The Glucose Oxidase Mechanism

INTERPRETATION OF THE pH DEPENDENCE*

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SUMMARY

The pH dependence of the steady state parameters of the glucose oxidase (EC 1.1.3.4, from Aspergillus niger) reaction was determined by O₂-monitored experiments over the entire pH range from 3 to 10 at 25°, with D-glucose as substrate. The data were fitted to a three-parameter steady state rate equation and the significance of the steady state parameters was examined by stopped flow half-reaction and turnover measurements at the extremes of the pH range used. The major conclusions from these studies can be summarized as follows.

1. At low pH, in the presence of halide, the maximum turnover number (kcat) is determined entirely by the rate of flavin reduction (k₆) in the reductive half-reaction. Furthermore, substrate combines only with an unprotonated form of the oxidized enzyme and the reductive half-reaction can be represented as follows.

$$H^+ E_0 \xrightleftharpoons[K_1]{K_{-1}} E_0 + S \xrightarrow{k_6} E_0 - S \xrightarrow{k_3} E_6 + \delta-lactone$$

Since k₆ and k₃ are both specifically decreased by halides at low pH values, it is probable that the turnover rate in the low pH range is also limited by k₆ in the absence of halide. The steady state absorption spectrum of E₀ - S is indistinguishable from the spectrum of E₀. This finding, together with the fact that removal of the 1-hydrogen from D-glucose is a rate-limiting process in flavin reduction is consistent with both a hydride transfer mechanism and a flavin-glucose adduct mechanism in which this adduct is relatively unstable and never accumulates significantly as a kinetic intermediate.

2. The importance of k₃ as a limiting first order process in turnover diminishes as the pH is raised. Thus, at pH 10 the major first order process in turnover is the breakdown of a species of oxidized enzyme, E₃', in the oxidative half-reaction. The rate of this process at pH 10.0 is 214 sec⁻¹, whereas k₃ has a value of 800 sec⁻¹.

3. The reduced enzyme exists in two kinetically significant states of ionization, E₆ and E₆⁻. The rapid reoxidation of E₆ with O₂ to regenerate E₀ is predominant at pH values less than 7. At pH values greater than 7, a much less rapid reaction of E₆⁻ with O₂ leading to the formation of E₆'O, becomes increasingly important. The species E₆'O is unreactive with glucose and it is the conversion of a protonated form of E₆'O to E₀ which principally governs k₆ at pH values greater than 7.

We present a complete kinetic scheme describing the effects of pH and discuss the possible chemical significance of the species E₆'O.

The oxidation of aldoses to the corresponding lactones catalyzed by glucose oxidase (EC 1.1.3.4) is shown in Equation 1.

$$R\xrightarrow{O_2} E_0 \xrightarrow{O_2} E_6 \xrightarrow{H_2O_2} \delta-lactone$$

**Equation 1**

The first rapid reaction studies were reported by Gibson, Swoboda, and Massey (1) and by Nakamura and Ogura (2). Recently, Bright and Appleby (3) have described the pH dependence of individual steps of the reaction in studies (with the enzyme from Penicillium notatum) which were based on the kinetic mechanism elaborated by Gibson et al. (1) for the Aspergillus niger enzyme. With β-D-glucose, at 25° and pH 5.5, the general mechanism of Gibson et al. reduced to

$$E_0 + S \xrightarrow{k_1} E_0 + P$$

$$E_0 + O_2 \xrightarrow{k_3} E_0 + H_2O_2$$

**Scheme 1**

The following abbreviations are used: E₀, total catalytically active enzyme expressed in terms of substrate-reducible FAD; E₆, catalytically active enzyme in which FAD is in the oxidized form; E₆', catalytically active enzyme in which FAD is in the reduced form; S, β-D-glucose. It should be noted that whereas previously (3) the mechanism for glucose oxidation at 25° was analyzed in terms of three steps and four essential ionizations, in these studies the mechanism has been considerably extended and modified. It has proved convenient to initially analyze turnover experiments in terms of the three parameters k₆, k₃ and k₆', and then examine the makeup of these parameters. As far as possible, the rate and equilibrium constant notation used previously (4) has been retained.
In contrast, Nakamura and Ogura deduced the following scheme for both the A. niger and P. notatum enzymes (2, 4) at pH 5.5 and 25°C.

\[ E_0 + S \xrightarrow{k_1} E_0 - S \xrightarrow{k_2} E_r + P \]  
(4)

\[ E_r + O_2 \xrightarrow{k_3} E_r + H_2O_2 \]  
(5)

Scheme 2

Both mechanisms require a single first order step involving an oxidized species of the enzyme. This species is identified as \( E_r \) in Scheme 1 and as \( E_0 - S \) in Scheme 2. The two mechanisms are homeomorphic with respect to their steady state turnover behavior.

From a combination of stopped flow and \( O_2 \)-monitored experiments, we show that the pH profiles of the A. niger enzyme are in substantial agreement with those of \( P. \) notatum reported by Bright and Appleby in the presence of 0.2 M KCl (3), but that the entire pH profile should be interpreted as a hybrid of Schemes 1 and 2.

**EXPERIMENTAL PROCEDURE**

**Materials**

Glucose oxidase (\( A. \) niger), analytical grade was obtained from Mann Research Laboratories. The enzyme was further purified by chromatography on Whatman DE-23 DEAE-cellulose. A column, 2.5 x 30 cm, was equilibrated with 0.05 M potassium phosphate, pH 6.0, and approximately 200 mg of the protein were applied in 20 ml. The column was washed with 500 ml of the same buffer and then subjected to a 2-litter linear potassium phosphate gradient (pH 6.0, from 0.05 to 0.30 M).

A sample from the center of the yellow enzyme band was homogeneous by standard disc gel electrophoresis and showed negligible catalase activity. A sensitive measurement of trace catalase activity in the purified glucose oxidase preparations indicated it to be present at noninterfering levels. Oxidase evolution as a result of catalase in the purified enzyme was measured in the following manner. An anaerobic solution of 10 \( \mu M \) glucose oxidase was incubated in the \( O_2 \) monitor at pH 7.0 in the presence of 10\(^{-4} \) M EDTA. \( H_2O_2 (10^{-4} \) M) was added to the anaerobic enzyme solution and \( O_2 \) evolution was measured. The rate of \( O_2 \) evolution was several orders of magnitude smaller than the rate of glucose oxidase by 10 \( \mu M \) glucose oxidase under turnover conditions, where the highest attainable concentration of \( O_2 \) is about 10 \( ^{-6} \) M. Furthermore, addition of \( CN^- \) to both turnover and stopped flow experiments was without effect.

The purified enzyme was stored either as a precipitate in ammonium sulfate (60 g/100 ml) solution or in 0.1 M phosphate, pH 6.0, at 4°C. Enzyme was routinely recovered and concentrated, or subjected to buffer exchange, by means of the Schleicher and Schuell collodion bag apparatus (Schleicher and Schuell Company, Keene, New Hampshire).

Anhydrous dextrose, chemically pure, was obtained from Fassottich Laboratories, Waukegan, Illinois, and all solutions were allowed 24 hours at 25°C to mutarotate to equilibrium. Under all conditions of turnover used, mutarotation is very slow compared with the total time required for the last experimental point to be taken. All concentrations of glucose are expressed as the amount of \( \beta \) anomer present.

The following buffers were used and contained 10\(^{-4} \) M EDTA: 0.01 M potassium citrate for the pH range 3.0 to 5.0, with the exception of fluoride solutions in which species such as HF\(^-\) are sufficient buffering agents; 0.01 M imidazole-HCl for the pH range 6.0 to 8.0; 0.01 M Tris-HCl for the pH range 7.0 to 9.0; 0.01 M L-leucine or L-alanine for pH 10.0. Buffer adjustments were made with HSO\(_4\) or KOH. All chemicals, including buffers, were of "analyzed" or "certified" grade. Water was commercially deionized and solutions routinely millipored before use.

Control of ionic strength was not necessary at the low buffer concentrations used. No specific buffer effects were observed.

**Methods**

**Enzyme Concentration**—All enzyme concentrations are expressed as molarity of catalytically active bound FAD. Active flavin was determined spectrophotometrically on a Cary model 15 with a differential molar extinction coefficient of 1.31 × 10\(^{4} \) M\(^{-1}\) cm\(^{-1}\) at 450 nm. This value was determined elsewhere (5) and represents the differential molar extinction coefficient between oxidized and reduced bound FAD of the enzyme from \( A. \) niger in 0.01 M potassium acetate, pH 5.5. This value is in good agreement with published spectra (6) and a reported extinction coefficient value of 1.4 × 10\(^{4} \) M\(^{-1}\) cm\(^{-1}\) for oxidized enzyme at 450 nm (7).

Typically the absorbance of solutions containing 1 to 10 \( \mu M \) bound FAD was measured in a standard 1-ml cuvette with 0.01 M potassium acetate, pH 5.5. A 1.0 M glucose solution, 10 \( \mu l \), was added via a Hamilton syringe, the solution mixed, and the cuvette top sealed with parafilm. The absorbance was then recorded for the reduced form and appropriate dilution corrections made. Accuracy was limited only by pipetting procedures and was within 1%.

Enzyme stability at the extremes of the pH range of interest was checked by means of \( O_2 \)-monitored turnover experiments. With 0.10 M glucose and partially air-saturated solutions, rates of 0.2 oxygen uptake were determined throughout a 15-min experiment in which greater than 90% of the \( O_2 \) was eventually consumed. Pores of \( E_r/O \) versus 1/0 were linear, indicating that \( E_r \) was conserved throughout the entire experiment. Since turnover data for kinetic analysis were gathered in less than 5 min in each case, none of the results is complicated by irreversible or slowly reversible effects of pH on the enzyme.

**Oxygen-monitored Turnover**—The Clark electrode, power source, and amplifier assembly were obtained from Yellow Springs Instrument Company, Yellow Springs, Ohio. The electrode chamber was thermostatted at 25°C and a 10-inch Beckman recordor was used. A 3-ml aliquot of the appropriate glucose solution was equilibrated by rapid stirring for 5 min with air or by bubbling \( O_2 \) into the chamber. The value used for \( O_2 \) solubility in pure water at 25°C and total pressure of 760 mm was 1.21 × 10\(^{-3} \) M (8). \( O_2 \) concentrations in dissolved air were referenced to this value and appropriate barometric corrections were made when significant. Low buffer and glucose concentrations were used when possible in order that the \( O_2 \) activities measured by the electrode reflect actual oxygen concentrations.

No corrections were made for salt effects on the solubility of \( O_2 \) in the halide experiments as the maximum concentration of salt was 0.1 M and would only amount to a maximum correction of...
3% (9). Additions of enzyme were made to the system through a groove in the electrode holder with Hamilton syringes. Since the data obtained are integral in nature, integrated rate equations for turnover were used. It was found that a simple three parameter equation (Equation 6) was adequate to fit all data at the 1% level for a given pH. Equation 6 is the integrated form of Equation 7.

\[ \log t = \log \left( \frac{1}{k_{\text{red}}} \right) \log \left( \frac{[\bar{S}]_0}{([\bar{S}]_0 - ([O_2] = [O_2]_0))} \right) + \left( \frac{1}{k_{\text{ox}}} \log ([O_2]_0)/([O_2]_0) + \left( \frac{1}{k_{\text{cat}}} \log ([O_2]_0)/([O_2]_0) \right) \right) \]

(6)

\[ E_{\text{T}}/V = \frac{1}{k_{\text{red}}}[S] + \frac{1}{k_{\text{ox}}}[O_2] + \frac{1}{k_{\text{cat}}} \]

(7)

These equations are based on a minimal mechanism involving two bimolecular steps and one monomolecular step in the turnover sequence. The integral data from measurements at 10 or more glucose concentrations, in which oxygen had been 80% depleted, were fitted by Equation 6 by optimizing the three rate parameters. A nonlinear regression program developed by Marquardt (10), SHARE 3049, was revised to accommodate weighting and adapted to the problem. Confidence interval algorithms are built into the program and are indicated in the appropriate figures by vertical bars. Studies with fluoride at low pH were essentially zero order in O2 and linear plots of E_{\text{T}}/V versus 1/[S] were hand calculated.

Stopped Flow Turnover Experiments—These experiments were carried out at pH 10 at 25° with the Gibson-Durrum stopped flow apparatus. Bound FAD was measured spectrophotometrically at 450 nm with slit widths less than 1.0 mm. The experiments were performed by a pH jump on the enzyme. The enzyme solutions were unbuffered at pH 7.8 and saturated with O2. The glucose solution was made anaerobic by bubbling deoxygenated N2 through the solution for 15 min. The integral traces of percentage of transmission versus time were converted to absorbance versus time curves, then integrated sectionally and totally by numerical quadrature. Appropriate treatment of the areas obtained appears elsewhere (5). The two principal equations used are 8 and 9.

\[ \int_0^\infty [E_{\text{ox}}] dt = [O_2]_0 \left( \frac{1}{k_{\text{red}}} [S] + 1/k' \right) \]

(8)

\[ \frac{E_{\text{T}}}{[E_{\text{ox}}]} = 1 + k_{\text{cat}} \left( \int_0^\infty [E_{\text{ox}}] dt - \int_0^\infty [E_{\text{red}}] dt \right) + 1 \]

(9)

where E_{ox} is the sum of all oxidized enzyme species and 1/k' is the sum of the reciprocals of all first order rate constants associated with oxidized enzyme species. These yield all parameters obtained from conventional steady state turnover monitored by product formation or substrate depletion.

Differential spectra of the intermediates associated with k_{cat} were obtained by observing initial absorbance of stopped flow experiments as a function of wave length. The steady state concentrations of these intermediates could be readily calculated from known values of rate constants and substrate concentrations.

Stopped Flow Half-reactions—All half-reactions were forced to pseudo first order reactions by maintaining S or O2 >> enzyme. The reductive half-reactions were carried out as follows. The enzyme was slightly buffered at pH 7.8 with 0.001 M L-leucine and was made anaerobic by purging the interior of the reservoir syringe with N2 while stirring internally with a small magnetic bar. After 30 min, 10 µM L-amino acid oxidase (5 µl per ml of glucose oxidase solution) were added and the syringe sealed onto the valve block of the stopped flow instrument. Deoxygenated N2 was bubbled through the buffered glucose solution syringe for 5 min and 10 µM glucose oxidase (5 µl per ml of glucose solution) were added. The solution was then sealed to the valve block. This procedure achieves and maintains anaerobiosis of the solutions extremely well.

The reoxidative half-reactions were carried out as follows. The enzyme side was unbuffered at pH 7.8 and the reservoir syringe swept with N2 as before. Mannose (1.0 M, 1 µl per ml of enzyme solution) was then added and the enzyme was slowly reduced. The O2 side was buffered and initially saturated with either air or O2. Variable O2 concentrations were achieved by carefully mixing air- or O2-saturated solutions with N2-saturated solutions by means of a three-way Hamilton valve. Excess mannose on the millimolar level does not interfere with the reoxidative half reaction as it reduces E_{red} at least two orders of magnitude more slowly than O2 oxidizes E_{ox} under the experimental conditions.

Electron Paramagnetic Resonance Experiment—A turnover reaction of several seconds duration was initiated by mixing 20 µM unbuffered glucose oxidase with 0.05 M glucose, pH 10.0. Both solutions were initially air saturated and mixed rapidly in a simple fashion through a "T" joint. The mixed solution was delivered directly into a standard 3-mm quartz tube immersed in liquid N2 by means of a small diameter, short polyethylene needle. This procedure effectively quenched the reaction by rapid freezing under conditions where the principal rate-limiting process of turnover is the combination of ER with O2. We estimate that 0.5 µM of O2 could have been detected in this experiment, which represents about 5% of the concentration of E_{red} present. Electron paramagnetic resonance spectra were obtained with a Varian E-3 instrument equipped with a variable temperature regulator. The cavity was regulated at 100°K. The following settings were employed: field center at 3280 gauss; field width ± 100 gauss; microwave power 20 milliwatts; modulation amplitude 0.1 to 5.0 gauss at 100 kHz; microwave frequency, 9.17 GHz. At the highest sensitivity, no signal corresponding to any free radical could be detected.

RESULTS

Oxygen-monitored Turnover—Fig. 1, 2, and 3 show, respectively, the pH profiles of k_{red}, k_{ox}, and k_{cat} as obtained from three parameter fits of 02-monitored turnover experiments. The integral data were fit to Equation 6 at the 1% level with O2 and glucose concentrations varied 5- and 10-fold, respectively. The pH profile for k_{red} (Fig. 1), the apparent bimolecular rate constant for the combination of glucose with enzyme, shows two distinct limbs in the absence of halide. The low pH limb is not characteristic of a simple ionization and may reflect nonspecific charge effects associated with several ionizations.
Fig. 1. The pH profile of $k_{red}$ (○, no halide; ●, in the presence of 0.1 M Cl$^-$). Evaluation of $k_{red}$ was carried out by nonlinear regression on Equation 6, using O$_2$-monitored turnover data at the indicated pH values. Confidence intervals indicated by horizontal bars. Temperature 25$^\circ$C; other conditions given under "Experimental Procedure."

Fig. 2. The pH profile of $k_{ox}$. Evaluation of $k_{ox}$ was carried out by nonlinear regression on Equation 6, using O$_2$-monitored turnover data at the indicated pH values. Confidence intervals indicated by horizontal bars. The solid line represents computed values of $k_{ox}$ using Equation 13 and constants from Table I. Temperature 25$^\circ$C; other conditions given under "Experimental Procedure."

Fig. 3 shows the pH profile of $k_{cat}$. This parameter represents the limiting turnover velocity, $v/V_E$, at infinite concentrations of both O$_2$ and glucose. It may reflect more than one first order process in turnover and has the form $(21/k_i)^{-1}$, where the $k_i$ are individual rate constants for all kinetically significant first order processes. It should be noted that $k_{cat}$ reaches a constant value of about 1000 sec$^{-1}$ at low pH in the absence of halide and falls off significantly at high pH. Halide specifically affects this constant at low pH and the effect of 0.10 M Cl$^-$ is indicated in Fig. 3. At low pH, this parameter has rather large confidence intervals because of its small contribution to the turnover equation, but is well correlated. The effect of halide on $k_{cat}$, which will prove to be important in the interpretation of the kinetic mechanism, was not investigated previously (3).
The parameters $k_{\text{red}}$ and $k_{\text{cat}}$ were also decreased in the presence of $F^-$ and $Br^-$ (measured by $O_2$-monitored turnover at pH 4.0 and 0.10 M total halide), the order of effectiveness being $F^- > Cl^- > Br^-$. As previously reported (3), sulfate showed no effect. Figs. 4 and 5 show the effect of $Cl^-$ on the profiles of $k_{\text{red}}$ and $k_{\text{cat}}$, respectively, at pH 3.0. It should be noted in Fig. 5 that it is not possible to isolate $k_{\text{cat}}$ in the low $Cl^-$ experiments as evidenced by the initial uncorrelated interval. This is attributed to the large initial decrease of $k_{\text{red}}$, relative to that of $k_{\text{cat}}$ which causes the turnover equation to be principally controlled by $k_{\text{red}}$ with the substrate concentrations used.

Stopped Flow Reductive Half-Reactions—Fig. 6 shows a comparison of the stopped flow reductive half-reaction data and $O_2$-monitored turnover experiments at pH 4.0 with 0.10 M total fluoride. Under these conditions, the turnover reaction is zero order in $O_2$ and Equation 7 reduces to Equation 10.

$$ET/V = 1/(k_{\text{red}}[S]) + 1/k_{\text{cat}}$$

The fact that an ordinate intercept is observed for the half-

FIG. 5. The $Cl^-$ dependence of $k_{\text{cat}}$ at pH 3.0. Evaluation of $k_{\text{cat}}$ at each $Cl^-$ concentration was carried out by non-linear regression on Equation 6 using $O_2$-monitored turnover data. Confidence intervals indicated by horizontal bars. Temperature 25°C; 0.01 M citrate buffer; other conditions given under “Experimental Procedure.”

FIG. 6. Comparison of stopped flow reductive half reaction data ($1/k_{\text{red, obs}}$, ×) with $O_2$-monitored turnover data ($ET/\bar{v}$, ○) at pH 4.0, 0.1 M total fluoride. The turnover data were analyzed according to Equation 10. Temperature 25°C; other conditions given under “Experimental Procedure.”

FIG. 7. Comparison of stopped flow reductive half-reaction data ($1/k_{\text{red, obs}}$, × in the presence of 0.01 M DL-leucine, ○ in the presence of 0.01 M DL-phenylalanine), stopped flow $E_T$-monitored turnover data ($ET/\bar{v}$, ■) and $O_2$-monitored turnover data ($ET/\bar{v}$, ●) at pH 10.0. The stopped flow and $O_2$-monitored turnover data were analyzed according to Equations 8 and 7, respectively, and were both obtained in the presence of 0.10 M DL-leucine. Temperature 25°C; other conditions given under “Experimental Procedure.” From the ordinate intercepts, $k_2$ and $k_{\text{cat}}$ are 800 ± 200 sec$^{-1}$ and 170 sec$^{-1}$, respectively. From the reciprocals of the slopes, $k_{\text{red}}$ is $1.1 \times 10^5$ M$^{-1}$ sec$^{-1}$.

FIG. 8. Stopped flow $E_T$-monitored turnover data, at pH 10 and with several glucose concentrations, plotted according to Equation 9. Temperature 25°C; 0.01 M DL-leucine; other conditions given under “Experimental Procedure.” The ordinate intercept of unity shows that no first order process in turnover involves a species of reduced enzyme. The value of $k_{\text{cat}}$ from the reciprocal of the slope is $1.2 \times 10^8$ M$^{-1}$ sec$^{-1}$.
reaction plot and is identical with that of turnover indicates that $k_{cat}$ is associated with an oxidized form of the enzyme and occurs in the reductive half-reaction. The simplest explanation is that the reductive half-reaction can be represented as Equation 4.

At high pH, the same comparison (pH 10.0, no halide) shown in Fig. 7, indicates that the slope of the stopped flow reductive half-reaction data is identical with that of both $O_2$-monitored turnover and stopped flow $E_0$-monitored turnover. However, the ordinate intercept of the half-reaction plot corresponds to 800 ± 200 sec⁻¹ which is considerably greater than the values corresponding to the ordinate intercepts of the turnover data. This indicates that at high pH there are one or more first order processes, in addition to $k_0$, which are kinetically significant in turnover.

**Stopped Flow $E_0$-Monitored Turnover**—The stopped flow turnover data of Fig. 7 obtained at pH 10.0 are plotted according to Equation 8 and agree well with the line corresponding to $O_2$-monitored turnover data plotted according to Equation 7. Both yield slopes of $1/k_{cat}$ and the same ordinate intercept (corresponding to 170 sec⁻¹). The stopped flow turnover intercept contains only those first order processes involving oxidized forms of the enzyme. The ordinate intercept of the turnover plot would contain all first order processes involving both oxidized and reduced intermediates. The fact that these two intercepts are equal indicates that there are no significant first order processes in turnover involving reduced enzyme. Furthermore, because of the agreement between the stopped flow turnover and $O_2$-monitored turnover experiments, it must be concluded that $E_0 - S$ and the other species of oxidized enzyme which participate in kinetically significant first order processes have the same extinction coefficient as $E_0$ at 450 μm, where the stopped flow turnover reactions are monitored.

The oxidative constant $k_{ox}$ may also be obtained from the stopped flow turnover data from plots of Equation 9. Such a plot is shown in Fig. 8 for several glucose concentrations. The slope of this plot is $1/k_{ox}$ and the intercept is unity if there are no significant first order processes involving reduced intermediates (5).

**Stopped Flow Oxidative Half-reaction**—Figs. 9 and 10 are double reciprocal plots of pH jump oxidative half-reaction data at pH 3.2 and 10.0, respectively. The plot for low pH yields a $k_{ox}$ value (1.8 × 10⁶ M⁻¹ sec⁻¹) which is identical with that from $O_2$-monitored turnover experiments. A possible intercept...


**DISCUSSION**

**Reductive Half-reaction at Low pH**—The most general expression for this sequence is Equation 4. This mechanism has been well established for the substrates 2-deoxyglucose and $p$-glucose with $p$-glucose $\text{H}^+$ ($1, 10$). It is important to note that the value of $k_2$ at high pH ($800 \pm 200 \text{ sec}^{-1}$) is close to the limiting value of $k_{sast}$ ($1000 \text{ sec}^{-1}$) at low pH and in the absence of halide. If we assign the value of $k_{sast}$ to $k_2$ at low pH, this would indicate that $k_2$ is nearly constant over the entire pH range studied. Bright and Appleby (3), in studies with 2-deoxyglucose, where $k_2$ is the only significant first order process in turnover, have shown that except for a slight decrease at pH values greater than 7, this parameter is essentially pH independent. These factors, coupled with the results from the halide experiments at low pH, support the assignment of the entire value of $k_{sast}$ (with a value of about 1000 sec$^{-1}$ in the absence of halide) to the flavin reduction step $k_2$ at pH values below 5. This value correlates rather well with a minimal value used by Bright and Gibson for $k_2$ of 1000 sec$^{-1}$ to achieve a deuterium isotope effect of 15 on $k_E$ at 3°C (11).

The parameter $k_{sast}$ is interpreted as $(k_2/k_3)/(k_{-1} + k_3)$, or, if the rapid equilibrium assumption is used, as $(k_2/k_3)/(k_{-1})$. In the absence of halide, the decrease of $k_{sast}$ at low pH can be attributed to the partition of oxidized enzyme into protonated and unprotonated forms (Equation 11) as concluded previously for the P. notatum enzyme (3).

$$E_0 + \text{H}^+ \xrightarrow{k_1} E_0\text{H}^+$$

(11)

The apparent value of $pK_1$ in the absence of halide (2.5 to 3.0) suggests the participation of a carboxylate group. The protonated species $E_0\text{H}^+$ either has little, or no, binding affinity for glucose.

The mechanism by which the substrate grouping

$$C=\text{X}$$

is converted to flavoprotein oxidase reactions is a matter of great current interest. The two extreme alternatives for a heterolytic oxidation-reduction mechanism can be visualized on the one hand as involving nucleophilic attack by $\text{XH}$ on the flavin nucleus followed by proton abstraction from carbon and electronic rearrangement, and on the other as hydride transfer from carbon to flavin. Both of these mechanisms could be assisted in principle by the general base catalysis which we find associated with $k_{sast}$ and both mechanisms have been discussed by us (3, 11) and by others (see Reference 12 for a recent discussion). The two possibilities can now be compared with the currently available experimental evidence as follows. For glucose oxidase, the flavin-substrate adduct hypothesis can be depicted as shown in Mechanism I, where $H-A$ represents either $\text{H}_2\text{O}$ or an acidic enzyme residue with $pK_1 > 10$.

$$E_0 - S \xrightarrow{k_2} E_0 + P$$

**Mechanism I**

$E_0 - S$ represents an encounter complex in which no covalent interaction between glucose and flavin has occurred. It is known that model compounds of $I$ are spectrally very different from $E_0$ and $E_1$ (14). The two pertinent observations which have to be accommodated are the kinetic isotope effect of between 10- and 15-fold which is associated with flavin reduction ($k_2$) in the

$^3$ Recent discussions of the substrate-flavin adduct mechanism in the case of model studies (Reference 13) emphasize nucleophilic attack on flavin at position C-4a, rather than at the 2-carbonyl position suggested previously (11) which is probably very unreactive. We have benefited from discussions with Dr. Gordon A. Hamilton on this point.
case of D-glucose-1-2H (11) and the fact that the steady state absorption spectrum of $E_0 - S$ plus $I$ (see Fig. 11) is indistinguishable from the spectrum of $E_0$. Mechanism I can only be reconciled with these observations if we assume rapid equilibrium between $E_0 - S$ and $I$, with $k_I/k_r \leq 0.1$ and $k_{II} \geq 10^4 \text{sec}^{-1}$. The expression for $k_2$ (which has a value of about 1000 sec$^{-1}$) is then $k_I k_{II}/k_r$, with $k_I \geq 10^8 \text{sec}^{-1}$ and $k_r \geq 10^6 \text{sec}^{-1}$. Intermediate $I$ would therefore not accumulate to any extent during turnover and the experimentally determined value of $k_2$ would exhibit the full kinetic isotope effect originating in proton removal from glucose, which is controlled by $k_{II}$. Thus, although we can place limitations on the possibility of a glucose-flavin adduct mechanism, our experimental data can neither establish nor rule out this mechanism.

The second alternative, namely hydride transfer from glucose, assisted by general base catalysis, is depicted in Mechanism II.

It is postulated in Mechanism II that the carboxylate anion important in both binding glucose and assisting hydride transfer within the $E_0 - S$ complex. The bracketed intermediates $II$ and $III$ are $E_0 - S$ complexes in which all binding interactions are satisfied with the exception of the carboxylate-glucose-1-hydroxyl interaction. Intermediate $II$ is not accumulated to any significant degree and favorable enzyme-substrate interaction in Intermediate $III$ cannot occur because the carboxyl anion is protonated. Intermediates $IV$ and $V$ are significant kinetic intermediates with identical spectrophotometric properties corresponding to those of $E_0$. Intermediate $IV$ represents the carboxylate-glucose-1-hydroxyl interaction, and Intermediate $V$ is the result of proton transfer from the glucose-1-hydroxyl to carboxylate (It is of interest in this regard that the product n-glucono-δ-lactone, which lacks a 1-hydroxyl group, is an extremely weak inhibitor (11)). Hydride transfer is then assumed to proceed to the electron sink of the flavin moiety. If one assigns $pK$ values of 2.5 for the carboxyl and 12.3 for the 1-hydroxyl of glucose (10), the equilibrium $V'/IV$ is about 10$^{-6}$. Since the observed value of $k_2$ is 10$^5 \text{sec}^{-1}$, which is based on a concentration of $(IV + V)$ for the intermediate undergoing the first order process $k_2$, the real value of $k_2$ would be 10$^8 \text{sec}^{-1}$. This is within the fundamental vibrational frequency range for a carbon-hydrogen-stretching motion. This interpretation is supported by the large substrate deuterium isotope effects (11, 16) and minor solvent deuterium isotope effects reported previously (11). Furthermore, Mechanism II is consistent with the steady state spectral data of Fig. 11, since Intermediates $IV$ and $V$ may reasonably be expected to have the spectral properties of $E_0$.

At low pH in the presence of halide, the inhibitory effect we observe is attributed to a general shutdown of active enzyme by binding of halide. This inactivation is presumed to be the result of the induction by halide of a minor conformational change which affects both the binding of substrate and flavin reduction. With the Cl$^-$ profiles of $k_{red}$ and $k_{cat}$ at pH 3.0, appropriate binding plots of $S/(Cl^-)$ versus $S$ (where $S = (k_{red} - Cl^-)/k_{cat}$ and represents a saturation function assuming $k_{red}$, ecx = 0) appeared nonlinear. This may reflect multiple binding or nonequivalent and interacting halide effects. It is reasonable to argue that protonation of the protein is required for halide binding as halide effects disappear at pH values greater than 6. All halide effects were observed to be reversible to the extent that complete recovery of enzyme which had been subjected to halides, as well as complete reduction of $E_0$ in stopped flow turnover and half-reactions, could be achieved.

The more pronounced effect of halide on $k_{red}$, as compared to the effect on $k_{cat}$, is evident from the halide parameter profiles in Figs. 4 and 5. This is attributed to the prefered binding of halide to $E_0H^+$ (Equation 11), resulting in a concerted action of $H^+$ and halide in decreasing $k_{red}$.

**Oxidative Half-reaction at Low pH**—The bimolecular oxidative constant, $k_{ox}$, does not show any halide effects. However, its pH profile displays two distinct limbs. The apparent lack of any halide effect at low pH is attributed to the fact that the oxidation of reduced flavin by $O_2$ is probably not catalyzed by the attached protein. This conclusion is suggested by the work of Gibson and Hastings (17) who obtained a bimolecular oxidation rate constant with the model system FMNH$_2$/O$_2$ which is almost identical with that for reduced glucose oxidase under comparable conditions.

The stopped flow oxidative half-reaction measurements at low pH agree quite well with those obtained from $O_2$-monitored turnover and no significant first order process was observed. Oxidative half-reactions in which reduced enzyme was incubated in $\text{H}_2\text{O}$ for several hours to exchange the hydrogen in positions N(1) and N(5) of the flavin showed no isotope effects at pH 3.0.

**Reductive Half-reaction at High pH**—The high pH drift of $k_{red}$, the apparent bimolecular rate constant for the combination of glucose with $E_0$, appears to reflect multiple ionizations. If rapid equilibrium is assumed for the Michaelis scheme of Equa-
tion 4, then the constant $k_{\text{red}}$ is interpreted as $k_{\text{red}}k_{-1}$. Any one of the three rate constants may be responsible for the drift. However, $k_9$ and $k_{\text{red}}$ have decayed by about the same amount at pH 10.0. Both parameters decrease to about 75% of their values at pH 5.0 and show a comparable slow drift in the pH range 5 to 8. Therefore, if we use $k_{\text{red}}$ as an empirical marker for the drift in $k_9$, we can calculate the contribution of $k_9$ to $k_{\text{cat}}$ at high pH values (see "Appendix B"). Upon dissection of the $k_9$ contribution from $k_{\text{cat}}$, the profile of the residual parameter shows a pH in the vicinity of 9. As shown earlier, the species associated with the profile of the residual part of $k_{\text{cat}}$ is not part of the reductive sequence but must be associated with the oxidative half-reaction.

Oxidative Half-reaction at High pH—Three features of the high pH experiments must be accounted for in any explanation. (a) The apparent pK of 7.5 associated with the $k_{\text{ox}}$ profile and the fact that there are two distinct species reacting with O2 as evidenced by the finite values of $k_{\text{ox}}$ at the pH extremes of the profile. (b) The apparent pK of 9 associated with the residual $k_{\text{cat}}$ profile. (c) The necessity for a first order process involving some species of oxidized enzyme ($E'_o$) in the oxidative half-reaction which decays to $E_o$. This step can not be an effective equilibrium process since $k_{\text{red}}$, which is associated with the $E_0$ species, does not portray a pH dependence which resembles the pH dependence of the residual part of $k_{\text{cat}}$. A minimal mechanism consistent with all aspects of the high pH experiments is as follows.

![Scheme 3](image)

Only those states of ionization of the enzyme which directly determine the rate of the subsequent chemical process are shown in Scheme 3. Furthermore, high pH effects on $k_4$ are neglected in this mechanism, but will be accounted for in the parameter analysis (see "Appendix B"). The species of $	ext{H}^+$ in the oxidative half-reaction which decays to $E_o$. This step can not be an effective equilibrium process since $k_{\text{red}}$, which is associated with the $E_0$ species, does not portray a pH dependence which resembles the pH dependence of the residual part of $k_{\text{cat}}$. A minimal mechanism consistent with all aspects of the high pH experiments is as follows.

$$E_0 \xrightleftharpoons[k_{-1}']{k_1} [S] \xrightarrow[k_2]{k_4} E_o + S \xrightarrow{H^+} [E_0^+] \xrightarrow{k_4} [E_0^+] \xrightarrow{H^+} E_o^- \xrightarrow{H_2O_2} K_4 \xrightarrow{k_4} [O_2] \xrightarrow{H^+} K_5 \xrightarrow{k_5} E_o^-$$

Scheme 3

Utilizing the steady state assumption, and rapid equilibrium for both the proton transfers and Michaelis complex, the turnover equation is

$$E_T/v = k_{-1}/(k_1k_2[S]) + (1 + K_{\text{app}}/[H^+])(k_4 + k'4K_{\text{app}}/[H^+])[O_2]$$

(12)

It should be noted that Equation 12 is indeterminate at $H^+ \rightarrow 0$ because of the third term. This is equivalent to locking all enzyme into $E'_0^-$ after one turnover. Comparing Equation 12 with the empirical three parameter turnover equation (Equation 7) we find

$$k_{\text{ox}} = (k_4 + k'4K_{\text{app}}/[H^+]/(1 + K_{\text{app}}/[H^+])$$

(13)

and

$$k_{\text{cat}} = k_9b(k_4 + k'4K_{\text{app}}/[H^+])/((b'4K_{\text{app}}/[H^+])(1 + K_{\text{app}}/[H^+])$$

(14)

The expression for $k_{\text{ox}}$ contains three parameters of which $k_4$ and $k'4$ are estimated as the limiting low and high pH values of the $k_{\text{ox}}$ profile. $K_{\text{app}}$ is initially estimated from the apparent pK of the $k_{\text{ox}}$ profile. With nonlinear regression, Equation 13 was fit between pH 5 and 10. With the values of $k_9$, $k'4$, and $K_{\text{app}}$ and fixed empirical values of $k_9$ calculated from the drift in $k_{\text{red}}$, the values of $K_4$ and $k'$4 were obtained by nonlinear regression on Equation 14. The parameters thus obtained were all heavily correlated in the regression analysis and are given in Table I. Fits of the data with the above parameters and empirical $k_9$ values yield a standard deviation of about 4% for the $k_{\text{ox}}$ profile and about 1% for the $k_{\text{cat}}$ profile. The calculated profiles of $k_{\text{ox}}$ and $k_{\text{cat}}$ are shown in Figs. 2 and 3, respectively by the solid line.

It is difficult to reconcile the large difference of magnitude in $K_{\text{app}}$ and $K_4$ as resulting from the same ionizable group of the reduced and oxidized isalloxazine moiety, respectively. $K_{\text{app}}$ is close to the accepted pK of 6 to 7 for the N(1) hydrogen of reduced isalloxazine (18). However, the anion would be expected to undergo oxidation by O2 faster than the neutral species, rather than slower as is observed.

A preferred interpretation is that the same ionizable group is involved in $K_{\text{app}}$ and $K_4$, but that $K_{\text{app}}$ is the product of an intramolecular equilibrium process coupled to a proton transfer equilibrium. This would displace the true $K_4$ in the following manner. For the system,

$$E_R \xrightarrow{K_4} E_R^- \xrightarrow{K_4'} E_R^{-}$$

the apparent equilibrium for $E_R \rightarrow E_R^{-}$ is of the form $E_R^-/E_R = K_4K_4'/[H^+]$. Thus $K_{\text{app}} = K_4K_4'$ and if we assume $K_4$ to be equal to $K_5$, then $K_4'$, the intramolecular equilibrium, is in the vicinity of 45. Returning to Scheme 3, the replacement of the $E_R \rightarrow E_R^{-}$ equilibrium with the sequence of Equation 15 yields the same form of steady state rate equation. Although a new intermediate, $E'_R^-$ has been added to the scheme, $K_4'$ is
large enough that significant amounts of \( E_F^{\prime} \), in comparison to \( E_R \) and \( E_R \), are never accumulated during turnover. This is apparent when the two different steady state rate equations are compared. The new term \((K_f[H^+]^{-1})(1+K_f')\) appears wherever the previous term \( K_{4,pp}/[H^+] \) appeared in Equation 12. The approximation \((1+K_f') \approx K_f'\) is valid as \( K_f' \) is at least 45.

It is appropriate here to compare the results of these studies with those obtained previously with the \( P. notatum \) enzyme (3). The studies are in agreement with respect to the conclusion that glucose must bind with a form of the enzyme which has an essential ionizable group in the basic state (see Scheme 3). Similarly, the fact that \( O_2 \) combines with a species of reduced enzyme containing an ionizable group in the acidic state was recognized previously. However, the greater precision of the present data, together with the wider pH range used, has shown additionally that the basic form of the reduced enzyme (\( E_F \) in Scheme 3) has low, rather than zero, reactivity toward \( O_2 \). The major discrepancy between this and the earlier report concerns the extent and consequences of the contribution of flavin reduction (controlled by \( k_b \)) to turnover. It should be noted that the magnitude of \( k_b \) (around 1000 sec\(^{-1}\)) in the absence of halide is such that it is difficult to evaluate this constant by the stopped flow half-reaction technique. This point was discussed previously (11) in considering the kinetics of oxidation of a glucose-1-\( ^5 \)H. For this substrate as well as 2-deoxyglucose (1), \( k_b \) is the only kinetically significant first order process in turnover. However, the exclusion of including \( F^- \) in the low pH experiments (Fig. 6) clearly shows that flavin reduction is the only significant first order process in turnover under these conditions. If this is also true for the \( P. notatum \) enzyme, then the assignment of a second ionization at the \( k_b \) step (4) for the oxidative half-reaction (to account for the acidic limb of the bell-shaped \( k_c(c) \) profile in the presence of 0.2 M Cl\(^-\)) would be incorrect. The high pH behavior of \( k_c(c) \) is dominated by \( pK_a \), as was concluded previously (3). The value of \( pK_a \), however, is larger than that deduced directly from the \( k_c(c) \) profile because of the failure to recognize the small contribution of \( k_b \) to \( k_c(c) \) at high pH values.

It is of interest to speculate about the chemical significance of the steps of the oxidative half-reaction. The \( pK \) of the \( \text{N}(3) \) hydrogen of free oxidized isoalloxazine is estimated to be 10 (19, 20). The \( pK \) of this hydrogen in the reduced form of isoalloxazine is not known, but it is reasonable to assume it is 9.1 (\( pK_1 \) and \( pK_5 \)) is close to 10 (19, 20). The \( pK \) of this hydrogen in the reduced form of isoalloxazine is estimated to be 10.0 in the glucose oxidase reaction under conditions where the \( k_b \) step is important in the binding and subsequent quenching of fluorescence of FAD bound to the glucose oxidase apoprotein.

As in the low pH studies, the stopped flow oxidative half-reaction at high pH showed no isotope effect upon exchanging the hydrogens of reduced flavin with solvent deuterium. Bright and Gibson did not detect any isotope effect in the oxidative half-reaction of glucose oxidase in their studies at pH 5.5 (11). Gray and Jones have reported large primary isotope effects for hydrogen abstraction from the imine position of ethylenimine by methyl radicals (23). The lack of a primary isotope affecting the oxidative half-reaction at all pH values supports the contention that this process is electron transfer to oxygen followed by rapid proton dissociation from the isoalloxazine positions \( \text{N}(1) \) and \( \text{N}(5) \) rather than hydrogen abstraction.

Recently, Massey \textit{et al.} have examined several flavoprotein reactions for the presence of superoxide radical (\( O_2^- \)) with ethylenediamine superoxide dismutase (24). They did not detect \( O_2^- \) in the glucose oxidase reaction at pH 8.5 by this method. Knowles \textit{et al.} have shown directly by rapid freezing electron paramagnetic resonance studies the production of \( O_2^- \) during catalysis of the oxidation of xanthine by molecular \( O_2 \) in the presence of xanthine oxidase (25). Our rapid freezing electron paramagnetic resonance experiments did not detect \( O_2^- \) at pH 10.0 in the glucose oxidase reaction under conditions where the predominant species controlling turnover is reduced enzyme. Therefore, it appears that \( O_2^- \), if produced, may be reduced to the level of peroxide before it can diffuse from the solvent cage of enzyme-bound flavin.

**APPENDIX A**

Define the fraction of total enzyme in the reduced form as \( \alpha = [E\_R]/[E\_T] \) and the fraction of total enzyme in the oxidized form (assuming only two such species) as \( ([E\_O] + [E\_O\_d])/[E\_T] \). At pH 3, \( E\_O\_d \) is predominantly \( E\_p\_S \), whereas at pH 10 \( E\_O \) is predominantly \( E\_O\_d \). Furthermore, let \( q \) be the mole fraction \( [E\_R]/([E\_T] + [E\_O\_d]) \) and \( q \) the mole fraction \( [E\_O\_d]/([E\_T] + [E\_O\_d]) \). Then the initial steady state absorbance in a stopped flow turnover experiment is given as follows

\[
A_s = [E\_T]\left(q \alpha + (1 - \alpha) \cdot (q \alpha + q \alpha^* \bar{\epsilon}_o)ight)
\]

After \( O_2 \) is depleted and turnover is complete \( A_s = [E\_T] \bar{\epsilon}_o \). Consequently, \( \Delta \bar{\epsilon} = [E\_T](1 - \alpha) \cdot (q \alpha \bar{\epsilon}_o + q \alpha^* \bar{\epsilon}_o) \). If \( \epsilon^* = \epsilon_o \), then \( \Delta \bar{\epsilon} \) is proportional to \( (\alpha - \epsilon_o) \) and can be normalized to the static difference spectrum \( E_o - E_r \) at all wave
lengths. If a high proportion of oxidized enzyme is in the form $E_0^+$ and its spectrum is significantly different from that of $E_0$, then the normalized stopped flow difference spectrum will deviate from that of $E_0 - E_0^-$. The deviation will depend on both $q_i/k_i$ and $\epsilon_{i*/i}$.

**APPENDIX B**

The simplest model for the high pH drift of $k_{ro}$, the apparent bimolecular rate constant for the combination of glucose and oxidized enzyme, is the following:

$$
E_0^+ + S \xrightleftharpoons{k_{f1}} (E_0^+ S)_0 \xrightleftharpoons{k_{f2}} E_0^+ + k(O_2)
$$

$$
E_0^- + S \xrightleftharpoons{k_{f1}} (E_0^- S)_0 \xrightleftharpoons{k_{f2}} E_0^- + k(O_2)
$$

It is assumed that only the electron transfer parameter $k_{f1}$ is affected by the ionization state of the enzyme. pH effects on $k_{ro}$ will be ignored as it separates independently from the parameters $k_{f1}$ and $k_{f2}$ in the turnover equation to be derived.

The above mechanism contains $i$ ionizations, therefore

$$
e = ([E_{ri}] + [E_{ri}] + \cdots [E_{ri}])k(O_2) = k(O_2) \sum_{i=0}^{r} E_{ri}
$$

and

$$
|E_T| = \sum_{i=0}^{r} \text{all species} = \sum_{i=0}^{r} \left([E_{ri}] + [E_{ri}] + [E_{ri}]\right)
$$

Considering a turnover loop for a specific ionization state and assuming steady state and rapid equilibria,

$$
[E_{ri}] = k_{f1}[E_{ri}]/[O_2]/k_{f2}
$$

$$
[E_{ri}] = k_{f1}[E_{ri}]/[O_2]/k_{f2}/[S]
$$

Substitution of Equations 3 and 4 into Equation 2 and dividing the result by Equation 1 yields

$$
\frac{|E_{ri}|}{e} = \sum_{i=0}^{r} \left(\frac{1}{k_{f1}} \cdot \frac{[E_{ri}]/[E_{ri}]}{[E_{ri}]}ight)
$$

The quotient $\frac{|E_{ri}|}{e}$ defines the fraction of reduced enzyme $q_i$ in the $i$th ionization state. The first two terms of Equation 5 have the same functional form aside from a constant factor $k_{i-1}/k_i$. The leading term is correlated with $1/k_{ro}$, the experimental bimolecular rate constant for oxidized enzyme and glucose obtained from turnover data, and the following term is $1/k_{o}$, an apparent first order flavin reduction step. $1/k_{o}$ at high pH is only one of the contributions to the turnover parameter $1/k_{ro}$ which includes all first order contributions to turnover. Since both $k_{ro}$ and the apparent flavin reduction rate constant $k_{o}$ are controlled by the same function $q_i/k_i$ with respect to pH, differences in $k_{ro}$ reflect similar changes in $k_{o}$.

This will allow dissection of the $k_{o}$ contribution from the $k_{ro}$ turnover parameter without resorting to difficult direct measurements of the stopped flow reductive half-reaction. In addition, for small changes in the values of $k_{o}$ value and a finite number of ionizations with different $pK$ values in the pH range 5 to 9, the dependence of the term

$$
\sum_{i=0}^{r} \frac{q_i}{k_i}
$$

as a function of pH would produce a continuous curve such as that noted for $k_{ro}$ at high pH.

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