Multiple Inhibition Analysis of *Aspergillus niger* Glucose Oxidase by D-Glucal and Halide Ions*

M. J. Rogers† and K. G. Brandt‡

ABSTRACT: Multiple inhibition analysis of *Aspergillus niger* glucose oxidase has been performed with two inhibitors, D-glucal and chloride ion, each of which is competitive with respect to the substrate D-glucose. Evidence is presented which demonstrates that the two inhibitors can bind simultaneously to the enzyme, with an average interaction constant, α, equal to 2.5. Similar analysis using bromide ion or iodide ion in place of chloride ion gives values of $\alpha = 2.0$ and 2.1, respectively. The results indicate that there is only minimal interaction between the substrate analog inhibitor, D-glucal, and halide ion, suggesting that although each is a competitive inhibitor with respect to D-glucose, the two inhibitors bind at different loci at the active site of the enzyme. The results are also consistent with an hypothesis that the competitive inhibition by chloride ion does not result from a direct steric interaction between chloride ion and the substrate.

D-Glucal and chloride ion have each been shown to be inhibitors of *Aspergillus niger* glucose oxidase (β-D-glucose: oxygen oxidoreductase, EC 1.1.3.4). D-Glucal, a substrate analog, is a competitive inhibitor with respect to D-glucose (Rogers and Brandt, 1971a). At ionic strengths between 0.5 and 0.6, chloride ion has also been shown to exhibit apparently competitive inhibition with respect to D-glucose (Rogers and Brandt, 1971b), although Weibel and Bright (1971) have recently reported an additional, less pronounced effect of 0.1 M chloride ion on the maximum turnover number at pH values below 5. Both chloride ion and D-glucal have been shown to bind to the oxidized form of glucose oxidase, as detected by perturbation of the visible absorption spectrum of the enzyme-bound FAD[1] (Rogers and Brandt, 1971a,b). For both chloride ion and D-glucal it was postulated that inhibition results from formation of a complex between the oxidized enzyme and the inhibitor which is unable to bind substrate. Excellent agreement was observed between the kinetically determined inhibition constant and the static, spectrophotometrically determined dissociation constant for these enzyme–inhibitor complexes.

It was noted (Rogers and Brandt, 1971a), however, that at pH 4.0 the dissociation constant for the enzyme–D-glucal complex was not affected by the presence of 0.2 M potassium chloride, suggesting that these two competitive inhibitors might be able to bind simultaneously to the oxidized enzyme. In this paper we report evidence demonstrating that such simultaneous binding of these two competitive inhibitors can occur. Multiple inhibition analysis (Yonetani and Theorell, 1964) indicates only minimal interaction between the two inhibitors and suggests that, although each is a competitive inhibitor, they bind at different loci at the active site of the enzyme.

Materials and Methods

A. niger glucose oxidase (lot GOP 8JA) was obtained from Worthington Biochemical Corp. and used without further purification (Rogers and Brandt, 1971a). Enzyme concentration was determined at 450 nm at pH 5.6 and 25° using a molar absorptivity of 1.41×10^4 M^{-1} cm^{-1} (Gibson *et al.*, 1964), and is reported as concentration of enzyme-bound FAD.

D-Glucal was prepared as described previously (Rogers and Brandt, 1971a). All other chemicals were reagent grade.

Steady-state kinetic measurements were carried out using a Yellow Springs Instrument Co. oxygen monitor as described previously (Rogers and Brandt, 1971a), in 0.2 M sodium acetate buffer (pH 4.5), containing 0.2 mM EDTA at 25°. Ionic strength was maintained constant with potassium sulfate and was 0.63 in the experiments with potassium chloride and potassium bromide and 0.60 in the experiments with potassium iodide. D-Glucal stock solutions were prepared in water and maintained at neutrality. In the experiments using potassium iodide a yellow color was observed to develop slowly on addition of enzyme to the reaction mixture. This color is believed to be due to reaction between iodide ion and hydrogen peroxide, the latter being a product of the enzymatic oxidation of glucose. During the short time required for kinetic measurements this side reaction appeared to be negligible.

Difference spectra and spectrophotometric titrations were carried out as described previously (Rogers and Brandt, 1971a). All spectral experiments were performed in 0.10 M sodium acetate buffer, pH 5.6, at 25°. The ionic strength was 0.11 M prior to addition of potassium chloride. No attempt was made to maintain constant ionic strength in the titration experiments, since it had been shown that ionic strength had virtually no effect on the spectrum of the enzyme-bound FAD (Rogers and Brandt, 1971b).

Results and Analysis of Data

The initial, steady-state rate of glucose oxidase catalyzed oxidation of D-glucose was measured in the presence of both

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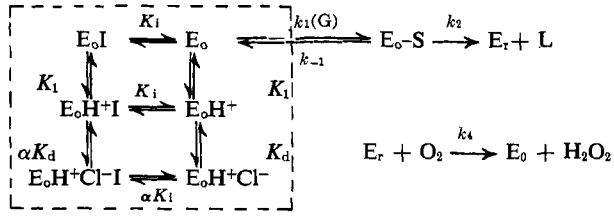
[1] Abbreviations used are: FAD, flavin-adenine dinucleotide; (E_T), total concentration of enzyme-bound FAD; E_o, enzyme in which the enzyme-bound FAD is in the oxidized form; E_r, enzyme in which the enzyme-bound FAD is in the reduced form; G, D-glucose; L, D-glucono-δ-lactone; I, D-glucal.

SCHEME I



chloride ion and D-glucal at pH 4.5. This pH was chosen to enhance chloride ion inhibition, which is pH dependent (Rogers and Brandt, 1971b). Data from a typical experiment are shown in Figure 1. Figure 1A shows the variation in the reciprocal of the turnover number as a function of D-glucal concentration in the absence of chloride ion and in the presence of four different concentrations of chloride ion. The ionic strength was maintained at a constant value throughout with potassium sulfate, which has previously been shown not to affect the turnover number of *A. niger* glucose oxidase (Rogers and Brandt, 1971b). It is apparent that a family of straight lines which intersect at a common point is obtained. The intersection point appears to fall on the abscissa. Similar results are observed in Figure 1B where the reciprocal of the turnover number is plotted against chloride ion concentration in the absence and presence of D-glucal. Again, a family of straight lines intersecting in a common point, apparently on the abscissa, is observed.

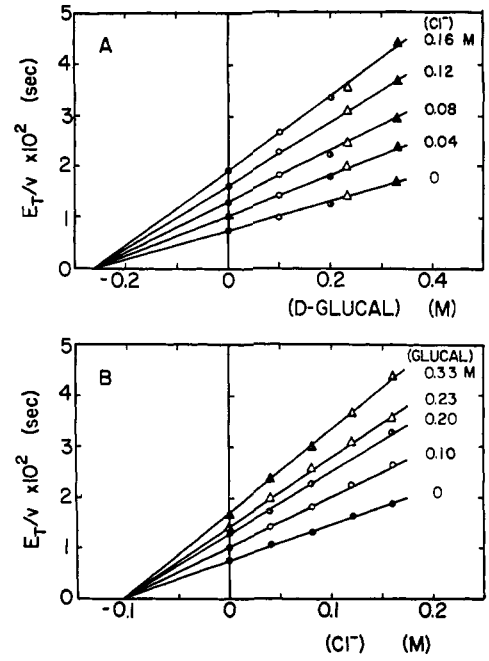


FIGURE 1: Multiple inhibition of glucose oxidase by chloride ion and D-glucal at pH 4.5 and 25°. Initial velocities were measured at 0.025 M D-glucose and 0.27 mM oxygen, using 26 nM enzyme-bound FAD. (A) Plot of the reciprocal of the initial turnover number *vs.* D-glucal concentration at the indicated fixed concentrations of chloride ion. (B) Plot of the reciprocal of the initial turnover number *vs.* chloride ion concentration at the indicated fixed concentrations of D-glucal.

It has been shown (Rogers and Brandt, 1971b) that the pH-dependent inhibition of *A. niger* glucose oxidase by chloride ion can be explained in terms of chloride ion binding to a protonated form of the oxidized enzyme with only the unprotonated form being reactive with the substrate D-glucose. The inability of the protonated form of the oxidized enzyme to interact with substrate has been previously proposed to explain the pH dependence of *P. notatum* glucose oxidase (Bright and Appleby, 1969) and *A. niger* glucose oxidase (Weibel and Bright, 1971). Inhibition of *A. niger* glucose oxidase by the substrate analog D-glucal appears to be pH independent (Rogers and Brandt, 1971a). This can be accounted for if D-glucal can bind equally well to either the protonated or unprotonated form of the oxidized enzyme. Scheme I incorporates these two mechanisms of inhibition and in addition considers the possibility of simultaneous binding of both D-glucal and chloride ion. The remainder of the kinetic mechanism[1] is the mechanism recently proposed by Weibel and Bright (1971) for the oxidation of D-glucose at this pH. The treatment of the inhibitor-binding equilibria is based on that of Yonetani and Theorell (1964) for multiple inhibition analysis of inhibitors of liver alcohol dehydrogenase.

In Scheme I, K_i is the dissociation constant for D-glucal from either E_oI or E_oH^+I, K_1 is the acid dissociation constant for E_oH^+ and E_oH^+I, and K_d is the dissociation constant for chloride ion from $E_oH^+Cl^-$. The parameter α is a constant which measures the interaction between chloride ion and D-glucal. α can vary between zero and infinity.

If $\alpha = \infty$, the two inhibitors exclude each other and do not bind simultaneously. If $1 < \alpha < \infty$, both inhibitors can bind simultaneously, but the presence of one inhibitor decreases the affinity of the enzyme for the other inhibitor. If $\alpha = 1$, there is no interaction between the two inhibitors and they can bind simultaneously and independently. If $0 < \alpha < 1$, the presence of one inhibitor increases the affinity of the enzyme for the other inhibitor.

The rate equation generated by the mechanism of Scheme I, assuming the species enclosed in dashed lines are in rapid equilibrium, has been derived using the combined steady-state and rapid-equilibrium method of Cha (1968), and is given in eq 1 in double-reciprocal form. When (Cl^-) is set equal

$$\frac{(E_T)}{v} = \frac{1}{k_2} + \frac{1}{k_4(O_2)} + \frac{1}{(G)}\left[\frac{k_{-1} + k_2}{k_1k_2}\right]\left[1 + \frac{(I)}{K_i} + \frac{(H^+)}{K_1} + \frac{(I)(H^+)}{K_iK_1} + \frac{(Cl^-)(H^+)}{K_dK_1} + \frac{(I)(Cl^-)(H^+)}{\alpha K_iK_dK_1}\right] \quad (1)$$

to zero, eq 1 reduces to the equation previously proposed to describe inhibition by D-glucal alone (eq 5 of Rogers and Brandt, 1971a) with the $1/(G)$ term in that equation multiplied by $(1 + (H^+)/K_1)$ to account for the pH dependence of substrate binding which was not specifically considered in that equation. When (I) is set equal to zero, eq 1 reduces to the equation previously proposed to describe inhibition by chloride ion alone (eq 10 of Rogers and Brandt, 1971b).

Equation 2 is obtained from eq 1 by setting $\alpha = \infty$ and rearranging to obtain an expression for $(E_T)/v$ as a function of chloride ion concentration. It is apparent that eq 2 predicts that varying the concentration of D-glucal will affect the ordinate intercept of a plot of $(E_T)/v$ *vs.* (Cl^-), but not the slope,

$$\frac{(E_T)}{v} = \left[\frac{1}{k_2} + \frac{1}{k_4(O_2)} + \frac{k_{-1} + k_2}{k_1k_2(G)}\left(1 + \frac{(I)}{K_i} + \frac{(H^+)}{K_1} + \frac{(I)(H^+)}{K_iK_1}\right)\right] + (Cl^-)\left[\frac{(k_{-1} + k_2)(H^+)}{k_1k_2K_dK_1(G)}\right] \quad (2)$$

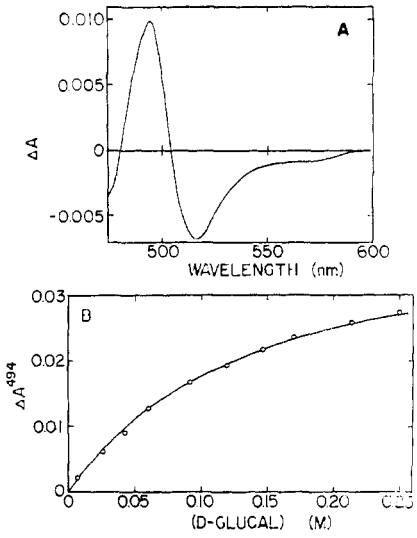


FIGURE 2: Spectral studies of the effect of D-glucal on the visible absorption spectrum of glucose oxidase in the presence of 0.6 M chloride ion. (A) Difference spectrum between (14.7 μM enzyme-bound FAD + 0.6 M KCl + 0.257 M D-glucal) and (14.7 μM enzyme-bound FAD + 0.6 M KCl). (B) Plot of ΔA^{494}, the absorbance change resulting from addition of D-glucal to a solution of 29.6 μM enzyme-bound FAD + 0.6 M KCl, *vs.* concentration of D-glucal added. The line drawn through the data is the theoretical line calculated for eq 5 using values of $K_{app} = 0.136$ M and $\Delta A^{494}_{max} = 0.042$.

so that a family of parallel lines will be obtained. Since the lines in Figure 1B are not parallel, it is concluded that $\alpha < \infty$ and that chloride ion and D-glucal can bind simultaneously to the enzyme.

When (Cl^-) is set equal to $-\alpha K_d(1 + K_I/(H^+))$, eq 1 predicts that $(E_T)/v$ will be independent of the concentration of D-glucal. Thus, knowing K_d, K_I, and (H^+), α can be calculated from the abscissa coordinate of the point of intersection of the lines in Figure 1B, which is equal to -0.106 M. Using the values of $K_d = 0.005$ M, and $pK_I = 3.7$ as determined previously (Rogers and Brandt, 1971b), a value of $\alpha = 2.9$ is obtained.

When (I) is set equal to $-\alpha K_i$, eq 1 predicts that $(E_T)/v$ will be independent of the concentration of chloride ion. From the abscissa coordinate of the point of intersection of the lines in Figure 1A, which is equal to -0.26 M, a value for α can be calculated if K_i is known. Using the average value of $K_i = 0.13$ M obtained previously (Rogers and Brandt, 1971a), a value of $\alpha = 2.0$ is obtained. The average of these two values is $\alpha = 2.5$, and indicates that there is only a small (negative) interaction between chloride ion and D-glucal when they are present simultaneously on the enzyme.

The value of $\alpha = 2.0$ is considered to be the more accurate. It depends on only one independently determined constant, K_i. The value of $\alpha = 2.9$ depends on both K_d and K_I, the former having been obtained by curve fitting and being the least accurate of the three dissociation constants.

It was noted above that in Figure 1 the intersection of the lines appears to fall on the abscissa. This would not ordinarily be expected. Equation 3 gives the expression for the value of

$$\left(\frac{(E_T)}{v}\right)_{intersection} = \frac{1}{k_2} + \frac{1}{k_4(O_2)} + \left[\frac{(1-\alpha)(k_{-1}+k_2)}{k_1k_2(G)}\right] \times \left[1 + \frac{(H^+)}{K_I}\right] \quad (3)$$

$(E_T)/v$ at this intersection. Using values of $k_2 = 10^3$ sec^{-1} and $k_4 = 1.95 \times 10^6$ M^{-1} sec^{-1} (Weibel and Bright, 1971), $k_1k_2/(k_{-1} + k_2) = 1.26 \times 10^4$ M^{-1} sec^{-1} and $pK_I = 3.7$ (Rogers and Brandt, 1971b), (G) = 0.025 M, $(O_2) = 0.27$ mM, and $\alpha = 2.0$, at pH 4.5 the value of $(E_T)/v$ at this intersection is calculated to be -0.0008 sec. This value is sufficiently small so as to be experimentally indistinguishable from zero.

Multiple inhibition analysis was also performed using potassium bromide or potassium iodide in place of potassium chloride. Bromide ion and iodide ion, like chloride ion, exhibit competitive inhibition with respect to D-glucose with *A. niger* glucose oxidase (Rogers and Brandt, 1971b). The apparent inhibition constants for bromide ion and iodide ion at pH 4.5 were 0.051 and 0.27 M, respectively. The multiple inhibition analysis for either bromide ion or iodide ion with D-glucal yielded intersecting lines (data not shown) similar to the results shown for chloride ion and D-glucal in Figure 1. Values of α for bromide ion or iodide ion with D-glucal were evaluated as described above for chloride ion. The average values obtained were $\alpha = 2.0$ (bromide ion) and $\alpha = 2.1$ (iodide ion).

In order to test the internal consistency of the parameters α, K_I, K_i, and K_d evaluated in this and the two previous papers (Rogers and Brandt, 1971a,b) for chloride ion and D-glucal, difference spectral titrations of the enzyme with D-glucal were performed in the presence of a constant concentration of chloride ion. Both D-glucal and chloride ion binding have been shown to result in a perturbation of the visible absorption spectrum of the enzyme-bound FAD of *A. niger* glucose oxidase (Rogers and Brandt, 1971a,b). Figure 2A shows the difference spectrum obtained at pH 5.6 between a solution containing 14.7 μM enzyme-bound FAD, 0.257 M D-glucal, and 0.6 M potassium chloride and a solution containing only 14.7 μM enzyme-bound FAD and 0.6 M potassium chloride. There is a small positive peak at 494 nm. A titration of the spectral change at 494 nm, ΔA^{494}, on addition of D-glucal in the presence of a constant concentration (0.6 M) of potassium chloride was carried out. To increase the total absorbance change resulting from D-glucal binding, the concentration of enzyme-bound FAD was increased to 29.6 μM for the titration. The results of the titration are shown in Figure 2B.

The equilibria involved are those enclosed in dashed lines in Scheme I representing the binding of D-glucal and chloride ion to E_o at constant pH in the absence of substrate. It can be shown (see Appendix) that ΔA^{494} is directly proportional to the sum of the concentrations of E_oI, E_oH^+I, and $E_oH^+Cl^-I$, as described by eq 4, where $\Delta\epsilon_{app}$ is defined in the Appendix. It can also be shown (see Appendix) that eq 5 describes the dependence of ΔA^{494} on the concentration of D-glucal, where K_{app} is defined by eq 6. K_{app} and ΔA^{494}_{max} were evaluated from

$$\Delta A^{494} = \Delta\epsilon_{app}[(E_oI) + (E_oH^+I) + (E_oH^+Cl^-I)] \quad (4)$$

$$\Delta A^{494} = \Delta A^{494}_{max}\{(I)/[(I) + K_{app}]\} \quad (5)$$

$$K_{app} = \frac{\alpha K_i[K_IK_d + K_d(H^+) + (Cl^-)(H^+)]}{\alpha K_IK_d + \alpha K_d(H^+) + (Cl^-)(H^+)} \quad (6)$$

a plot (not shown) of $1/\Delta A^{494}$ *vs.* $1/(I)$; a least-squares fit gives $K_{app} = 0.136$ M and $\Delta A^{494}_{max} = 0.042$. In this analysis the concentrations of free D-glucal, (I), and free chloride ion, (Cl^-), were assumed to be equal to the total concentrations added, since they were each much greater than the concentration of enzyme active sites.

A theoretical value of K_{app} was calculated from eq 6 for this experiment, which was performed at pH 5.6. Using values of $(H^+) = 2.5\ \mu M$, $(Cl^-) = 0.6$ M, $\alpha = 2.0$, $pK_1 = 3.7$ and $K_d = 0.005$ M (Rogers and Brandt, 1971b), and $K_i = 0.13$ M (Rogers and Brandt, 1971a), the value obtained is $K_{app}^{calcd} = 0.19$ M. This value is in reasonable agreement with the experimental value, demonstrating the internal consistency of the values obtained for α, K_1, K_d, and K_i in this and previous papers (Roger and Brandt, 1971a,b).

Discussion

The steady-state kinetic results shown in Figure 1 have been analyzed above in terms of the model shown in Scheme I which permits the simultaneous binding of both chloride ion and D-glucal to the enzyme. Chloride ion and D-glucal have each been shown previously to exhibit competitive inhibition with respect to D-glucose with *A. niger* glucose oxidase (Rogers and Brandt, 1971a,b). The formulation of the rather complex binding equilibria shown in Scheme I yields a rate equation which adequately predicts the inhibitory effect of either chloride ion or D-glucal alone on the enzyme. It also allows for the formation of a ternary enzyme–chloride ion–D-glucal complex. The parameter α describes the extent of interaction between the two competitive inhibitors in the postulated ternary complex (Yonetani and Theorell, 1964). The observation of intersecting lines demonstrates that, to the extent that Scheme I is an adequate model, both chloride ion and D-glucal can bind simultaneously. The average interaction constant evaluated from Figure 1 for chloride ion and D-glucal is $\alpha = 2.5$. The smallness of this value indicates minimal interaction between the two inhibitors.

D-Glucal is a structural analog of the substrate D-glucose and it is reasonable to assume that it inhibits by directly competing with D-glucose for the active site. That is, D-glucal bound to the active site sterically prevents D-glucose from binding. In contrast, the competitive inhibition observed for chloride ion has been interpreted (Rogers and Brandt, 1971b) in terms of binding to an inactive protonated form of the oxidized enzyme, E_oH^+, thereby shifting the equilibrium between E_o and E_oH^+ away from E_o with a resultant decrease in the concentration of the active E_o species. Thus chloride ion inhibition has been interpreted as *not necessarily* resulting from direct steric interaction between chloride ion and D-glucose. The hypothesis that E_o but not E_oH^+ is able to interact with D-glucose has been previously proposed for both *P. notatum* glucose oxidase (Bright and Appleby, 1969) and *A. niger* glucose oxidase (Weibel and Bright, 1971).

In terms of these two molecular interpretations of the mechanisms of inhibition by D-glucal and chloride ion, it would be expected that the two inhibitors might be able to bind simultaneously to the enzyme. Such simultaneous binding was initially considered as a result of the observation that 0.2 M potassium chloride did not affect the observed inhibition constant for D-glucal at pH 4.0 (Rogers and Brandt, 1971a), despite the fact that 0.2 M chloride ion should have resulted in 93% saturation of the chloride ion binding site on the oxidized enzyme.

It seems reasonable to postulate, therefore, that chloride ion and D-glucal do not interact sterically with one another on the enzyme surface. Rather the small negative interaction indicated by $\alpha > 1$ may result from some small conformational difference at the active site which occurs when one inhibitor binds, which slightly reduces the affinity of the enzyme for the other inhibitor.

Such an hypothesis is supported by the results of multiple inhibition analysis using bromide ion or iodide ion in place of chloride ion. At pH 4.5, the apparent inhibition constants for chloride, bromide, and iodide ions are 0.037, 0.051, and 0.27 M, respectively. Despite the approximately sevenfold increase in the apparent inhibition constant in this series, the average interaction constant for the various halide ion: D-glucal pairs is relatively insensitive to the halide ion used. Thus with chloride, bromide, and iodide ions, the average values of α are 2.5, 2.0, and 2.1, respectively. The ionic radius of the halide ions increases from 1.81 to 2.20 Å between chloride ion and iodide ion (Weast, 1969). If the value of $\alpha > 1$ obtained for chloride ion were due entirely to a direct steric interaction between bound D-glucal and chloride ion, steric interaction between bound D-glucal and the larger iodide ion should be significantly greater. Yet α is affected only slightly by varying the halide ion.

In the introduction it was noted that Weibel and Bright (1971) have reported an effect of chloride ion on the maximum turnover number of *A. niger* glucose oxidase. This effect appears to require that chloride ion bind to the E_o–S complex and alter k_2. Under our experimental conditions, however, chloride ion exhibited apparently competitive inhibition with respect to both D-glucose and 2-deoxy-D-glucose (Rogers and Brandt, 1971b). Chloride ion binding to the oxidized form of the enzyme was demonstrated by static, difference spectral studies, and that binding quantitatively accounted for the observed inhibition (Rogers and Brandt, 1971b). The observations reported in this paper are consistent with the hypothesis that chloride ion and D-glucal, a substrate analog, can bind simultaneously to the oxidized form of *A. niger* glucose oxidase. These observations therefore support the idea of a ternary complex between oxidized enzyme, *substrate*, and chloride ion, although under our experimental conditions no kinetic evidence for that ternary complex was obtained.

In summary, the two competitive inhibitors halide ion and D-glucal can bind simultaneously to *A. niger* glucose oxidase with only a small negative interaction between them in the resulting ternary complex. While not demonstrated conclusively, it seems likely that this negative interaction is not steric. Regardless of the nature of the interaction, it is clear that halide ion and D-glucal bind at different loci at the active site. This observation lends further support to the hypothesis (Rogers and Brandt, 1971b) that the pH-dependent chloride ion inhibition, although apparently competitive with respect to D-glucose, does not result from direct steric exclusion of the substrate from the active site.

Appendix

In order to derive eq 4, 5, and 6 in the text, it is necessary to write the expressions for the absorbance of the reference and sample solutions. To simplify the nomenclature, let E = oxidized enzyme, H = proton, C = chloride ion, and I = D-glucal.

The reference solution contains only oxidized enzyme and chloride ion at constant pH. Only two equilibria are involved:

$$E \overset{K_1}{\rightleftharpoons} EH \overset{K_d}{\rightleftharpoons} EHC$$

where

$$K_1 = \frac{(E)(H)}{(EH)},\quad K_d = \frac{(EH)(C)}{(EHC)} \tag{1a}$$

Now

$$A_{ref} = \epsilon_E(E) + \epsilon_{EH}(EH) + \epsilon_{EHC}(EHC) \quad (2a)$$

and

$$(E_T) = (E) + (EH) + (EHC) \quad (3a)$$

where A = absorbance, ϵ_i = molar absorptivity of species i, and (E_T) = total concentration of enzyme-bound FAD. Substitution of eq 1a into eq 2a and 3a and combining the resulting equations gives

$$A_{ref} = \left[\frac{\epsilon_E + \epsilon_{EH}\frac{(H)}{K_1} + \epsilon_{EHC}\frac{(C)(H)}{K_dK_1}}{1 + \frac{(H)}{K_1} + \frac{(C)(H)}{K_dK_1}}\right](E_T) = \epsilon_{app}(E_T) \quad (4a)$$

where ϵ_{app} is a constant at constant pH and chloride ion concentration.

The sample solution contains oxidized enzyme, chloride ion, and D-glucal, so the equilibria involved are those enclosed in dashed lines in Scheme I. The additional dissociation constants needed are

$$K_i = \frac{(E)(I)}{(EI)} = \frac{(EH)(I)}{(EHI)}, \alpha K_d = \frac{(EHI)(C)}{(EHCI)} \quad (5a)$$

In the sample solution

$$A_{sam} = \epsilon_E(E) + \epsilon_{EH}(EH) + \epsilon_{EHC}(EHC) + \epsilon_{EI}(EI) + \epsilon_{EHI}(EHI) + \epsilon_{EHCI}(EHCI) \quad (6a)$$

and

$$(E_T) = (E) + (EH) + (EHC) + (EI) + (EHI) + (EHCI) \quad (7a)$$

Substitution of the equilibrium expressions into eq 6a gives

$$A_{sam} = \left[\epsilon_E + \epsilon_{EH}\frac{(H)}{K_1} + \epsilon_{EHC}\frac{(C)(H)}{K_dK_1}\right](E) + \left[\epsilon_{EI} + \epsilon_{EHI}\frac{(H)}{K_1} + \epsilon_{EHCI}\frac{(C)(H)}{\alpha K_dK_1}\right](EI) \quad (8a)$$

Since

$$(E) + (EH) + (EHC) = \left[1 + \frac{(H)}{K_1} + \frac{(C)(H)}{K_dK_1}\right](E) \quad (9a)$$

and

$$(EI) + (EHI) + (EHCI) = \left[1 + \frac{(H)}{K_1} + \frac{(C)(H)}{\alpha K_dK_1}\right](EI) \quad (10a)$$

substitution of eq 9a and 10a into eq 8a gives

$$A_{sam} = \epsilon_{app}[(E) + (EH) + (EHC)] + \epsilon'_{app}[(EI) + (EHI) + (EHCI)] \quad (11a)$$

where

$$\epsilon'_{app} = \frac{\epsilon_{EI} + \epsilon_{EHI}\frac{(H)}{K_1} + \epsilon_{EHCI}\frac{(C)(H)}{\alpha K_dK_1}}{1 + \frac{(H)}{K_1} + \frac{(C)(H)}{\alpha K_dK_1}} \quad (12a)$$

Therefore, the absorbance difference between the two solutions is given by

$$\Delta A = A_{sam} - A_{ref} = -\epsilon_{app}[(E_T) - (E) - (EH) - (EHC)] + [(EI) + (EHI) + (EHCI)]\epsilon'_{app} \quad (13a)$$

which, on substitution of eq 7a, gives

$$\Delta A = (\epsilon'_{app} - \epsilon_{app})[(EI) + (EHI) + (EHCI)] = \Delta\epsilon_{app}[(EI) + (EHI) + (EHCI)] \quad (14a)$$

which is identical with eq 4 in the text.

Defining

$$K_{app} = \frac{(I)[(E) + (EH) + (EHC)]}{[(EI) + (EHI) + (EHCI)]} \quad (15a)$$

substitution of eq 7a and 14a gives

$$K_{app} = \frac{(I)\left[(E_T) - \frac{\Delta A}{\Delta\epsilon_{app}}\right]}{\frac{\Delta A}{\Delta\epsilon_{app}}} = \frac{(I)\left[1 - \frac{\Delta A}{\Delta\epsilon_{app}(E_T)}\right]}{\frac{\Delta A}{\Delta\epsilon_{app}(E_T)}} \quad (16a)$$

which can be rearranged to

$$\Delta A = \Delta A_{max}\left[\frac{(I)}{K_{app} + (I)}\right] \quad (17a)$$

where

$$\Delta A_{max} = \Delta\epsilon_{app}(E_T) \quad (18a)$$

Substitution of eq 9a and 10a into eq 15a and simplifying gives

$$K_{app} = \frac{(E)(I)}{(EI)}\left[\frac{\alpha[K_1K_d + K_d(H) + (C)(H)]}{\alpha K_1K_d + \alpha K_d(H) + (C)(H)}\right] = \frac{\alpha K_i[K_1K_d + K_d(H) + (C)(H)]}{\alpha K_1K_d + \alpha K_d(H) + (C)(H)} \quad (19a)$$

Equations 17a and 19a are identical with eq 5 and 6, respectively, in the text.

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Reevaluation of the Activation of Bovine Chymotrypsinogen A*

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ABSTRACT: α-Chymotrypsin is the only activated form of bovine chymotrypsinogen A which has been found to self-associate to a significant extent at low pH, in agreement with early studies by Schwert (Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655). Variability in the dimerization constants of this enzyme has been shown to be due to the presence of small amounts of contaminating materials in the preparations, removable by active-site affinity chromatography. The dimerization assay, amino-terminal amino acid residue determinations, and disc gel electrophoresis have been used to investigate some of the intermediates in the activation of chymotrypsinogen A. A new species of chymotrypsin has been isolated and partially characterized. It is called κ-chymotrypsin and it has cystine (assumed), isoleucine (determined), and threonine (determined) as amino-terminal residues. It is produced from trypsin-free δ-chymotrypsin at pH 3.1 and room temperature in 48 hr and does not reversibly dimerize at low pH. κ-Chymotrypsin can be converted to both γ-chymotrypsin and α-chymotrypsin by variation of the crystallization conditions. Under conditions of classical slow activation (pH 7.5, 5°, 48 hr) trypsin-free δ-chymotrypsin is relatively stable and shows only about 50% conversion to electrophoretically different species. These results support the activation scheme of Wright *et al.* (Wright, H. T., Kraut, and Wilcox, P. E. (1968), *J. Mol. Biol. 37*, 363) that α-chymotrypsin is formed from neochymotrypsinogens rather than from δ-chymotrypsin. It is desirable, however, to insert κ-chymotrypsin into this scheme between δ- and γ-chymotrypsin.

Bovine chymotrypsinogen A can be activated to yield several active chymotrypsins A. α-Chymotrypsin, the first to be discovered (Kunitz and Northrup, 1935), has been well studied. β- and γ-chymotrypsins (Kunitz, 1938) can also be crystallized from "slow" (0.01% trypsin) activation mixtures. π- and δ-chymotrypsins (Jacobsen, 1947) result from the "rapid" (3% trypsin) activation of chymotrypsinogen.

The peptide-bond cleavages associated with the activation of chymotrypsinogen are well established (Dreyer and Neurath, 1955; Rovery *et al.*, 1955, 1957; Hartley, 1964). The essential step in the activation is a tryptic cleavage at the Arg_{15}–Ile_{16} bond (Oppenheimer *et al.*, 1966). Limited tryptic digestion of chymotrypsinogen results in the formation of π-chymotrypsin (Jacobsen, 1947) which is rapidly converted to δ-chymotrypsin by hydrolysis of the bond Leu_{12}–Ser_{13} (Rovery *et al.*, 1955; Hartley, 1964).

In the formation of α- or γ-chymotrypsin, four peptide bonds are split with the release of two dipeptides: Ser_{13}–Arg_{14} and Thr_{147}–Asn_{148} (Hartley, 1964). Recent crystallographic examinations (Matthews *et al.*, 1967; Cohen *et al.*, 1970; Davies *et al.*, 1969) have not revealed differences in amino acid sequence or any major structural difference, although the crystal habits of the two forms are quite different.

Recently, Wright *et al.* (1968) published a modification of Desnuelle's (1960) scheme for the genesis of the various forms of chymotrypsins (Figure 1). In the scheme of Wright *et al.* (1968), α-chymotrypsin is not derived directly from δ-chymotrypsin by autocatalytic degradation, but only by activation of neochymotrypsinogens (Rovery *et al.*, 1957). The immediate product of autocatalytic splitting of Thr_{147}–Asn_{148} from δ-chymotrypsin is proposed to be γ-chymotrypsin.

This investigation was initiated for several reasons. First, we wished to determine which, if any, of the activation schemes is correct by a characterization of the product formed after limited autolysis of δ-chymotrypsin. Second, the results of Bettelheim and Neurath (1955) showed that when a rapid activation mixture was allowed to autolyze at 5° for 56 hr, the product had the same carboxyl-terminal residues as α-chymotrypsin, but had only 0.21 residue of amino-terminal alanine/mole. We were interested in this enzyme since it might have been an intermediate between δ- and γ-chymotrypsins in the Wright *et al.* (1968) scheme. Third, if the activation mechanism of Wright *et al.* (Figure 1) is correct, α-chymotrypsin produced from δ-chymotrypsin might yield a more homogeneous α-chymotrypsin preparation than is available commercially.

We have been successful in isolating a new form of chymotrypsin, κ-chymotrypsin; and we propose that it is an intermediate in the scheme of Wright *et al.* (1968, Figure 1). In

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