 7.1 in 0.1 M phosphate is −0.068 ± 0.013 V. This is 0.14 V positive of E_m for unbound FMN (Draper & Ingraham, 1968) and is 0.02 V positive of E_m for the substrate couple glyoxalate/glycolate (Clark, 1960). Interactions between FMN and the apoprotein of glycolate oxidase produce an oxidant that can react with glycolate in a thermodynamically feasible reaction.

Jorns and Hersh (1976) reported an E_m of −0.170 V at pH 7 for glycolate oxidase substituted with 5-deaza-FMN. Unbound 5-deaza-FMN has an E_m of −0.278 V; therefore, the E_m of the 5-deazaflavin was shifted positive by 0.108 V when bound to apoenzyme. The magnitude of the positive shift of E_m is 0.032 V less for 5-deaza-FMN-substituted glycolate oxidase than for native glycolate oxidase. It may be that the apoprotein of glycolate oxidase has a higher ratio of binding constants for the oxidized and reduced forms of 5-deaza-FMN than for the oxidized and reduced forms of FMN. An alternative explanation is that E_m for 5-deaza-FMN-substituted glycolate oxidase is altered by the presence of the redox couple pyruvate/lactate. Binding of substrate analogues (competitive inhibitors) can drastically change the thermodynamics of electron transfer in D-amino acid oxidase (Van den Berghe-Snorek & Stankovich, 1985), and similar effects were reported here for glycolate oxidase in the presence of oxalate. Oxalate binding to glycolate oxidase affects the pK_a at the N(3) position of FMN in $EF1_{ox}$ (Schuman & Massey, 1971a) and shifts $E_1^{\circ\prime}$ negative by 0.04 V. This indicates tighter binding of oxalate to $EF1_{ox}$ than to $EF1^{-}\cdot$. Oxalate had little effect upon $E_2^{\circ\prime}$. This indicates equivalent (perhaps zero) binding to $EF1^{-}\cdot$ and $EF1_{red}H^{-}$.

Although 0.1 M phosphate has been shown to activate glycolate oxidase at pH 7 and 0.1 M chloride is inhibitory (Schuman & Massey, 1971a), there is no obvious correlation of these kinetic properties with the redox potentials of glycolate oxidase. E_m in 0.1 M phosphate at pH 7.1 (−0.068 V) is more negative than E_m in 0.1 M chloride (−0.038 V). There is, however, an obvious effect of conducting the experiments in 5 mM chloride. For example, E_m at pH 8.2 in 5 mM chloride is −0.066 V whereas in 0.1 M chloride E_m is −0.106 V and in 0.1 M phosphate E_m is −0.104 V.

Scheme I



Many other flavoprotein oxidases exhibit positive shifts in E_m compared to unbound flavin. These include glucose oxidase (Stankovich et al., 1978), thiamin oxidase (Gomez-Moreno et al., 1979), lactate oxidase (Stankovich & Fox, 1983), L-amino acid oxidase (Stankovich & Fox, 1984), and D-amino acid oxidase (Van den Berghe-Snorek & Stankovich, 1985). For these enzymes, $E_1^{\circ\prime}$ is usually more positive than $E_2^{\circ\prime}$, and with the exception of glucose oxidase, large amounts of flavoprotein radical are thermodynamically stabilized. In contrast, glycolate oxidase exhibits large positive shifts of both $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$. Therefore, less than 40% of $EF1^{-}\cdot$ is thermodynamically stabilized by glycolate oxidase.

The marked positive shift in $E_1^{\circ\prime}$ in the flavoprotein oxidases is consistent with the proposal (Massey & Hemmerich, 1980) that the flavin binding sites of the flavoprotein oxidases have a common structural feature, an interaction between a positively charged group on the polypeptide and the N(1)—C(2)=O locus of the flavin. In the case of D-amino acid oxidase, the positive group has been identified as an active site arginine (Fitzpatrick & Massey, 1983). When that bond is disrupted, as when benzoate binds to D-amino acid oxidase, $E_1^{\circ\prime}$ shifts negative, so that $E_1^{\circ\prime}$ for benzoate-bound D-amino acid oxidase is similar to that of unbound flavin (Van den Berghe-Snorek & Stankovich, 1985).

Both $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ of glycolate oxidase are pH-dependent over the pH range 7–9 (Figure 2). In contrast, $E_1^{\circ\prime}$ for L-amino acid oxidase (Stankovich & Fox, 1984) or D-amino acid oxidase (Van den Berghe-Snorek & Stankovich, 1985) is independent of pH in this range. The FMN of $EF1_{ox}$ is negatively charged over a portion of the pH range 7–9 whereas the flavins of other oxidases are uncharged in the oxidized state in this pH range. Schuman and Massey (1971a) obtained a pK_a of 8.0 for FMN of $EF1_{ox}$ on the basis of pH-dependent spectral changes. These spectral changes were also observed for the glycolate oxidase preparations used here, but there are no breaks in the potential–pH plots for glycolate oxidase (Figure 2) and the red, anionic radical is observed from pH 7 to pH 9.

The unique spectral and redox properties of glycolate oxidase suggest the following picture of the active site (Scheme I). A positively charged amino acid residue is assumed to interact at the N(1)—C(2)=O locus, consistent with the common structural feature of the oxidases (Massey & Hemmerich, 1980). A second positively charged amino acid is shown interacting with the flavin at the N(3)—C(4)=O locus (Jorns et al., 1983). These positively charged amino acids may cause the unusually low pK_a of 8.0 at N(3) of $EF1_{ox}$. They also contribute to the marked positive shifts of the redox potentials of glycolate oxidase.

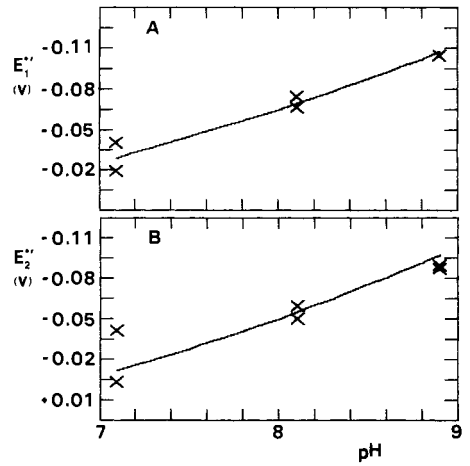


FIGURE 4: pH dependence of $E^{\circ\prime}$ for free glycolate oxidase. The lines were drawn by computer simulation of Scheme I with the values $pK_{Fl,0}$ = 8, $pK_{B,0}$ = 6, $pK_{Fl,1}$ = 5, $pK_{B,1}$ = 7.5, $pK_{Fl,2}$ = 5, and $pK_{B,2}$ = 7. Numerical subscripts refer to the oxidation state of the flavin; 0 = oxidized, 1 = radical, 2 = fully reduced. The subscript Fl refers to flavin, and B refers to base 3. The data points (×) are $E_1^{\circ\prime}$ values (A) and $E_2^{\circ\prime}$ values (B) obtained in the presence of 5 mM Cl^-.

The first electron transfer to glycolate oxidase involves different forms of $EF1_{ox}$ at pH 7 and at pH 9 (Scheme I) because of the pK_a or 8.0 at N(3) of FMN. However, the anion radical is stabilized over the entire pH range. At pH 9, transfer of the first electron requires transfer of a proton to FMN because $EF1_{ox}$ is already anionic. At pH 7, transfer of the first electron to $EF1_{ox}$ does not involve transfer of a proton to FMN, yet the redox potential for this transfer is pH-dependent (Figure 2). The proton must bind to a third amino acid near the FMN. This amino acid is designated by the letter B in Scheme I. The affinity of this amino acid for protons must be dependent upon the oxidation state of the flavin. It is unprotonated in $EF1_{ox}$ but has a pK_a of approximately 7.5 in $EF1^{-}\cdot$ (Figure 4). Proton uptake by this base can, therefore, complement that of FMN during the first electron transfer to glycolate oxidase. Conversion of $EF1^{-}\cdot$ to $EF1_{red}H^-$ is accompanied by proton transfer to the flavin from pH 7 to pH 9.

In the literature of flavins and flavoproteins, there are examples of redox-state-dependent changes of protonation of basic groups near the flavin that do not necessarily cause changes in spectral properties (Matthews & Williams, 1976; Williamson & Edmondson, 1985; Ohnishi et al., 1981). However, the proposed interaction of the C(4) carbonyl with a positively charged species from protein in glycolate oxidase (Jorns et al., 1983) appears to be unique among the flavoprotein oxidases. Resonance Raman studies (Visser et al., 1983) have shown that lactate oxidase is hydrogen-bonded only at the C(2) carbonyl. Studies with 4-thioflavin show that in D-amino acid oxidase the C(4) carbonyl position of the flavin is accessible to solvent, suggesting that this position of flavin is not hydrogen bonded to enzyme (Massey et al., 1984). In contrast, resonance Raman and X-ray studies show that flavodoxin from *Megasphera elsdenii* has hydrogen bonds at the C(4) carbonyl as well as at the N(1) and N(5) positions (Visser et al., 1983; Mayhew & Ludwig, 1975). Therefore, with regard to hydrogen bonding at the C(4) carbonyl, glycolate oxidase is more like the dehydrogenases and less like the other oxidases. The CD spectrum of glycolate oxidase is also more similar to flavodoxin than to the FMN-containing oxidase lactate oxidase. Although CD data are difficult to relate to specific structures, these data taken together with electrochemical and resonance Raman data suggest that the glycolate oxidase structure is different from those of other oxidases and is similar to that of the dehydrogenases. If this difference is due to the postulated C(4) bond and the positioning of a third base at the active site, this would be most clearly shown by resonance Raman spectroscopy (Dutta et al., 1977; Kitagawa et al., 1979; Schmidt et al., 1983; Visser et al., 1983).

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SUPPLEMENTARY MATERIAL AVAILABLE

Details of potentiometric titrations of indicator dyes (Table II) and glycolate oxidase (Table III) and extinction coefficients used in eq 1 (Table IV) (3 pages). Ordering information is given on any current masthead page.

Registry No. Cl^-, 16887-00-6; P_i, 14265-44-2; glycolate oxidase, 9028-71-1; oxalate, 144-62-7.

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β-Glucosidase: Substrate, Solvent, and Viscosity Variation as Probes of the Rate-Limiting Steps†

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ABSTRACT: The second-order rate constants (k_{cat}/K_m) for the β-glucosidase-catalyzed hydrolysis of aryl β-D-glucopyranosides show a bell-shaped dependence on pH. The pK_as that characterize this dependence are 4.4 ($\Delta H_{ion} \approx 0$) and 6.7 ($\Delta H_{ion} \approx 0$). In D_2O these pK_as are increased by 0.5 (±0.1) unit, but there is no solvent isotope effect on the pH-independent second-order rate constant. Nath and Rydon [Nath, R. L., & Rydon, H. N. (1954) *Biochem. J. 57*, 1-10] examined the kinetics of the β-glucosidase-catalyzed hydrolysis of a series of substituted phenyl glucosides. We have extended this study to include glucosides with phenol leaving groups of pK_a < 7. Brønsted plots for this extended series were nonlinear for both k_{cat}/K_m and k_{cat}. Brønsted coefficients for those compounds with leaving groups of pK_a > 7 (for k_{cat}/K_m) or pK_a > 8.5 (for k_{cat}) were nearly equal to −1.0, indicating substantial negative charge buildup on the leaving group in the transition state. The nonlinearity indicates an intermediate in the reaction. This was confirmed by partitioning experiments in the presence of methanol as a competing glucose acceptor. A constant product ratio, [methyl glucoside]/[glucose], was found with aryl glucoside substrates varying over 16 000-fold in reactivity (V/K), indicative of a common intermediate. Viscosity variation (in sucrose-containing buffers) was used to probe the extent to which the β-glucosidase reactions are diffusion-controlled. The results suggest that while k_{cat}/K_m may be limited by the association of the enzyme with the more reactive substrates (leaving group pK_a < 7), k_{cat} is not limited by product dissociation.

Glucosidases are widespread in nature, efficiently catalyzing the hydrolysis of various glycosides and oligosaccharides. Although β-glucosidase (EC 3.2.1.21) from sweet almond was one of the earliest enzymes investigated (Wohler & Liebig, 1837; Fischer, 1898), its mechanism of action is still far from clearly understood. Some indirect evidence suggests that β-glucosidase undergoes a double-displacement mechanism. Eveleigh and Perlin (1969) have shown that the reaction proceeds with retention of configuration at the anomeric carbon (C-1), and it is known that it is the glucosyl C–O bond that is cleaved (Bunton et al., 1954; Rosenberg & Kirsch, 1981). The β-glucosidase-catalyzed hyrolysis of phenyl β-glucopyranoside shows essentially no secondary deuterium kinetic isotope effect on V/K (Dahlquist et al., 1969). The simplest, but not only (Knier & Jencks, 1980), explanation of this is that there is no hybridization change at C-1 in the rate-limiting step (i.e., an S_N2 type mechanism). More recently, Weber and Fink (1980) have demonstrated "burst" kinetics with PNPGlc[1] at subzero temperatures. This is consistent with an intermediate in the β-glucosidase reaction. This work was undertaken in order to further probe the nature of this putitive intermediate.

Because of the rather broad specificity of β-glucosidase [see Dale et al. (1985)], it is ideally suited for structure–reactivity studies. Indeed, Nath and Rydon (1954), using a series of 21 substituted phenyl glucosides (leaving group pK_a > 7), persuasively demonstrated a structural sensitivity of the β-glucosidase reaction similar to that of the alkaline hydrolysis reaction. We report here an extension of this study to include phenyl glucosides with more acidic leaving groups. We also report the applications of solvent kinetic isotope effects, pH

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[1] Abbreviations: PNPGal, *p*-nitrophenyl β-D-galactopyranoside; PNPGlc, *p*-nitrophenyl β-D-glucopyranoside; DNPGal, dinitrophenyl β-D-galactopyranoside; DNPGlc, dinitrophenyl β-D-glucopyranoside; EDTA, (ethylenedinitrilo)tetraacetic acid; MES, 4-morpholineethanesulfonate; PIPES, 1,4-piperazinediethanesulfonic acid; SKIE, solvent kinetic isotope effect.

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