stant of 0.042 M for D-glucose from the oxidized enzyme–D-glucose complex at 3° and pH 5.6. The dissociation constant for D-glucal from the oxidized enzyme–D-glucal complex at pH 5.6 and 25° reported in this paper is 0.15 M. One of the theories suggested to explain the rate enhancement of an enzyme-catalyzed reaction over the corresponding uncatalyzed reaction is that part of the substrate binding energy is utilized to distort the substrate into a conformation resembling the transition state for the reaction (*cf.* Jencks, 1969). Thus the X-ray diffraction studies of lysozyme suggest that the hexose ring of the substrate which is bound at the site where bond cleavage occurs must be bound in a strained half-chair conformation, facilitating reaction (Phillips, 1967). A corollary of this theory is that an inhibitor which is frozen in the conformation of the transition state for the normal catalytic reaction should bind more strongly to the active site than the substrate itself, since none of the binding energy would have to be used to strain the analog (*cf.* Jencks, 1969). Recent examples supporting this concept have been reported by Lee (1969) for β-D-galactopyranosidase and Wolfenden (1969) for cytidine deaminase. Since D-glucal appears to bind less tightly to the oxidized form of glucose oxidase than does D-glucose, it would not appear that strain induced in D-glucose driving carbon atom 1 toward a planar sp^2 hybridization as is found in the product lactone plays a significant role in catalysis by glucose oxidase.

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Interaction of Halide Ions with *Aspergillus niger* Glucose Oxidase*

M. J. Rogers‡ and K. G. Brandt†

ABSTRACT: The inhibition of *Aspergillus niger* glucose oxidase by halide ions has been studied. The inhibition by chloride ion appears to be competitive with respect to D-glucose and uncompetitive with respect to oxygen. Similar apparent competitive inhibition by chloride ion with respect to the substrate 2-deoxy-D-glucose was observed. Chloride ion binding to the oxidized form of the enzyme was shown to result in a perturbation of the visible absorption spectrum of the flavin–adenine dinucleotide prosthetic group of the enzyme. Dissociation constants were calculated from difference spectral data by titrating the enzyme with chloride ion. The dissociation constants so obtained are in good agreement with the kinetically determined inhibition constants. Chloride ion binding was shown to be markedly pH dependent, being stronger at more acid pH values. The data were quantitatively fit to a model which proposes that chloride ion binds to a protonated form of the oxidized enzyme. The acidic group has a pK_1 of 3.7 and the pH-independent dissociation constant for chloride ion binding to the protonated species has a value of K_d = 0.005 M. Bromide ion and iodide ion also exhibit apparently competitive inhibition with respect to D-glucose. At pH 4.5 the apparent inhibition constants for chloride, bromide, and iodide are 0.037, 0.051, and 0.27 M, respectively.

The pH dependence of the kinetics of oxidation of several monosaccharides by *Penicillium notatum* glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) has been reported by Bright and Appleby (1969). In the course of their work they noted that the enzyme was subject to specific halide ion effects and that, more particularly, chloride ion

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affected the pK of an ionizable group on the protein which appears to control the activity of the enzyme. In an accompanying paper (Rogers and Brandt, 1971a), it has been noted that the binding of D-glucal, a competitive inhibitor of *Aspergillus niger* glucose oxidase, was apparently insensitive to the presence of 0.2 M chloride ion at pH 4.0.

In this paper are reported kinetic and spectrophotometric studies of the interaction of halide ions with *A. niger* glucose oxidase. Inhibition of *A. niger* glucose oxidase by chloride ion has been studied by steady-state kinetic techniques at selected pH values in the range pH 4.0–6.2. The inhibition by chloride ion appears to be competitive with respect to D-glucose or 2-deoxy-D-glucose and uncompetitive with respect to oxygen. The binding of chloride ion to the oxidized form of the enzyme has also been studied spectrophotometrically at selected pH values in the range pH 3.5–6.2. The binding of chloride ion to the oxidized form of *A. niger* glucose oxidase is markedly pH dependent, in contrast to the pH independence of D-glucal binding (Rogers and Brandt, 1971a).

While this paper was in preparation, a study of the pH dependence of the kinetics of oxidation of D-glucose by *A. niger* glucose oxidase as reported (Weibel and Bright, 1971). In their study they have observed an effect of chloride ion on the apparent bimolecular rate constant for reduction of the enzyme-bound FAD by D-glucose and a less pronounced effect on the maximum turnover number.

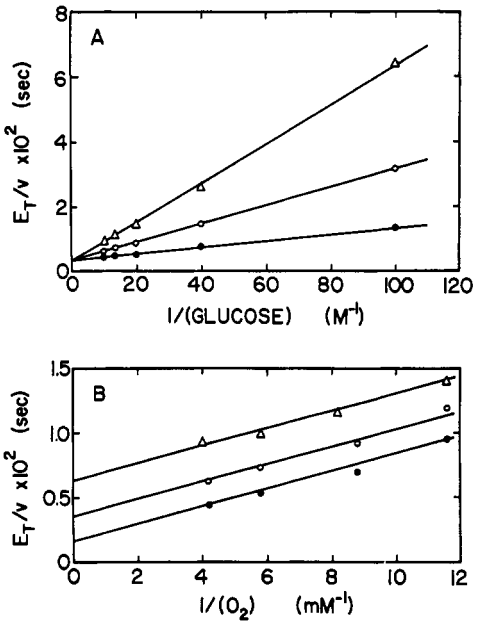

FIGURE 1: Effect of chloride ion on the kinetics of glucose oxidase catalyzed oxidation of D-glucose at pH 4.0 and 25°. (A) (O_2) = 0.27 mM. (B) (D-Glucose) = 0.1 M. Potassium chloride concentration: (●) zero, (○) 0.032 M, and (△) 0.081 M.

Materials and Methods

A. niger glucose oxidase preparations used were the same as those used in the preceding paper (Rogers and Brandt, 1971a). Enzyme concentration was calculated from the absorbance at 450 nm, using a molar absorptivity of 1.41×10^4 M^{-1} cm^{-1} (Gibson *et al.*, 1964), and are reported as concentration of enzyme-bound FAD.[1]

D-Glucose was obtained from Fisher Scientific Co. and 2-deoxy-D-glucose from Calbiochem. Reagent grade inorganic salts and buffers were used throughout.

Steady-state turnover experiments were performed at 25° as described previously (Rogers and Brandt, 1971a) using a Yellow Springs Instrument Co. oxygen monitor. Concentration of enzyme-bound FAD was between 0.01 and 0.1 μM. At a particular pH care was taken to maintain constant ionic strength. At pH 5.6 a decrease in ionic strength (using potassium sulfate) from 0.52 to 0.06 had no effect on the observed turnover number with D-glucose. At pH 4.0 and 6.2, five- to ninefold variations in the ionic strength (using potassium sulfate) affected the observed turnover number by only ±5%.

Difference spectra and spectrophotometric titrations were performed at 25° as described previously (Rogers and Brandt, 1971a). Concentration of enzyme-bound FAD was between 10 and 30 μM. In these experiments no attempt was made to maintain the ionic strength of the enzyme solution in the reference beam equal to that in the sample beam. In a separate experiment it was observed that increasing the ionic strength from 0.081 to 0.48 with potassium sulfate had virtually no effect on the spectrum of the enzyme at pH 5.0. There was no detectable effect at 502.5 nm, the wavelength at which the chloride ion titrations were carried out, and less than 0.005 A unit decrease at shorter wavelengths.

Buffers used in the kinetic experiments were as follows: pH 4.0–5.6, 0.1 M sodium acetate; pH 6.2, 0.1 M sodium cacodylate. Ionic strength was adjusted with potassium sulfate. The total ionic strength at each pH was: pH 4.0, 0.56, pH 4.1, 0.56; pH 4.3, 0.56; pH 4.5, 0.57; pH 4.9, 0.58; pH 5.6, 0.52; pH 6.2, 0.87. The higher ionic strength at pH 6.2 was due to the high concentration of chloride ion necessary to produce significant inhibition at that pH. All buffers in these experiments contained 0.16 mM EDTA.

In the spectrophotometric studies of chloride ion binding, the buffers used were as follows: pH 3.5–5.6, 0.1 M sodium acetate, pH 6.2, 0.1 M sodium cacodylate. In each case the ionic strength prior to addition of chloride ion was adjusted to 0.1 M with potassium sulfate.

Results

Chloride Ion Inhibition. Figure 1 shows the effect of potassium chloride at pH 4.0 on the reciprocal of the steady-state turnover number, $(E)_T/v$, of D-glucose oxidation catalyzed by *A. niger* glucose oxidase. Since potassium sulfate at similar concentrations had no effect on the kinetics, it is assumed that the chloride ion is the inhibitor. Chloride ion inhibition appears to be competitive with respect to D-glucose and uncompetitive with respect to oxygen. Similar inhibition patterns were observed with 2-deoxy-D-glucose as the substrate, as shown in Figure 2.

The apparent inhibition constant for chloride ion, K_i^{app}, was evaluated from the slopes of the lines in Figure 1A, assuming chloride ion increased the slope of the line obtained in the absence of chloride ion by the factor $(1 + (Cl^-)/K_i^{app})$, as would be expected for a competitive inhibitor (Mahler and Cordes, 1966). At pH 4.0 the value obtained is K_i^{app} = 0.015 M. Similar analysis of the data in Figure 2 gives a value of K_i^{app} = 0.016 M at pH 4.0

[1] Abbreviations used are: FAD, flavin-adenine dinucleotide; (E_T), total concentration of enzyme-bound FAD; E_o, enzyme in which the enzyme-bound FAD is in the oxidized form; E_r, enzyme in which the enzyme-bound FAD is in the reduced form; G, D-glucose; S, substrate; P, product.

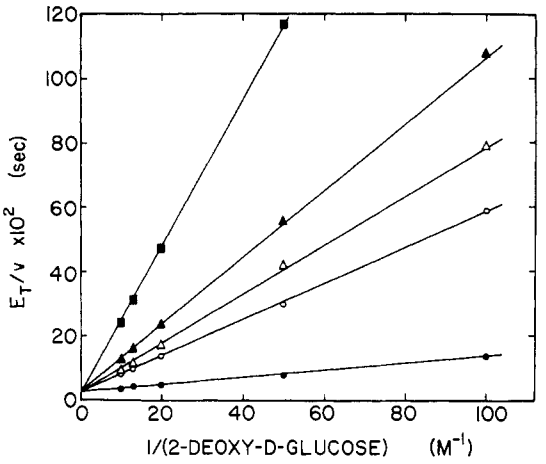

FIGURE 2: Effect of chloride ion on the kinetics of glucose oxidase catalyzed oxidation of 2-deoxy-D-glucose at pH 4.0 and 25°. (O_2) = 0.27 mM. Potassium chloride concentration: (●) zero, (○) 0.081 M, (△) 0.106 M, (▲) 0.16 M, and (■) 0.32 M.

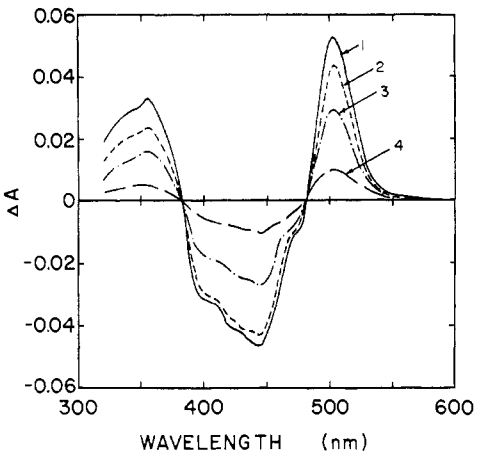

FIGURE 4: pH dependence of the chloride ion difference spectrum at 0.5 M potassium chloride and 25°. The concentration of enzyme-bound FAD has been normalized to 20.6 μM for each difference spectrum. Curve 1, pH 4.0; curve 2, pH 4.8; curve 3, pH 5.6; curve 4, pH 6.2.

Apparently competitive inhibition by chloride ion with respect to D-glucose was also observed at pH's 4.3, 5.6, and 6.2. In contrast to the previously reported (Rogers and Brandt, 1971a) pH independence of the inhibition constant for D-glucal with *A. niger* glucose oxidase, the inhibition constant for chloride ion is pH dependent. The values of K_i^{app} determined from these kinetic studies are plotted as pK_i^{app} *vs.* pH in Figure 3 (open circles).

Spectrophotometric Studies of Chloride Ion Binding. When chloride ion is added to the oxidized form of *A. niger* glucose oxidase, the spectrum of the enzyme-bound FAD undergoes a red shift. The difference spectrum can be explained by postulating the formation of a complex between chloride ion and the oxidized enzyme, E_o.[1] Figure 4 shows the difference spectra resulting from addition of 0.5 M potassium chloride to glucose oxidase at pH's 4.0, 4.8, 5.6, and 6.2. Potassium sulfate at similar concentrations had no effect on the absorbance at 502.5 nm, indicating that the difference spectra are due to chloride ion rather than either ionic strength, *per se*, or potassium ion. A similar red shift has been reported (Swoboda and Massey, 1966) to reversibly occur when the pH of a solution of *A. niger* glucose oxidase is adjusted to pH 3.1 with HCl, which results in addition of about 1 mM chloride ion.

The shape of the difference spectrum due to the presence of 0.5 M chloride ion is seen to be unaffected by pH, but the magnitude of the absorbance difference at constant chloride ion concentration is pH dependent, being smaller at more alkaline pH. Measurement of the absorbance difference at 502.5 nm at constant pH as a function of chloride ion concentration permits the evaluation of the apparent dissociation constant for the presumed E_o:Cl^- complex at that pH. If it is assumed that the formation of the complex at a particular pH is described by eq 1 and 2,[1] and that the measured

$$E_o + Cl^- \rightleftharpoons E_o{:}Cl^- \tag{1}$$

$$K_d^{app} = \frac{(E_o)(Cl^-)}{(E_o{:}Cl^-)} \tag{2}$$

absorbance difference, ΔA, at a particular chloride ion concentration is proportional to the concentration of E_o:Cl^- complex, treatment of the equations after the manner of Benesi and Hildebrand (1949) yields eq 3. This equation assumes $(Cl^-)_T \gg (E_T)$, where $(Cl^-)_T$ and E_T are the total analytical concentrations of chloride ion and enzyme-bound

$$\frac{1}{(Cl^-)_T} = \frac{(E_T)\Delta\epsilon}{K_d^{app}}\frac{1}{\Delta A} - \frac{1}{K_d^{app}} \tag{3}$$

FAD, respectively. $\Delta\epsilon$ is the proportionality constant between ΔA and the concentration of E_o:Cl^- complex. Equation 3 predicts that a plot of $1/(Cl^-)_T$ *vs.* $1/\Delta A$ will be a straight line with ordinate intercept equal to $-1/K_d^{app}$. Figure 5 shows typical data obtained from these experiments at pH 4.0 and 4.8. Similar linear plots were obtained at pH's 3.5, 5.2, 5.6, and 6.2. The values of K_d^{app} so obtained are plotted in Figure 3 (filled circles) as pK_d^{app} *vs.* pH. It can be seen that the static spectrophotometric values of pK_d^{app} are pH dependent and in reasonable agreement with the kinetically determined values of pK_i^{app}.

pH Dependence of D-*Glucose Oxidation in the Absence of Chloride Ion.* Gibson *et al.* (1964) have shown that, at con-

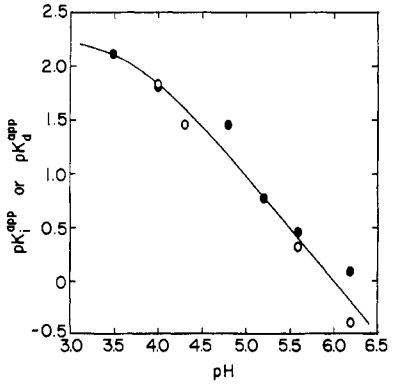

FIGURE 3: Effect of pH on pK_i^{app} (○) and pK_d^{app} (●) for chloride ion binding. The line drawn through the data is the theoretical line calculated from eq 15 in the text assuming K_d = 0.005 M and pK_i = 3.7.

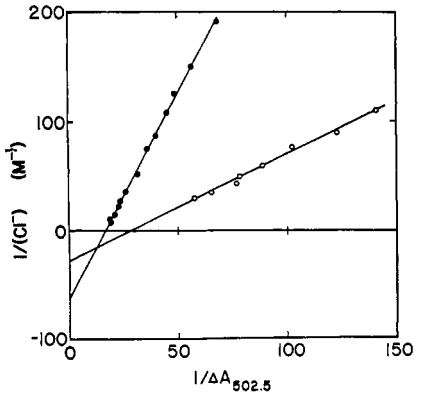

FIGURE 5: Benesi–Hildebrand plots according to eq 3 of chloride ion difference spectral titrations at 502.5 nm. (●) pH 4.0, 28 μM enzyme-bound FAD; (○) pH 4.8, 31 μM enzyme-bound FAD.

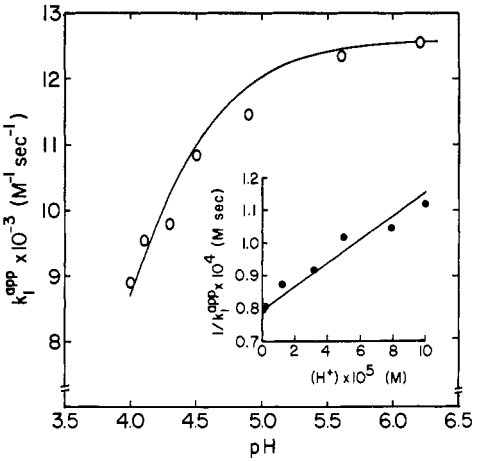

FIGURE 6: pH dependence of k_1^{app} for D-glucose oxidation, determined at 0.27 mM oxygen and 25°. The line drawn through the data is the theoretical curve calculated from eq 12 using values of $k_1k_2/(k_{-1} + k_2)$ = 12,600 M^{-1} sec^{-1} and pK_1 = 3.7. Inset: plot of $1/k_1^{app}$ *vs.* (H^+) according to eq 12.

stant pH, the minimal mechanism[1] given in eq 4 and 5 satisfies the steady-state and stopped-flow kinetic data for D-glucose oxidation by *A. niger* glucose oxidase at pH 5.6 and

$$E_o + G \xrightarrow{k_1^{app}} E_r + \text{lactone} \quad (4)$$

$$E_r + O_2 \xrightarrow{k_4} E_o\text{–}P \xrightarrow{k_5} E_o + H_2O_2 \quad (5)$$

25°. The numbering of the rate constants corresponds to Bright and Appleby (1969). The steady-state rate equation generated by this mechanism is given in reciprocal form in eq 6. The slope of a plot of $(E)_T/v$ *vs.* 1/(G) will therefore be equal to $1/k_1^{app}$, the reciprocal of the apparent bimolecular

$$\frac{(E_T)}{v} = \frac{1}{k_5} + \frac{1}{k_1^{app}(G)} + \frac{1}{k_4(O_2)} \quad (6)$$

rate constant for reduction of the enzyme bound FAD by D-glucose.

The mechanism of eq 4 and 5 has been used by Bright and Appleby (1969) to analyze the pH dependence of D-glucose oxidation catalyzed by *P. notatum* glucose oxidase. They have reported that k_1^{app} for *P. notatum* glucose oxidase is pH dependent, decreasing at acid pH following the protonation of a group on the enzyme with pK_1 = 5.0. Their studies were performed in the presence of 0.2 M potassium chloride. They also observed that k_1^{app} for D-mannose was similarly affected by pH, but that in the absence of chloride ion the pK_1 was 4.0.

Chloride ion has been shown above to be an apparently competitive inhibitor with respect to D-glucose. Consequently k_1^{app} for D-glucose was determined as a function of pH in the absence of chloride ion with the *A. niger* enzyme by determining the effect of pH on the slope of plots of $(E_T)/v$ *vs.* 1/(G) in accordance with eq 6. Figure 6 shows that k_1^{app} is pH dependent in the range pH 4.0–6.2 in the absence of chloride ion. These experiments were carried out at relatively high ionic strength (approximately 0.56, except at pH 6.2, where the ionic strength was 0.87) since evaluation of pK_i^{app} and pK_d^{app} required the use of high chloride ion concentration.

During the preparation of this paper, Weibel and Bright (1971) reported kinetic studies of the pH dependence of *A. niger* glucose oxidase catalyzed oxidation of D-glucose. Their results show that the apparent bimolecular rate constant for enzyme-bound FAD reduction by D-glucose (which corresponds to k_1^{app} in eq 4) was pH dependent in the absence of chloride ion. They have also reported a pronounced effect of chloride ion on this rate constant below pH 5.

Inhibition of D-Glucose Oxidation by Bromide and Iodide Ions. The oxidation of D-glucose catalyzed by *A. niger* glucose oxidase is also inhibited by bromide and iodide ions at pH 4.5. Figure 7 shows plots of $(E_T)/v$ *vs.* halide ion concentration at each of two concentrations of D-glucose. The results are consistent with competitive inhibition (Dixon, 1953) as was shown above for chloride ion. The apparent inhibition constants at pH 4.5 calculated from the abscissa coordinate of the intersection point (Dixon, 1953) for bromide ion and iodide ion are 0.051 and 0.27 M, respectively.

Discussion

The current mechanism of *A. niger* glucose oxidase catalyzed oxidation of D-glucose or 2-deoxy-D-glucose at 25° in the absence of halide ions over the range pH 3.5–6.2 is shown in eq 7 and 8.[1] This mechanism is based on that of

$$E_oH^+ \rightleftharpoons^{K_1} E_o \underset{k_{-1}}{\overset{k_1(s)}{\rightleftharpoons}} E_o\text{–}S \xrightarrow{k_2} E_r + \text{lactone} \quad (7)$$

$$E_r + O_2 \xrightarrow{k_4} E_o + H_2O_2 \quad (8)$$

Gibson *et al.* (1964) as modified by Weibel and Bright (1971) for the acidic pH range where the experiments reported in this paper were performed. The mechanism is identical with that previously proposed by Nakamura and Ogura (1962, 1968). It assumes that the oxidized enzyme can exist in both a protonated and an unprotonated state, with only the latter being capable of binding substrate. K_1 is the acid dissociation constant for E_oH^+.

The data on chloride ion inhibition reported in this paper can be accommodated by replacing eq 7 with eq 9, where K_d is the pH-independent dissociation constant for chloride ion

$$E_oH^+Cl^- \rightleftharpoons^{K_d} E_oH^+ \rightleftharpoons^{K_1} E_o \underset{k_{-1}}{\overset{k_1(s)}{\rightleftharpoons}} E_o\text{–}S \xrightarrow{k_2} E_r + \text{lactone} \quad (9)$$

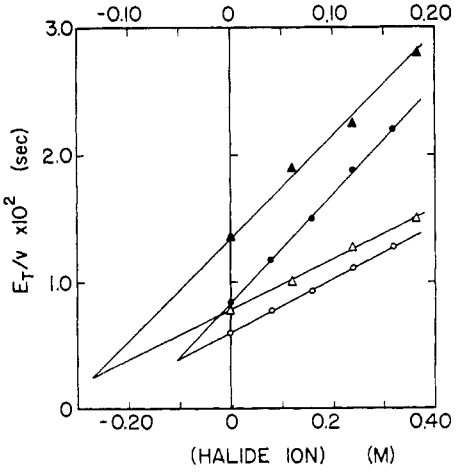

FIGURE 7: Effect of bromide ion and iodide ion on the kinetics of D-glucose oxidation at pH 4.5 and 25°. Oxygen concentration was 0.27 mM. The circles and top abscissa scale are for potassium bromide, ionic strength 0.63 with K_2SO_4: (●) 0.026 M D-glucose and (○) 0.052 M D-glucose. The triangles and bottom abscissa scale are for potassium iodide, ionic strength 0.60 with K_2SO_4: (▲) 0.010 M D-glucose and (△) 0.024 M D-glucose.

from $E_oH^+Cl^-$. Interaction of chloride ion with E_oH^+ has been proposed by Bright and Appleby (1969) for *P. notatum* glucose oxidase. Assuming E_o, E_oH^+, and $E_oH^+Cl^-$ are in rapid equilibrium, the steady-state rate equation for the mechanism in eq 8 and 9 is given in reciprocal from in eq 10. Equation 10 predicts that chloride ion concentration will

$$\frac{(E_T)}{v} = \frac{1}{(S)}\left[\left(\frac{k_{-1}+k_2}{k_1k_2}\right)\left(1+\frac{(H^+)}{K_1}\left[1+\frac{(Cl^-)}{K_d}\right]\right)\right] + \frac{1}{k_4(O_2)} + \frac{1}{k_2} \quad (10)$$

affect only the slope of a plot of $(E_T)/v$ *vs.* $1/(S)$ and only the ordinate intercept of a plot of $(E_T)/v$ *vs.* $1/(O_2)$, as observed in Figures 1 and 2.

The slope of a plot of $(E_T)/v$ *vs.* $1/(S)$ is given by eq 11. With D-glucose as the substrate, this slope corresponds to $1/k_1^{app}$ in eq 6. In the absence of chloride ion, eq 11 reduces

$$\frac{1}{k_1^{app}} = \left(\frac{k_{-1}+k_2}{k_1k_2}\right)\left(1+\frac{(H^+)}{K_1}\left[1+\frac{(Cl^-)}{K_d}\right]\right) \quad (11)$$

$$\frac{1}{k_1^{app}} = \left(\frac{k_{-1}+k_2}{k_1k_2}\right)\left(1+\frac{(H^+)}{K_1}\right) \quad (12)$$

to eq 12. According to eq 12, a plot of $1/k_1^{app}$ *vs.* (H^+) should be linear, as shown in the inset of Figure 6 for D-glucose. The values of $k_1k_2/(k_{-1}+k_2)$ and pK_1 calculated from the line drawn through the data in the inset of Figure 6 are $k_1k_2/(k_{-1}+k_2)$ = 12,600 M^{-1} sec^{-1} and pK_1 = 3.7. This value of $k_1k_2/(k_{-1}+k_2)$, which corresponds to the apparent bimolecular rate constant for enzyme-bound FAD reduction by D-glucose, is in reasonable agreement with values reported previously (Weibel and Bright, 1971; Bright and Appleby, 1969; Bright and Gibson, 1967; Gibson *et al.*, 1964). The value of pK_1 determined in this work with *A. niger* glucose oxidase in the absence of chloride ion is significantly higher than the value of pK_1 = 2.5–3.0 obtained in the absence of chloride ion using D-glucose as substrate with the *A. niger* enzyme by Weibel and Bright (1971). This difference may possibly be due to the much higher ionic strength used in this work as compared with that of Weibel and Bright.

At constant pH, comparison of eq 11 and 12 shows that the slope of a plot of $(E_T)/v$ *vs.* $1/(S)$ should be increased by chloride ion by the factor $(1 + (Cl^-)/K_i^{app})$, with K_i^{app} being given in eq 13. Equation 13 predicts that K_i^{app}, the apparent

$$K_i^{app} = K_d\left(1+\frac{K_1}{(H^+)}\right) \quad (13)$$

inhibition constant for chloride ion, will be pH dependent as it was shown to be in Figure 3. This effect of chloride ion on the slope of a plot of $(E_T)/v$ *vs.* $1/(S)$ which is observed in Figure 1A is in agreement with the effect of 0.1 M chloride ion on the apparent bimolecular rate constant for enzyme-bound FAD reduction by D-glucose reported by Weibel and Bright (1971).

The mechanism of eq 8 and 9 requires that at a given pH, chloride ion should increase the slope of a plot of $(E_T)/v$ *vs.* $1/(S)$ with 2-deoxy-D-glucose as substrate by the same factor as with D-glucose as substrate. This was observed to be the case at pH 4.0. The values of K_i^{app} calculated from the data in Figures 1A and 2 are identical within experimental error.

The inhibitory effect of chloride ion reported in this paper has been interpreted in terms of a mechanism which postulates that chloride ion binds to a protonated form of the oxidized enzyme. This has recently been suggested by Weibel and Bright (1971) to explain the effect of 0.1 M chloride ion on the pH dependence of the kinetics of oxidation of D-glucose by *A. niger* glucose oxidase. The spectrophotometric studies reported in this paper document such binding. Chloride ion perturbs the spectrum of the enzyme-bound FAD of *A. niger* glucose oxidase as shown in Figure 4. This perturbation is most simply explained as resulting from chloride ion binding. The concentration dependence of the difference spectrum is described quantitatively by eq 1, 2, and 3. Furthermore, the absence of an effect of potassium sulfate on the spectrum of the enzyme-bound FAD suggests that the chloride ion effect is not a simple ionic strength effect. Indeed, the chloride ion difference spectrum is quite similar to the difference spectrum which is produced by the binding of benzoate to D-amino acid oxidase (Veeger *et al.*, 1966).

Based on the partial mechanism of eq 9, the chloride ion binding equilibrium of eq 1 can now be written as

$$E_oH^+Cl^- \overset{K_d}{\rightleftharpoons} E_oH^+ \overset{K_1}{\rightleftharpoons} E_o \quad (14)$$

from which it follows that

$$K_d^{app} = \frac{[(E_o)+(E_oH^+)](Cl^-)}{(E_oH^+Cl^-)} = K_d\left(1+\frac{K_1}{(H^+)}\right) \quad (15)$$

According to eq 15, the value of K_d^{app} calculated from the difference spectral titrations such as those shown in Figure 3 should be identical with the K_i^{app} determined kinetically at a given pH. Figure 3 shows that the agreement between these two constants is satisfactory. The line drawn through the data in Figure 3 is calculated for eq 15 using pK_1 = 3.7 (the value determined from the pH dependence of k_1^{app} for D-glucose oxidation) and K_d = 0.005 M. The fit of the data in Figure 3 to this theoretical line is satisfactory, demonstrating that the mechanism of eq 8 and 9 adequately explains both the pH dependence and the chloride ion dependence of the ki-

netics of oxidation of D-glucose as well as the difference spectral results.

The difference spectrum (corresponding to a red shift) which results when chloride ion binds to *A. niger* glucose oxidase has been noted above to be similar to that observed when benzoate binds to D-amino acid oxidase. A red shift also results when FAD binds to lipoamide dehydrogenase apoenzyme, and has been correlated in the latter case (Veeger *et al.*, 1966) with a decreased exposure of the FAD to the aqueous solvent. The fact that chloride ion binding at pH 4.0 produces a red shift, together with the observation (Rogers and Brandt, 1971b) that chloride ion and D-glucal can bind simultaneously to the enzyme, suggests an explanation for the blue shift observed (Rogers and Brandt, 1971a) to accompany D-glucal binding at pH 4.0 in the presence of 0.2 M potassium chloride. Since chloride ion and D-glucal can bind simultaneously to glucose oxidase, if chloride binding at pH 4.0 decreases the polarity of the environment of the enzyme-bound FAD relative to free enzyme, the simultaneous presence of D-glucal and its hydroxyl groups may partially offset the effect of chloride ion. This would result in an *apparently* more polar environment in the enzyme–chloride–glucal ternary complex, producing an apparent blue shift relative to the enzyme–chloride complex.

The apparent inhibition constant for halide ion is dependent on the halide ion used. The value of K_i^{app} for chloride ion at pH 4.5, calculated from eq 13 using values of $pK_1 = 3.7$ and $K_d = 0.005$ M is $K_i^{app} = 0.037$ M. This value compares with values of 0.051 and 0.27 M for bromide and iodide ions, respectively, at pH 4.5 (Figure 7). These results agree with the observation of Weibel and Bright (1971) that the order of effectiveness of 0.1 M halide ion in decreasing the apparent bimolecular rate constant for enzyme-bound FAD reduction by D-glucose is $F^- \gg Cl^- \approx Br^-$.

The nature of the group characterized by pK_1 cannot be unequivocally determined based on kinetic data alone. It could reasonably be a carboxyl group, as Weibel and Bright (1971) have suggested. They have postulated that this carboxyl group may be involved in both binding of substrate and as a general base.

An interesting aspect of the mechanism of eq 8 and 9 is that it is not necessary to postulate that halide ions sterically block D-glucose from the active site, as is usually proposed for competitive inhibitors. Rather, the effect of halide ion binding is envisioned as simply decreasing the fraction of oxidized enzyme existing in the unprotonated form at any pH. As is clear from eq 10, competitive inhibition by chloride ion with respect to D-glucose is still predicted, despite the possible absence of direct steric interaction between chloride ion and substrate. In this regard one might then predict that halide ion binding should not prevent the binding of a substrate analog which does not exhibit pH-dependent binding. Such simultaneous binding of halide ion and D-glucal, which is a competitive inhibitor substrate analog of D-glucose, is documented in an accompanying paper (Rogers and Brandt, 1971b).

It is possible to speculate that the halide ion binding site, which is probably not the hypothetical (uncharged) protonated carboxyl group, may be a positively charged side chain, *e.g.*, an ϵ-amino group of a lysine residue. If this positively charged group were close enough to the carboxyl group to interact with it when the carboxyl is in the unprotonated state, protonation of the carboxyl would destroy the interaction and free the positively charged group near the active site so that chloride ion could bind. Although this model is highly speculative, it does offer at the molecular level a reasonable basis for explaining the observations reported in this paper.

Weibel and Bright (1971) have also reported an effect of 0.1 M chloride ion on the maximum turnover number (which corresponds to k_2 in eq 9) at pH <5 for *A. niger* glucose oxidase. This effect on k_2, which they find to be less pronounced than the effect on k_1^{app}, would require that chloride ion also affect the ordinate intercept of a plot of $(E_T)/v$ *vs.* $1/(S)$. Such an effect was not observed in Figures 1 and 2, although in the case of D-glucose it is possible that the effect would not be outside experimental error at the oxygen concentration used, since at air saturation with D-glucose $1/k_4(O_2) > 1/k_2$. However, at air saturation with 2-deoxy-D-glucose as substrate $1/k_2 > 1/k_4(O_2)$, and an effect of chloride ion on the ordinate intercept in Figure 2 would be expected based on the data of Weibel and Bright (1971). We are unable to account for this difference except to note that the experiments reported in Figures 1 and 2 were performed in a different buffer system and at a much higher ionic strength than those of Weibel and Bright. If chloride ion binding is significantly decreased at high ionic strength, it is possible that the effect of chloride ion on the ordinate intercept is within our experimental error at the chloride ion concentrations used in this work.

It seems reasonably clear however, that chloride ion can bind specifically to the oxidized form of *A. niger* glucose oxidase, and that such binding alters the environment of the enzyme-bound FAD resulting in a perturbation of its visible absorption spectrum.

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