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Interaction of D-Glucal with *Aspergillus niger* Glucose Oxidase*

M. J. Rogers† and K. G. Brandt‡

ABSTRACT: D-Glucal, a structural analog of D-glucose, is an inhibitor of *Aspergillus niger* glucose oxidase. The inhibition is competitive with respect to D-glucose and uncompetitive with respect to oxygen. In the presence of D-glucal the visible absorption spectrum of the enzyme-bound flavin-adenine dinucleotide in the oxidized form of the enzyme is perturbed. A model is proposed to account for these observations which postulates that D-glucal binds at the active site of the oxidized form of glucose oxidase. The inhibition constant for D-glucal is pH independent over the pH range from 3.8 to 7.5 with an average value of 0.13 M. Comparison of this value with the dissociation constant for the oxidized enzyme–D-glucose complex suggests that D-glucal is not a transition-state analog for glucose oxidase. The pH dependence of the Michaelis constant for 2-deoxy-D-glucose with *A. niger* glucose oxidase was determined in the absence of chloride ion and found to increase at acidic pH by less than twofold between pH 7.0 and 4.0.

Glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) from *Aspergillus niger*, a flavoprotein, catalyzes the oxidation of β-D-glucose by molecular oxygen to give D-glucono-δ-lactone and hydrogen peroxide (Bentley, 1963). The enzyme is highly specific for β-D-glucose, but a few other monosaccharides, *e.g.*, 2-deoxy-D-glucose, D-mannose, and D-galactose, exhibit low activity as substrates (Bentley, 1963). The kinetics of the enzyme-catalyzed reaction have been studied by both steady-state and stopped-flow methods (Gibson *et al.*, 1964; Bright and Gibson, 1967; Nakamura and Ogura, 1968).

Probably due to the remarkable substrate specificity of glucose oxidase, an unreactive competitive inhibitor of the enzyme has not been reported. The availability of such an inhibitor can be of considerable use in studying reaction mechanisms. By using a competitive inhibitor of D-amino acid oxidase, Koster and Veeger (1968) have shown that the mechanism is ordered sequential rather than Ping-Pong. Furthermore, the inhibition constant of a competitive inhibitor should be a simple dissociation constant, in contrast to the Michaelis constant for a substrate which is a complex constant composed of rate constants for both binding and catalytic steps. Consequently, to the extent that inhibitor binding reflects substrate binding, it is possible to separate the effects of pH and other external variables on substrate binding from their effects on subsequent reaction steps.

Lee (1969) has reported that D-galactal (Figure 1A) is a potent inhibitor of β-D-galactopyranosidases. This report prompted us to test D-glucal (Figure 1B) as an inhibitor of glucose oxidase. We have found that D-glucal, a glucose analog, is an inhibitor of *A. niger* glucose oxidase. This paper reports kinetic and spectrophotometric studies of the interaction of D-glucal with glucose oxidase.

Materials and Methods

Lyophilized glucose oxidase from *A. niger* (lots GOP 8AA and GOP 8JA) was obtained from Worthington Biochemical Corp. and used without further purification. Turnover numbers at air saturation extrapolated to infinite D-glucose concentration at 25°, pH 5.6, were 361 sec^{-1} (GOP 8AA) and 293 sec^{-1} (GOP 8JA). These values compare favorably with the value of 356 sec^{-1} calculated for these conditions from the kinetic data of Bright and Gibson (1967) for their enzyme preparations. Assays for contaminating catalase showed only insignificant amounts which did not affect the kinetics. Enzyme concentration, calculated as enzyme-bound FAD[1] concentration, was determined spectrophotometrically at 450 nm using a molar absorptivity of 1.41×10^4 $M^{-1}cm^{-1}$ (Gibson *et al.*, 1964).

D-Glucal was prepared from triacetyl-D-glucal (Aldrich Chemical Co.) based on the procedure of Shafizadeh and Stacey (1952). D-Glucal was obtained as a colorless, hygroscopic solid, mp 58–59° (lit. (Shafizadeh and Stacy, 1952) mp 57–

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[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; L, D-glucono-δ-lactone; I, D-glucal.

59°). These D-glucal preparations were judged to be at least 98% pure, based on thin-layer chromatography on silica gel. In chloroform–methanol (v/v, 6:1) the D-glucal showed an R_F of 0.25, with a minor impurity appearing at an R_F of 0.44. Triacetyl-D-glucal exhibits an R_F of 0.71 under identical conditions. Due to difficulty in recrystallizing D-glucal, and in view of the fact that kinetic and spectrophotometric data were very reproducible from preparation to preparation, this degree of purity was deemed acceptable.

D-Glucose was obtained from Fisher Scientific Co. and 2-deoxy-D-glucose from Calbiochem. β-Methyl D-glucoside was obtained from Calbiochem, fructose from Matheson Coleman & Bell, sucrose from Baker Chemical Co., and *i*-inositol (*meso*-inositol) from Nutritional Biochemical Co.

Turnover experiments were performed on a Yellow Springs Instrument Co. biological oxygen monitor connected to a Sargent Model SRLG recorder. Standard conditions were 25° and 0.2 M sodium acetate buffer (pH 5.6), adjusted to 0.2 M ionic strength with sodium chloride, containing 0.16 mM EDTA. Equilibrium mixtures of α and β anomers of D-glucose were used; glucose concentrations indicated represent total analytical concentration. Initial oxygen concentration was estimated using a value for Henry's law constant of 3.33×10^7 (Washburn, 1928). At 25° the concentration of oxygen in a solution equilibrated with air was calculated to be 0.27 mM. Reaction was initiated by addition of enzyme to give a final concentration of enzyme-bound FAD between 1 and 20 nM. Velocities were calculated from slopes drawn to the oxygen depletion curve at a particular oxygen concentration. The oxygen concentration was determined from the per cent saturation assuming the initial oxygen concentration given above. For these experiments the D-glucal stock solutions were prepared in 0.2 M sodium chloride and adjusted to pH 7.0 to avoid exposure of the labile double bond to acid.

For turnover experiments at other pH values, the following buffers were used: pH 3.8, 4.0, 4.5, and 5.5, 0.2 M sodium acetate; pH 6.5, 0.2 M sodium cacodylate or 0.133 M sodium phosphate; pH 7.5, 0.067 M sodium phosphate. Ionic strength was adjusted as indicated with sodium chloride or potassium sulfate, and each solution contained 0.16 mM EDTA.

Spectrophotometric experiments were performed with a Beckman DB-G spectrophotometer equipped with a Sargent SRLG recorder. The recorder was used in the linear mode with the scale expanded so that full scale corresponded to either 10 or 20% transmittance. Enzyme and D-glucal were dissolved in 0.02 M sodium acetate buffer. At pH 5.6, the buffer was adjusted to ionic strength 0.02 with sodium chloride. At pH 4.0, it was necessary to add 0.2 M sodium chloride to prevent enzyme precipitation. All solutions were filtered through 0.45 μ Millipore filters before use. The glucal solution was checked periodically by thin-layer chromatography, and no breakdown products could be detected during the time required for the experiment. EDTA was omitted in these experiments due to reports that millimolar concentrations of EDTA catalyze the photoreduction of glucose oxidase (Massey and Palmer, 1966).

The difference spectra reported in Figure 3 were obtained using a set of four matched cuvets with 1.0 cm-light path in tandem fashion. In the titration experiments only two cuvets were used. Equal volumes of glucose oxidase solution at the desired concentration were pipetted into the two cuvets, and the spectrophotometer adjusted to 100% T at the desired wavelength. Successive aliquots of D-glucal stock solution were added to the cuvet in the sample beam and an equal volume of buffer added to the cuvet in the reference beam. The per cent transmittance was recorded after each addition. The absorbance was calculated for each point and corrected for dilution to give ΔA which was used in the calculations.

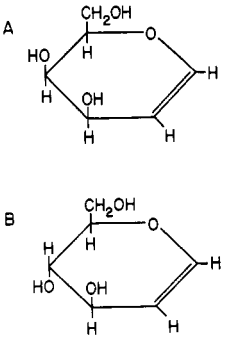

FIGURE 1: (A) Structure of D-galactal and (B) structure of D-glucal.

A Durrum-Gibson stopped-flow spectrophotometer thermostatted with a Lo-Temptrol constant-temperature unit was used for the stopped-flow experiments. A solution of glucose oxidase (10–20 μM enzyme-bound FAD) in buffer was mixed in the instrument with a solution of D-glucal in the same buffer. At pH 5.6, 0.02 M sodium acetate adjusted to 0.02 M ionic strength with sodium chloride was used.

Anaerobic studies of the interaction of D-glucal with glucose oxidase were performed in Thunberg cuvets made anaerobic by alternately evacuating and flushing with nitrogen or argon. Prepurified nitrogen was further purified by passing it over hot copper turnings and then bubbling it through a sodium vanadate solution (Meites and Meites, 1948). Argon was purified by bubbling it through chromous sulfate. A solution of D-glucal was added from the side arm. All experiments were performed in 0.02 M sodium acetate buffer (pH 5.6), adjusted to 0.02 M ionic strength with sodium chloride.

Results

Steady-State Turnover Experiments. Figure 2 shows the results of steady-state turnover experiments at pH 5.6 to determine the effect of D-glucal on the kinetics of D-glucose oxidation. Inhibition by D-glucal is competitive with respect to D-glucose and uncompetitive with respect to oxygen. From the slopes in Figure 2A, a K_i of 0.13 M was calculated.

Sucrose, *i*-inositol, β-methyl D-glucoside, and fructose were also tested as inhibitors to check for possible nonspecific effects of carbohydrates by measuring the initial velocity of D-glucose oxidation under standard conditions at a single D-glucose concentration of 0.025 M and air saturation. Since the Michaelis constant for D-glucose under these conditions is 0.11 M (Gibson *et al.*, 1964), the conditions are suitable for sensitive detection of inhibition. Defining the initial velocity observed under these conditions in the absence of inhibitor as 100, addition of the following concentrations of these compounds resulted in the indicated relative initial velocities: 0.15 M sucrose, $v = 108$; 0.097 M *i*-inositol, $v = 100$; 0.16 M β-methyl D-glucoside, $v = 106$; 0.16 M fructose, $v = 103$. In a comparable experiment 0.13 M D-glucal resulted in $v = 61$. It seems reasonable to conclude that none of these other compounds acts as an inhibitor. The small stimulation observed with fructose, β-methyl D-glucoside, and sucrose may be due to slight contamination with D-glucose.

Spectrophotometric Experiments. If D-glucal binds to the oxidized form of glucose oxidase, a perturbation of the spec-

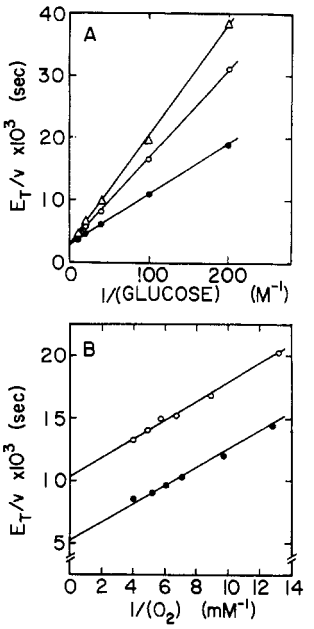

FIGURE 2: Lineweaver–Burk plots showing the effect of D-glucal on the kinetics of oxidation of D-glucose by glucose oxidase at 25°, pH 5.6. (A) Reciprocal of the initial turnover number at 0.27 mM oxygen *vs.* the reciprocal of D-glucose concentration. D-Glucal concentrations: (●) none, (○) 0.079 M, and (△) 0.133 M. (B) Reciprocal of the initial turnover number at 0.02 M D-glucose *vs.* the reciprocal of the oxygen concentration. D-Glucal concentration: (●) none and (○) 0.11 M.

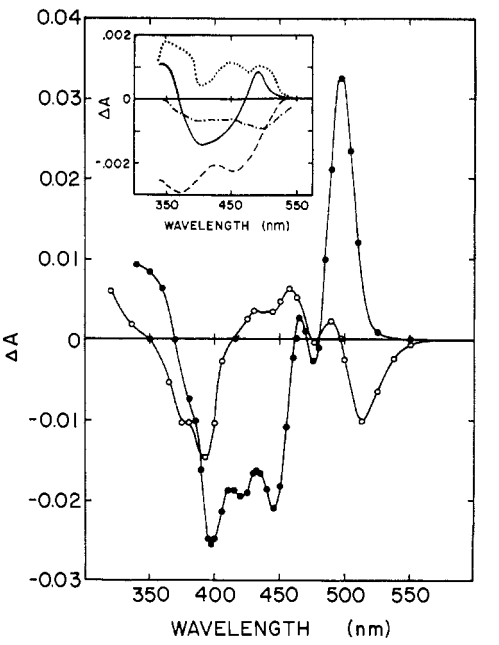

FIGURE 3: Difference spectra between glucose oxidase plus D-glucal and glucose oxidase. (●) 0.3 M D-glucal, 17 μM enzyme-bound FAD, in 0.02 M sodium acetate buffer, pH 5.5, 22°; (○) 0.27 M D-glucal, 17 μM enzyme-bound FAD, in 0.02 M sodium acetate buffer (pH 4.0), containing 0.2 M KCl, 25°. Inset: difference spectra measured at 25°, pH 5.6, resulting from addition to 16 μM enzyme-bound FAD: (····) 0.33 M fructose, (——) 0.33 M β-methyl D-glucoside, (——·——) 0.1 M *i*-inositol, and (----) 0.33 M sucrose.

trum of the enzyme-bound FAD might be expected. Such a perturbation is observed and the difference spectrum at pH 5.5 between glucose oxidase plus 0.3 M D-glucal and uncomplexed glucose oxidase is shown in Figure 3 (filled circles). Assuming this difference spectrum results from a simple binding interaction between D-glucal and the enzyme, one can write

$$E_o + I \rightleftharpoons E_o{:}I \tag{1}$$

$$K_d = \frac{(E_o)(I)}{(E_o{:}I)} \tag{2}$$

E_o is oxidized enzyme, *i.e.*, enzyme with the enzyme-bound FAD in the oxidized state, and I is D-glucal. If $(I)_T \gg (E_o)_T$, where the subscript T denotes the total analytical concentration of a species and assuming $\Delta A = \Delta\epsilon_M (E_o{:}I)$, treatment of the data after the method of Benesi and Hildebrand (1949) gives eq 3. ΔA, the difference in absorbance at a given wavelength between a solution of glucose oxidase plus D-glucal and

$$\frac{1}{(I)_T} = \frac{(E_o)_T \Delta\epsilon_M}{K_d} \frac{1}{\Delta A} - \frac{1}{K_d} \tag{3}$$

a solution containing the same total concentration of glucose oxidase in the absence of D-glucal, was measured as a function of $(I)_T$, and K_d was evaluated from a plot of $I/(I)_T$ *vs.* $I/\Delta A$ according to eq 3, as shown in Figure 4 (filled circles). The data conform to a straight line, as required by eq 3, and give $K_d = 0.15$ M at pH 5.6, in good agreement with the kinetically determined K_i of 0.13 M.

Evidence has been presented above that β-methyl D-glucoside, *i*-inositol, fructose, and sucrose at concentrations of about 0.1 M do not inhibit glucose oxidase. It was also found that these compounds do not significantly perturb the spectrum of the enzyme-bound FAD as shown in the inset of Figure 3. Note that the ordinate scale in the inset is four times that in the main figure.

The effect of D-glucal on the spectrum of glucose oxidase was also investigated at pH 4.0. In order to prevent precipitation of the enzyme at this pH at the high enzyme concentrations required for this experiment, it was necessary to do the experiment in the presence of 0.2 M sodium chloride. Figure 3 (open circles) shows the difference spectrum which results when D-glucal binds to glucose oxidase at pH 4.0. It is apparent that the difference spectrum is opposite in sign to that obtained at pH 5.6 and that the wavelength of the maxima and minima are shifted. Specifically, the long-wavelength peak is now at 513 nm and is the result of a blue shift. Titration of the difference spectrum at 513 nm as a function of D-glucal concentration gives a dissociation constant of 0.22 M as shown in Figure 4 (open circles), comparing favorably with the kinetically determined K_i of 0.17 M measured at pH 4.0 (see below).

pH Dependence of K_i. If K_i is in fact the dissociation constant of the oxidized enzyme–D-glucal complex, the study of its pH dependence will be relevant to the interpretation of the pH dependence of K_m for substrates. We have determined K_i for D-glucal at selected pH values in the range pH 3.8–7.5. It was assumed that D-glucal was a competitive inhibitor with respect to D-glucose throughout this pH range, as it was demonstrated to be at pH 5.6, and K_i was evaluated from plots of $1/v$ *vs.* $(I)_T$ as described by Dixon (1953). The initial velocity was measured at 0.24 mM oxygen at two different D-glucose concentrations, 0.025 and 0.051 M, in each case with no D-glucal present and also in the presence of 0.06 and 0.12

TABLE I: Effect of pH on K_i for D-Glucal and K_m for 2-Deoxy-D-glucose.

pH	K_i for D-Glucal[a] (M)	K_m for 2-Deoxy-D-glucose: This Work[b] (M)	Bright and Appleby (1969)[c] (M)	Bright and Appleby (1969) Cor According to Eq 7 (M)
3.8	0.18			
4.0	(0.17)	0.040	0.670	0.059
4.3		0.037	0.350	0.031
4.5	0.16 (0.12)			
5.5	(0.12)			
5.6	0.13	0.032	0.045	0.026
6.2		0.028	0.032	0.027
6.5	0.08 (0.15)			
7.0		0.027	0.029	0.027
7.5	0.11 (0.11)			

[a] All buffers were adjusted to ionic strength 0.2 with either NaCl or K_2SO_4, the values in parentheses being obtained using K_2SO_4. [b] Ionic strength was adjusted to between 0.5 and 0.6 with K_2SO_4. No chloride ion was present. [c] Calculated according to eq 15 of Bright and Appleby (1969) using their best fit parameters of K_m = 0.0286 M and pK_1 = 5.35.

M D-glucal. At all pH's the data conformed to the pattern expected for competitive inhibition, namely, intersection of lines determined at the two different D-glucose concentrations at a point above the abscissa and to the left of the ordinate. The values of K_i so obtained are recorded in column 2 of Table I. It appears that there is no significant effect of pH on K_i in this pH region. These values were obtained using sodium chloride to adjust the ionic strength. Chloride ion affects the kinetics of both *Penicillium notatum* glucose oxidase and *A. niger* glucose oxidase at acidic pH (Bright and Appleby, 1969; Weibel and Bright, 1971; Rogers and Brandt, 1971a). When K_i for D-glucal was determined at selected pH values between 4.0 and 7.5 using potassium sulfate to adjust the ionic strength, not only was no effect of pH on K_i observed, as can be seen from the values in parentheses in column 2 of Table I, but the values were identical within experimental error to those obtained in the presence of chloride ion.

pH Dependence of K_m for 2-Deoxy-D-glucose. 2-Deoxy-D-glucose is the only substrate of glucose oxidase which exhibits a saturation effect when the kinetics of reduction of the enzyme-bound FAD are studied by stopped-flow methods under anaerobic conditions (Gibson *et al.*, 1964). The steady-state K_m for 2-deoxy-D-glucose has been interpreted by Gibson *et al.* (1964) as the dissociation constant of the enzyme substrate complex, although Bright and Appleby (1969) have interpreted their data differently based on chloride ion effects. If the K_m for this substrate is simply the dissociation constant for the enzyme–2-deoxy-D-glucose complex, it might be expected that the pH dependence of K_m for 2-deoxy-D-glucose would be the same as the pH dependence of K_i for D-glucal. The pH independence of K_i for D-glucal with *A. niger* glucose oxidase contrasts sharply, therefore, with the marked pH dependence of K_m for 2-deoxy-D-glucose with *P. notatum* glucose oxidase reported by Bright and Appleby (1969).

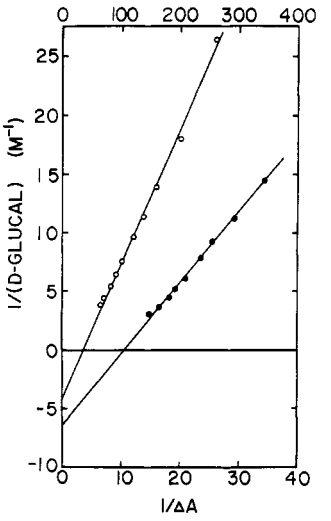

FIGURE 4: Benesi–Hildebrand plots according to eq 3 of titration of the difference spectra of Figure 3 as a function of D-glucal concentration. (●) pH 5.6, 22°, 33 μM enzyme-bound FAD, ΔA measured at 497.5 nm, bottom abscissa scale; (○) pH 4.0, 25°, 26 μM enzyme-bound FAD, ΔA measured at 513 nm, top abscissa scale. Other conditions as described in Figure 3.

K_m for 2-deoxy-D-glucose with *A. niger* glucose oxidase was therefore evaluated in the absence of chloride ion from the abscissa intercepts of plots of the reciprocal of the initial turnover number *vs.* the reciprocal of the 2-deoxy-D-glucose concentration at selected pH values between 4.0 and 7.0 at 0.27 mM oxygen. It was verified that with the *A. niger* enzyme the initial turnover numbers at 0.17 mM oxygen varied less than 10% from those obtained at 1.0 mM oxygen. It can therefore be assumed that the apparent K_m for 2-deoxy-D-glucose evaluated at 0.27 mM oxygen corresponds closely to the K_m at infinite oxygen concentration. The values so obtained for K_m in the absence of chloride ion are reported in column 3 of Table I. K_m increases gradually between pH 7.0 and 4.0. The extent of increase is consistent with the observation (Rogers and Brandt, 1971a) that D-glucose binding decreases under these conditions following protonation of a group on the enzyme with pK_1 = 3.7. For comparison, column 4 of Table I gives values of K_m^{app} for 2-deoxy-D-glucose obtained by Bright and Appleby (1969) with *P. notatum* glucose oxidase. These values have been calculated using eq 4 (which is eq 15 of Bright and Appleby (1969)) using their best-fit parameters of K_m = 0.0286 M and pK_1 = 5.35.

$$K_m^{app} = K_m(1 + (H^+)/K_1) \quad (4)$$

Bleaching of the Enzyme. During the course of the spectral work it was observed that when high concentrations of enzyme and D-glucal were mixed at pH 5.6 and allowed to stand, the characteristic yellow color of the enzyme-bound FAD was bleached. The effect could apparently be reversed by oxygen, since vigorous mixing of the solution in an aerobic atmosphere resulted in restoration of the yellow color. Enzyme which had been bleached and reoxidized several times showed no loss of catalytic activity.

The bleaching of the enzyme-bound FAD was followed under anaerobic conditions by observing the decrease in absorbance at 450 nm upon addition of D-glucal. No bleaching was observed during 90 min under anaerobic conditions in the absence of D-glucal. A short lag was generally ob-

served due to incomplete removal of oxygen. The overall bleaching reaction is biphasic, involving an initial, relatively fast portion which appears to be dependent on D-glucal concentration, followed by a very slow portion which is apparently independent of D-glucal concentration. The slow phase is accelerated about fourfold by light. As a first approximation the data were plotted as the logarithm of the per cent total absorbance change remaining against elapsed time and "apparent" first-order rate constants were calculated from the slopes of the two segments of the plots. At final concentrations of 0.32 M D-glucal and 4.2 μM enzyme-bound FAD, the rate constants for the fast and slow parts of the reaction were 1.21 and 0.012 min^{-1}, respectively. Similar results were obtained when the experiment was repeated using 25.7 μM enzyme-bound FAD. Extrapolation to infinite D-glucal gives a maximum value of 2.2 min^{-1} for the rate constant for the fast portion. Bleaching of the enzyme-bound FAD by D-glucal is therefore very slow compared to bleaching by D-glucose, the latter occurring with a pseudo-first-order rate constant of 60,000 min^{-1} at 0.1 M D-glucose and 27° (Gibson *et al.*, 1964).

Stopped-Flow Kinetic Experiments. In order to set some limits on the speed of the steps involved in D-glucal binding to glucose oxidase, an attempt was made to observe the kinetics of the process using stopped-flow techniques. At pH 5.6 and 25°, when enzyme and D-glucal were mixed in the stopped-flow apparatus to give final concentrations of 12 μM and 0.1 M, respectively, no time-dependent change in light transmittance was observed at 497.5 nm. From the data of Figure 2 and a molar absorptivity of the enzyme of 5.25×10^3 M^{-1} cm^{-1} at 497.5 nm, a 21-mV signal change was expected (100% T corresponded to an 800-mV signal). The per cent transmittance immediately after mixing corresponded to that expected for the complex. It appears, therefore, that binding is complete during the dead time of the apparatus, approximately 5 msec.

Discussion

The observation that D-glucal is a competitive inhibitor with respect to D-glucose and uncompetitive with respect to oxygen can be accommodated by the mechanism shown in Scheme I. This mechanism is simply the kinetic scheme proposed recently by Weibel and Bright (1971) for *A. niger* glucose oxidase with an additional hypothesis that a dead-end complex can form between E_o and D-glucal. The numbering of

SCHEME I

$$E_o + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_o\text{-}G \xrightarrow{k_2} E_r + L$$

$$E_r + O_2 \xrightarrow{k_4} E_o + H_2O_2$$

$$E_o + I \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_o{:}I$$

the rate constants is based on Weibel and Bright (1971). The protonic equilibria have been omitted for the sake of simplicity, and a discussion of the pH dependence and its bearing on this work is presented later (see below). This mechanism yields the steady-state rate equation given in eq 5 in double-reciprocal form, where $K_i = k_6/k_5$. This equation correctly predicts the observed inhibition pattern.

$$\frac{(E_T)}{v} = \frac{1}{k_2} + \frac{1}{(G)}\left(\frac{k_{-1} + k_2}{k_1 k_2}\right)\left(1 + \frac{(I)}{K_i}\right) + \frac{1}{k_4(O_2)} \quad (5)$$

The bleaching of the enzyme-bound FAD of glucose oxidase under anaerobic conditions indicates that D-glucal may not be an entirely unreactive competitive inhibitor, but that it may in some way reduce the flavin. It is possible that the bleaching results from a small amount of D-glucose or 2-deoxy-D-glucose present as contaminant in the D-glucal preparations. If that is the case, the bleaching does not complicate the above interpretation of the inhibition by D-glucal.

If the bleaching is due to reduction of the enzyme-bound FAD of glucose oxidase by D-glucal itself, an additional step must be added to the mechanism given in Scheme I. The resulting mechanism[1] is shown in Scheme II, where I′ is an as yet unidentified product.

SCHEME II

$$E_o + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_o\text{-}G \xrightarrow{k_2} E_r + L$$

$$E_r + O_2 \xrightarrow{k_4} E_o + H_2O_2$$

$$E_o + I \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_o{:}I \xrightarrow{k_7} E_r + I'$$

The fact that bleaching was observed initially in the titration experiments only at high enzyme and high D-glucal concentrations is readily explained by the aerobic conditions under which the titration experiments were carried out. Only when the rate of flavin bleaching and subsequent oxygen consumption for reoxidation of the reduced enzyme-bound FAD exceeded the rate of oxygen diffusion into the unstirred cuvet would bleaching be observed. Since the rate of oxygen utilization should increase with fractional saturation of the enzyme by D-glucal and also with the total enzyme concentration, only at high concentrations of D-glucal and enzyme would the bleaching be significant.

The steady-state rate equation generated by the mechanism of Scheme II is given in double-reciprocal form in eq 6. This equation reduces to eq 5 if (a) $k_1k_2(G)/(k_{-1} + k_2) \gg k_5k_7 \times (I)/(k_6 + k_7)$ and (b) $k_6 \gg k_7$. The fraction $k_1k_2/(k_{-1} + k_2)$ corresponds to the apparent bimolecular rate constant for enzyme-bound FAD reduction by D-glucose and has a value of 1.26×10^4 $M^{-1}sec^{-1}$ (Gibson *et al.*, 1964). If k_7 corresponds to the fast portion of the enzyme-bound FAD bleaching by D-glucal observed under anaerobic conditions and $(k_6 + k_7)/$

$$\frac{(E_T)}{v} = \frac{1}{k_2}\left[\frac{\frac{k_1k_2}{k_{-1} + k_2}(G)}{\frac{k_1k_2}{k_{-1} + k_2}(G) + \frac{k_5k_7}{k_6 + k_7}(I)}\right] + \frac{1 + \frac{k_5}{k_6 + k_7}(I)}{\frac{k_1k_2}{k_{-1} + k_2}(G) + \frac{k_5k_7}{k_6 + k_7}(I)} + \frac{1}{k_4(O_2)} \quad (6)$$

k_5 is taken to be 0.13 M, the value for K_i calculated from inhibition studies, it can be shown that inequality (a) is indeed valid even at 0.005 M D-glucose.

In order to decide whether the K_i determined from the inhibition and difference spectral experiment is a simple dissociation constant (k_6/k_5) or a steady-state constant ($(k_6 + k_7)/k_5$), the relative size of k_6 and k_7 must be considered. Since the maximum value of k_7 estimated above was 2.2 min^{-1} and since the spectral change resulting from D-glucal binding appeared to be complete within the dead time of the stopped-flow apparatus (about 5 msec), it is concluded that $k_6 \gg k_7$ and that K_i does indeed represent a simple dissociation constant for the enzyme–D-glucal complex.

The difference spectral data are also consistent with the hypothesis that D-glucal binds to the oxidized enzyme in the vicinity of the FAD. The spectral titration gives a value for the dissociation constant for the complex which agrees with the kinetically determined inhibition constant at pH 5.6; the spectrally determined value at pH 4.0 is in agreement with the kinetically determined value at pH 4.0.

At pH 5.6 the difference spectrum which results when D-glucal binds to glucose oxidase indicates a red shift of the FAD spectrum and is similar to that observed for the binding of competitive inhibitors to other flavoproteins (Veeger *et al.*, 1966). The red shift and the overall difference spectrum resulting from the interaction between D-glucal and glucose oxidase is remarkably similar to the difference spectrum observed for the binding of FAD to lipoamide dehydrogenase apoenzyme (Veeger *et al.*, 1966). This red shift has been correlated with a decrease in the exposure of the FAD to the aqueous solvent. That the effect of D-glucal on the spectrum is not a general solvent perturbation effect (Herskovits and Laskowski, 1962) but is a specific effect due to the binding of D-glucal at the active site is supported by the agreement between the kinetic and spectral dissociation constants and the observation of only minor spectral perturbations resulting from similar concentrations of sucrose, *i*-inositol, β-methyl D-glucoside, and fructose.

The change in the nature of the difference spectrum resulting from D-glucal binding at pH 4.0 relative to that at pH 5.6 is quite striking. At pH 4.0 D-glucal binding produces a blue shift of the enzyme-bound FAD spectrum. The spectrum obtained at pH 4.0 was obtained in the presence of 0.2 M sodium chloride to prevent enzyme precipitation which occurs at low ionic strength at this pH. Interpretation of the altered difference spectrum at pH 4.0 in the presence of 0.2 M sodium chloride is discussed in an accompanying paper which considers the effect of chloride ion on *A. niger* glucose oxidase (Rogers and Brandt, 1971a).

It is interesting to note, however, that the K_d for D-glucal calculated from the spectral titration at pH 4.0 in the presence of 0.2 M sodium chloride agrees well with the kinetically determined K_i for D-glucal at the same pH in the absence of chloride ion. In an accompanying paper (Rogers and Brandt, 1971a) chloride ion is shown to be a competitive inhibitor with respect to D-glucose and 2-deoxy-D-glucose with *A. niger* glucose oxidase, with an apparent dissociation constant of 0.015 M at pH 4.0. The observation that the interaction of D-glucal with glucose oxidase is not affected by 0.2 M chloride ion, which should give greater than 90% saturation of the chloride ion binding site at pH 4.0, suggests that chloride ion and D-glucal may be able to bind to *A. niger* glucose oxidase simultaneously. Such simultaneous binding of chloride ion and D-glucal is demonstrated in an accompanying paper (Rogers and Brandt, 1971b).

K_i for D-glucal inhibition of *A. niger* glucose oxidase is apparently pH independent in the pH range between 3.8 and 7.5. K_m for 2-deoxy-D-glucose with *A. niger* glucose oxidase exhibits a gradual increase with decreasing pH in the range of pH 7.0–4.0 in the absence of chloride ion. As noted above, this increase is consistent with the pH dependence of D-glucose binding (Rogers and Brandt, 1971a) under similar conditions. The complex constant $k_1k_2/(k_{-1} + k_2)$ for D-glucose oxidation has been observed to be pH dependent with both the *A. niger* enzyme (Weibel and Bright, 1971; Rogers and Brandt, 1971a) and the *P. notatum* enzyme (Bright and Appleby, 1969). This pH dependence has been interpreted as a direct effect on the binding of D-glucose to the oxidized enzyme. The pK_a of the group affecting substrate binding has been found to be less than 4.0 in the *A. niger* enzyme. It has been suggested for both the *P. notatum* enzyme (Bright and Appleby, 1969) and the *A. niger* enzyme (Weibel and Bright, 1971) that binding of substrate to enzyme may involve interactions between a carboxylate group at the active site and the hydroxyl group on carbon atom 1 of the substrate. The pH dependence of substrate binding would then be due to protonation of this carboxylate group. It seems reasonable to expect that the impossibility of an interaction between this postulated enzyme-carboxylate group and D-glucal (which lacks the hydroxyl group on carbon atom 1) may result in pH-independent binding of D-glucal.

The difference in the results presented above for the pH dependence of K_m for 2-deoxy-D-glucose with *A. niger* glucose oxidase and the corresponding values of Bright and Appleby (1969) with *P. notatum* glucose oxidase may simply be due to an inherent difference in the enzymes from the two fungi. There is an alternative explanation, however. Bright and Appleby carried out their experiments in the presence of 0.2 M potassium chloride. At ionic strengths between 0.5 and 0.6, chloride ion exhibits competitive inhibition with respect to both D-glucose and 2-deoxy-D-glucose (Rogers and Brandt, 1971a). The inhibition constant for chloride ion is pH dependent, varying from 7.8 mM at pH 3.5 to about 1.0 M at pH 6.0. Weibel and Bright (1971), working at low ionic strength, have recently reported that 0.1 M chloride ion also affects the maximum turnover number of the *A. niger* enzyme with D-glucose as the substrate, but they have not evaluated inhibition constants for this effect. Bright and Appleby (1969) have reported that chloride ion at low pH inhibits *P. notatum* glucose oxidase. Assuming that the inhibition constants for chloride ion obtained with the *A. niger* enzyme are applicable to the *P. notatum* enzyme, the K_m^{app} values of Bright and Appleby can be corrected for the competitive component of chloride ion inhibition using eq 7. In eq 7, K_m^{app} is the apparent Michaelis constant for 2-deoxy-D-glucose reported by Bright and Appleby (1969) in the presence of 0.2 M chloride ion at a

$$K_m = K_m^{app} \Big/ \left(1 + \frac{(Cl^-)}{K_{Cl}^{app}}\right) \qquad (7)$$

specified pH and K_{Cl}^{app} is the apparent inhibition constant for chloride ion at that pH for the *A. niger* enzyme (Rogers and Brandt, 1971a). These corrected values are recorded in column 5 of Table I. It can be seen that these corrected values compare favorably with the values obtained for *A. niger* glucose oxidase in the absence of chloride ion (column 3 in Table I).

Finally one can compare the relative values of K_i for D-glucal and the dissociation constant for D-glucose binding. Bright and Gibson (1967) have estimated a dissociation con-

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*

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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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