Active Site Generation of a Protonically Unstable Suicide Substrate from a Stable Precursor: Glucose Oxidase and Dibromonitromethane†

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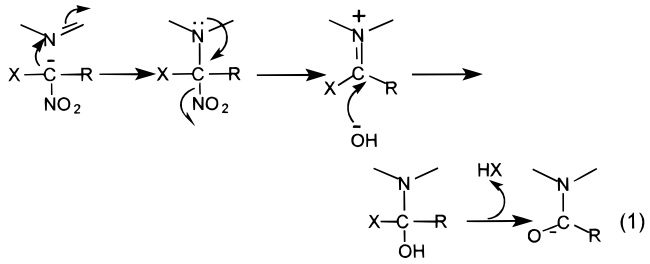
ABSTRACT: Bromonitromethane is an inefficient suicide substrate for glucose oxidase (in contrast to the case of $CH_3CCl{=}NO_2^-$ and D-amino acid oxidase) because, in the enzyme−substrate encounter step, the required ionization states of enzyme (EH_0^+, p$K_a \sim 3.5$) and substrate ($CHBr{=}NO_2^-$, p$K_a \sim 8.3$) cannot be highly populated simultaneously. Because reprotonation of $CHBr{=}NO_2^-$ is rapid at the pH value used for the assay of glucose oxidase, presentation of the enzyme with the preformed anion could not be exploited in this case. We circumvent this difficulty by allowing the enzyme to reductively dehalogenate $CHBr_2NO_2$, thereby generating the desired protonically unstable suicide substrate in situ ($E_r + CHBr_2NO_2 \rightarrow E_o + CHBr{=}NO_2^- + HBr + H^+$). Irreversible inactivation of the enzyme, because of the formation of a dead-end N-5 formylflavin adduct, is more than 100-fold faster when $CHBr{=}NO_2^-$ is generated in situ than when it is externally applied. The remaining competitive fates of $CHBr{=}NO_2^-$ at the active site are protonation and release or oxidation to HCOBr (or $HCONO_2$). Strong support for these conclusions comes from (1) the brisk evolution of $CH_3CBr{=}NO_2^-$ (which is too bulky to act further as an efficient suicide substrate) from the enzyme-catalyzed reductive debromination of $CH_3CBr_2NO_2$, (2) the 1:1 stoichiometry of enzyme inactivation, and (3) the identification of the modified flavin as 5-formyl-1,5-dihydro-FAD.

Starting with the observation that the anion of nitromethane ($CH_2{=}NO_2^- \times {}^-CH_2NO_2$) irreversibly inhibits its own oxidation by D-amino acid oxidase, we have used nitroalkanes extensively to probe flavoenzyme reaction mechanisms and as vehicles for suicide inactivation (*1*, *2*). Our major findings of relevance in this paper (which are certainly valid for D-amino acid oxidase and are applicable in large part to other flavoprotein oxidases) are summarized in Scheme 1.

The important facts of turnover in process A of Scheme 1 are that the enzyme, in a specific ionization state, accepts only the ionized nitroalkane in the bimolecular encounter step and that electron transfer is mediated by a series of three N-5 covalent flavin−sustrate adducts.

Irreversible inactivation can take one of two paths. Process B requires an external nucleophile such as cyanide to convert the iminium adduct to a dead-end tetrahedral species (*1*). Process C, on the other hand, is a genuine suicide pathway requiring no external (nonsolvent) reagent. The gist of pathway C is the inclusion of a second geminal leaving group, such as halide, in the starting nitroalkane (eq 1), which results in a dead-end N-5 acyl adduct (*2*).



Chloronitroethane anion ($CH_3CCl{=}NO_2^-$) reacts with D-amino acid oxidase as a suicide substrate according to the mechanism of eq 1 (*2*).

The question we address here is the design of nitroalkane-based suicide substrates for glucose oxidase. This is not a trivial matter because, unlike D-amino acid oxidase and L-amino acid oxidase, the required ionizable residue on glucose oxidase that controls substrate encounter has to be in its conjugate acid state (HE_0^+)[1] with a pK_a of ∼3.5. Thus, for the nitroalkane anion with pK_a values of >8, the product term $[E_oH^+]$[nitroalkane anion] in the rate equation for both catalytic turnover and suicide inactivation can never be large (*3*).[2] Furthermore, reprotonation of halonitroalkane anions tends to be fast, and we cannot exploit slow reprotonation as we have frequently done previously to probe mechanisms of flavoprotein reactions (*1*, *2*).

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH_2$, reduced FAD; E_0, oxidized glucose oxidase without reference to catalytically relevant protonation states; HE_0^+, protonated form of oxidized glucose oxidase that is reduced by nitroalkanes; E_r or E-$FADH_2$, fully reduced glucose oxidase; E_I, glucose oxidase inactivated by covalent modification of enzyme-bound FAD; tris, tris(hydroxymethyl)aminomethane.

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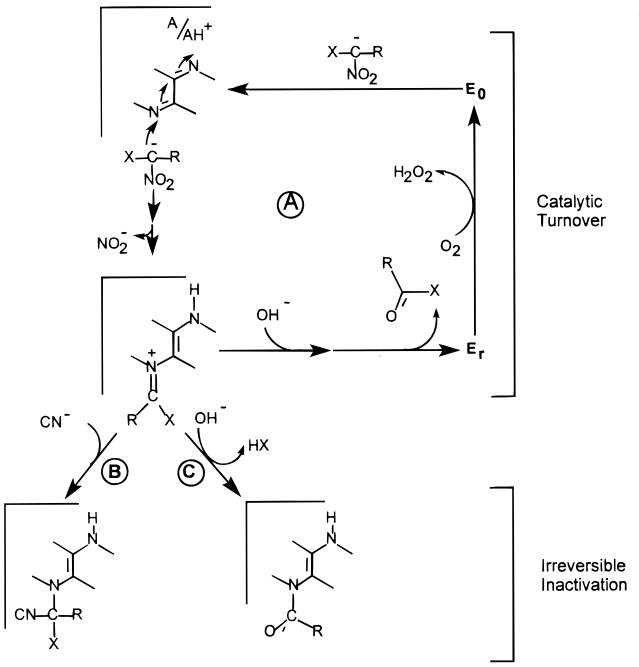
Scheme 1



The strategy that we adopt and describe herein is to allow the enzyme, through reductive dehalogenation of dihalonitromethane, to generate protonically unstable halonitromethane anion as a transient and reactive suicide substrate within the active site.

EXPERIMENTAL PROCEDURES

Materials. Aspergillus niger glucose oxidase type V from Sigma Chemical Co. (St. Louis, MO) was dialyzed against 0.1 M potassium phosphate buffer prior to use. The ratio of A_{274} to A_{450} was 10.2. Anions of nitroalkanes were prepared by dissolving 1 mmol of the respective nitroalkane in 1 mL of 1 N KOH. Stock solutions (1 M) of the nitroalkanes were prepared in methanol. Br_2 and *N*-bromosuccinimide were from Aldrich Chemical Co. (Milwaukee, WI). $^{13}CH_3NO_2$ (99.7% ^{13}C) was from Sigma Chemical Co.

CH_2BrNO_2 was synthesized from $NaCH_2NO_2$ and Br_2. $NaCH_2NO_2$ was prepared by addition of 6.1 g of CH_3NO_2 to 6.8 g of sodium ethoxide in 30 mL of absolute ethanol with stirring at 0 °C over the course of 30 min. The white precipitate was collected by filtration and washed with ethyl ether to yield 7.3 g of $NaCH_2NO_2$. $NaCH_2NO_2$ (7.3 g) was slowly added at 0 °C to 50 mL of CCl_4 containing 14 g of Br_2. After the mixture had been stirred for 15 min, excess Br_2 was purged from the mixture by bubbling air through the solution. The precipitated NaBr was removed by filtration. The product was purified by fractional distillation to yield 6.6 g (54% yield) of a clear liquid with a bp_{15} of 48−50 °C [bp_{12} = 42 °C (*4*)]. Proton NMR in $C^2H_3O^2H$ with TMS as a standard gave for CH_2BrNO_2 a δ of 5.98 ppm (s).

$CHBr_2NO_2$ was synthesized from $NaCHBrNO_2$ and Br_2 by the same procedure to give 3.4 g (41%) of a clear liquid with a bp_{20} of 62−64 °C [bp_{15} = 56−60 °C (*4*)]. ^1H NMR (in $C^2H_3O^2H$ with trimethylsilane as a standard) gave a δ of 7.85 ppm (s) for Br_2CHNO_2.

$CH_3CHBrNO_2$ was synthesized from $NaCH_3CHNO_2$ and Br_2 to give a fraction with a bp_{20} of 54−55 °C [bp_{10} = 48 °C (*4*)] in 24% yield. ^1H NMR (in $C^2H_3O^2H$ with trimethylsilane as a standard) gave for $CH_3CHBrNO_2$ δs of 6.3 [J = 6.4 Hz (q)] and 2.14 ppm [J = 6.4 Hz (d)].

CBr_3NO_2 was synthesized from CH_3NO_2 and *N*-bromosuccinimide. Thus, 0.01 mol of KOH was added with stirring at room temperature to a mixture of 3 g of CH_3NO_2 and 27 g of *N*-bromosuccinimide in 30 mL of H_2O. An additional 0.03 mol of KOH was added over a 3 h period. The product separated as a heavy liquid, and was purified in 23% yield by fractional distillation with a bp_{20} of 86−88 °C [bp_{20} = 89−90 °C (*4*)]. The liquid froze as a pale orange solid at 0 °C and exhibited a δ of 53.9 ppm, as determined by ^{13}C NMR (in $C^2H_3O^2H$ with $C^2H_3O^2H$ as a standard).

$CH_3CBr_2NO_2$ was synthesized from $CH_3CH_2NO_2$ and *N*-bromosuccinimide in a manner analogous to that described above for the synthesis of CBr_3NO_2. At the end of the reaction, the pH of the mixture was adjusted to 3.0 with HCl. The product separated as a heavy oil that was purified in 13% yield by fractional distillation. The bp_{20} was 70 °C [bp_{11} = 57−57.5 °C (*4*)], and ^1H NMR (in $C^2H_3O^2H$ with trimethylsilane as a standard) gave a δ of 2.96 ppm (s).

$^{13}CH_2BrNO_2$ was synthesized from $^{13}CH_3NO_2$ and Br_2. $^{13}CH_3NO_2$ (1 g) was added to 16 mL of 1 N KOH at 0 °C. Br_2 (2.6 g) was rapidly added over the course of 1 min to this solution with stirring. After 30 min, the pH of the solution was adjusted to 3.0 with HCl. The product was extracted into dichloromethane and purified by fractional distillation to give 0.8 g of a light yellow liquid with a bp_{20} of 45−47 °C. $^{13}CH_2BrNO_2$ (50 mM) inactivated glucose oxidase (60 μM) in 0.01 M potassium acetate containing 0.1 mM EDTA at pH 5.0 with a pseudo-first-order rate constant of 0.0018 s^{-1}, whereas the comparable value with 50 mM $^{12}CH_2BrNO_2$ as the inactivator was 0.0019 s^{-1}.

Kinetic and Spectral Measurements. Kinetic and spectral measurements were made, unless indicated otherwise, in 0.1 M potassium acetate containing 0.1 mM EDTA at pH 5.0 and 25 °C. Enzymatic activity was monitored through O_2 consumption detected with a Yellow Springs polarographic electrode. Enzyme concentrations were expressed in terms of the amount of flavin that can be reduced by 25 mM glucose [$\Delta\epsilon_{455}$ = 12 800 M^{-1} cm^{-1} (*5*)]. Spectra and optically monitored kinetics were recorded on a Cary 118 and Gilford recording spectrophotometer. Reactions were temperature equilibrated to 25 °C.

Purification of the Modified Flavin from the Inactivated Enzyme. A typical large-scale preparation of the flavin adduct for ^{13}C NMR measurements was generated as follows. To 12 mL of a glucose oxidase solution containing 6.9 μmol of enzyme-bound FAD was added 50 μL of $^{13}CH_2BrNO_2$ aerobically at 23 °C. After 100 min, the excess inactivator was removed by applying the inactivated enzyme to a 2.5 cm × 25 cm column of Sephadex G-25 resin which had been equilibrated in deionized H_2O. The protein fractions were pooled (16 mL), then diluted to 32 mL with H_2O, and placed in a boiling water bath for 6 min to precipitate the apoenzyme. The mixture was cooled to 5 °C, and the precipitated protein was removed by centrifugation. The volume of the supernatant solution was reduced to 5 mL by

[2] Glucose oxidase is slowly reduced by neutral nitromethane. However, the reduction of the protonated enzyme by the nitronate of nitroethane is 10^5-fold faster than the reduction of the enzyme by the neutral nitroethane (*3*).

rotary evaporation at 40 °C. Freshly distilled acetone (10 mL) was added to this solution, and additional precipitated protein was removed by centrifugation. The sample was taken to dryness under reduced pressure at 40 °C. This residue was dissolved in 3 mL of ^2H_2O and adjusted to a pH meter reading of 4.8 with ^2HCl. After filtration, the solution was taken to dryness under reduced pressure. The residue was redissolved in 0.4 mL of ^2H_2O and placed in a 5 mm Wilmad precision tube for the ^{13}C NMR measurements. Spectroscopy indicated that this sample contained 2.4 μmol of modified FAD and 0.4 μmol of unmodified FAD. These values were calculated on the basis of an ϵ_{317} of 6×10^3 M^{-1} cm^{-1} (*2*) for the adduct and an ϵ_{445} of 11.4×10^3 M^{-1} cm^{-1} (*5*) for FAD at pH 5.0.

Product Analysis. Nitrite was assayed by a chromogenic diazo coupling reaction (*6*). Bromide was assayed potentiometrically with an Orion bromide electrode and a single-junction reference electrode. Nitrite and bromide concentrations were determined by comparison with standard curves. Protein did not interfere with either of these assay procedures.

^1H and ^{13}C NMR spectra were recorded on a IBM/Bruker WP200SY instrument. For ^{13}C NMR spectra of the flavin adduct, the instrument settings included a spectrometer setting of 50.32 MHz, a sweep width of 11 904 Hz, a repetition time of 5 s, and a pulse width of 90°. For broad-band decoupled spectra, a power of approximately 1.5 W was applied. A total of 10 000 transients were accumulated.

Reactivation of CH_2BrNO_2-Inactivated Glucose Oxidase. CH_2BrNO_2-inactivated glucose oxidase was reactivated at room temperature in a 1 cm path length quartz cuvette. The solution of inactivated glucose oxidase was placed 1.0 in. from a source of 355 nm light (Minerallight model UVSL-25 lamp). Prior to illumination, the enzyme was freed of excess inactivator by column chromatography on Sephadex G-25 resin that had been equilibrated with 0.1 M potassium phosphate and 0.1 mM EDTA at pH 5.0. Photolysis of the protein-free adduct was similar to that described for the inactivated enzyme. All solutions were aerobic during the reactivation reaction.

Analysis of Data. The time courses for bleaching of the flavin absorbance of glucose oxidase by halonitroalkanes were single-exponential functions (eq 2)

$$\Delta A_{450}(t) = \Delta A_{450,\infty} e^{-k_{obs}t} \tag{2}$$

where k_{obs} is the pseudo-first-order rate constant characterizing the reaction and $\Delta A_{450,\infty}$ the maximal absorbance change. The dependence of the pseudo-first-order rate constant on the concentration of halonitroalkane (L) was described by a linear function (eq 3)

$$k_{obs}([L]) = k_2[L] \tag{3}$$

where k_2 is the bimolecular rate constant for the reaction of halonitroalkane with glucose oxidase.

Time courses for reprotonation of the nitronate were described by an equation analogous to eq 1 in which k_1 is a first-order rate constant independent of nitronate concentration (eq 4a).

$$\Delta A_{240}(t) = A_1 e^{-k_1 t} \tag{4a}$$

Time courses for the transient formation of the nitronate (A_{240}) from $CH_3CBr_2NO_2$, glucose oxidase, and glucose in the presence of 100 mM sodium acetate were described by a double-exponential function (eq 4b)

$$\Delta A_{240}(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \tag{4b}$$

where A_1 and A_2 are the amplitudes of the two phases and k_1 and k_2 the respective pseudo-first-order rate constants.

The time courses of oxygen consumption $[O_2(t)]$ during the inactivation of glucose oxidase were described by a first-order function (eq 5)

$$[O_2(t)] = \Delta[O_2]_\infty e^{-k_{obs}t} + [O_2]_\infty \tag{5}$$

where $\Delta[O_2]_\infty$ is the total amount of oxygen consumed during the inactivation process, $[O_2]_\infty$ the oxygen concentration at the end of the reaction, and k_{obs} the pseudo-first-order rate constant describing the process. The dependence of k_{obs} on the concentration of halonitroalkane was described by eq 6

$$k_{obs}([L]) = \frac{k[L]}{k + [L]} \tag{6}$$

where k is the maximal value for the rate constant of inactivation and K the concentration of ligand that gave 50% of the maximal rate of inactivation.

The inactivation of glucose oxidase by dibromonitromethane ([L]) was described by eq 7

$$\% \text{ activity remaining}([L]) = 100 - \frac{100}{a}[L] \qquad [L] < a$$
$$\% \text{ activity remaining}([L]) = 0 \qquad [L] > a \tag{7}$$

where a is the concentration of L that completely inactivated the enzyme.

The inactivation of glucose oxidase by tribromonitromethane was described by the logistic function (eq 8).

$$\% \text{ activity remaining}([L]) = \frac{100K^n}{K^n + [L]^n} \tag{8}$$

The appropriate equation was fitted to the data by nonlinear least squares using SigmaPlot from Jandel Scientific (Corte Madera, CA). The standard error in the least significant digit of the parameter is presented in parentheses.

RESULTS

Monobromonitroalkane Is a Slow-Reacting Suicide Substrate of Glucose Oxidase. Glucose oxidase is slowly inactivated by the $CH_2BrNO_2/CHBr{=}NO_2^-$ equilibrium mixture ($pK = 8.3$) at pH 5.0, and concomitantly, the enzyme-bound flavin is reduced to a species that is recalcitrant to oxidation by O_2. The time course for anaerobic bleaching of the flavin absorbance at 450 nm is a first-order process (Figure 1). The pseudo-first-order rate constants are linearly dependent on the concentration of the $CH_2BrNO_2/CHBr{=}NO_2^-$ mixture (inset of Figure 1). Thus, the apparent bimolecular rate constant for this reaction (k_2) at pH 5.0 is 0.0365(1) M^{-1} s^{-1}. Several features of this reaction are noteworthy. First, the inactivated enzyme has less than 1%

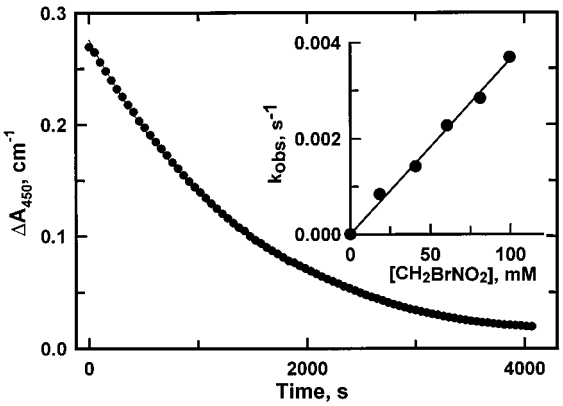


FIGURE 1: Kinetics for inactivation of glucose oxidase by CH_2BrNO_2. The absorbance of the enzyme-bound flavin of glucose oxidase (23 μM) was bleached by 20 mM CH_2BrNO_2 (lower trace). The value of the pseudo-first-order rate constant for this reaction was 0.00067(1) s^{-1} (eq 2). The values of pseudo-first-order rate constants for this reaction were linearly dependent upon the concentration of CH_2BrNO_2 (inset). The slope of the line was 3.65(1) $\times$ 10^{-2} M^{-1} s^{-1} (eq 3). After treatment with CH_2BrNO_2 for 100 min, the enzyme lost 99% of its catalytic activity with glucose as a substrate. Solutions were initially equilibrated with atmospheric O_2 (240 μM). O_2 was not consumed (<5 μM) during the inactivation of 51 μM glucose oxidase by 20 mM CH_2BrNO_2.

of the specific activity of native glucose oxidase. Second, inactivation of 51 μM enzyme by 20 mM CH_2BrNO_2 resulted in the consumption of less than 5 μmol of O_2. Thus, the partition ratio[3] is less than 0.1. Third, inactivation of 1 equiv of glucose oxidase by excess CH_2BrNO_2 generates 1.1 equiv of bromide and 1.2 equiv of nitrite. Finally, the value of the inactivation rate constant is very small.

Mutually Exclusive Ionization States in Enzyme and Substrate during Suicide Reactions of Monobromonitroalkane. In previous studies with nonhalogenated nitroalkanes as alternative reductive substrates for flavoprotein oxidases, we have exploited the sluggish rates of equilibration ($t_{1/2}$ > 1 min) of the ionization states of the potential substrates to determine which ionization state is enzymatically reactive (*1*−*3*). Thus, depending upon the ionization state of the reactive form of the substrate, the anion generates either a burst or lag in product formation as the anion comes to protonic equilibrium with the buffer. Unfortunately, CH_2BrNO_2, a carbon acid with a p*K* of 8.3 (*7*), protonates rapidly ($t_{1/2}$ ~ 3 s) in 0.025 M potassium acetate at pH 5.0. Consequently, the determination of the enzymatically reactive ionization state of CH_2BrNO_2 for glucose oxidase is not straightforward. However, if glucose oxidase is challenged with preformed 1 mM $CHBr{=}NO_2^-$ at pH 5 for 10 min (which is sufficient time for protonic equilibration of the nitro/anion pair to be attained), the enzyme loses 15% of its activity. This contrasts to only a 3% loss of activity when the enzyme is reacted with a 1 mM $CH_2BrNO_2/CHBr{=}NO_2^-$ equilibrium mixture for 10 min. These results suggest that the anion ($CHBr{=}NO_2^-$) is the inactivating species.

If inactivation of glucose oxidase by preformed anion is described by a scheme (eq 9) in which glucose oxidase (protonated and unprotonated enzyme) and H^+ compete for $CHBr{=}NO_2^-$ to give inactivated enzyme (E_I) or CH_2BrNO_2, then the apparent bimolecular rate constant for inactivation (k_I) can be calculated from eq 10 (where *f* is the fraction of enzyme activity remaining and $[CHBr{=}NO_2^-]_0$, the initial concentration of $CHBr{=}NO_2^-$, is much greater than the concentration of enzyme).

$$\begin{array}{ccc} HE_0^+ + CHBr{=}NO_2^- & \xrightarrow{k_I} & E_I \\ \downarrow k_H[H^+] & & \\ CH_2BrNO_2 & & \end{array} \qquad (9)$$

$$\ln(f) = \frac{-k_I[BrCH{=}NO_2^-]_0}{k_H[H^+]} \qquad (10)$$

We computed a k_I of 37 M^{-1} s^{-1} when f = 0.85 and $[CHBr{=}NO_2^-]_0$ = 1 mM and a value for the pseudo-first-order rate constant for reprotonation of the anion ($k_H[H^+]$) of 0.23 s^{-1} (corresponding to a $t_{1/2}$ of 3 s).

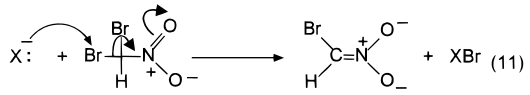
As noted previously, the bimolecular rate constant for inactivation of glucose oxidase by the $CH_2BrNO_2/CHBr{=}NO_2^-$ mixture at pH 5.0 is 0.0365 M^{-1} s^{-1} (Figure 1). Because the p*K* for the ionization of CH_2BrNO_2 is 8.3, the fraction of the total nitroalkane present as the nitronate at pH 5.0 is only 0.0005. If the nitronate were the only inactivating species, the bimolecular rate constant for inactivation of the oxidase by the anion would be calculated from these data to be 73 M^{-1} s^{-1}. Considering the uncertainty in the values of the parameters that are used for these calculations (i.e., the p*K* value and the rate constant for protonation of the anion), the calculated bimolecular rate constants of 37 and 73 M^{-1} s^{-1} are consistent with the hypothesis that the oxidase is inactivated by the nitronate.

To gain insight into the ionic state of glucose oxidase required for inactivation, the pseudo-first-order rate constants for inactivation of glucose oxidase by the $CH_2BrNO_2/CHBr{=}NO_2^-$ equilibrium mixture were determined at pH 5.0 and 6.1. If the inactivation process were analogous to the reduction of glucose oxidase by $CH_3CH_2NO_2$ (*3*), then $CHBr{=}NO_2^-$ would react with the protonated state of the oxidized enzyme (HE_0^+). If HE_0^+ were the catalytically active form of the enzyme with a p*K* of 3.5 (*3*) and $CHBr{=}NO_2^-$ were the active form of the substrate with a p*K* of 8.3 (*7*), the pseudo-first-order rate constant for inactivation would be independent of pH in the range of 3.5−8.3. The latter was confirmed experimentally by determining that the pseudo-first-order rate constants for inactivation of the enzyme by a 20 mM $CH_2BrNO_2/CHBr{=}NO_2^-$ equilibrium mixture were 7.6 $\times$ 10^{-4} and 8.2 $\times$ 10^{-4} s^{-1} at pH 5.0 and 6.1, respectively. The bimolecular rate constant for inactivation with the reagents in their correct protonation states (i.e., HE_0^+ and $CHBr{=}NO_2^-$) was calculated to be 2.4 $\times$ 10^3 M^{-1} s^{-1} from the measured bimolecular constant for inactivation at pH 5.0 (Figure 1) and the p*K* values for the enzyme (3.5) and substrate (8.3), which indicate that 3% of the enzyme and 0.05% of the substrate are in their correct protonation states.

Enzymatic Reductive Debromination of Dibromonitroalkanes. Whereas the intrinsic rate of inactivation of HE_0^+ by $CHBr{=}NO_2^-$ is fast (corresponding to a $t_{1/2}$ of ~0.3 s with 1 mM $CHBrNO_2^-$), the experimentally observed rate

[3] The partition ratio is the number of moles of product formed per mole of enzyme inactivated (*11*).

(corresponding to a $t_{1/2}$ of ~1500 s with a 1 mM CH_2BrNO_2/$CHBr{=}NO_2^-$ mixture) is slow because the anion is extensively (and rapidly) protonated at any pH value for which a significant fraction of the enzyme is in the protonated state. We considered the possibility of enzymatic generation of $CHBr{=}NO_2^-$ at the active site from a suitable precursor to circumvent this problem. Nucleophilic attack on the bromine in CH_2BrNO_2 generates $CH_2{=}NO_2^-$ (*8*, *9*). Substitution of $CHBr_2NO_2$ for CH_2BrNO_2 should result in the transient formation of $CHBr{=}NO_2^-$ as shown in eq 11.



(11)

Thus, if the reduced flavin of E_r were the nucleophile $X:^-$, in the general case, $RCBr{=}NO_2^-$ should be generated enzymatically at the active site of the oxidase through the reductive debromination of $RCBr_2NO_2$.

In accordance with this prediction, we found that $CH_3CBr_2NO_2$ oxidizes reduced glucose oxidase (Figure 2A). This reaction was monitored by the characteristic absorbance changes of the enzyme-bound flavin at 450 nm as the enzyme cycles between the oxidized and reduced states. Thus, 5 mM D-glucose rapidly reduced the enzyme with concomitant bleaching of the flavin absorbance at 450 nm, whereas 5 mM $CH_3CBr_2NO_2$ had no effect on the flavin absorbance. However, simultaneous addition of these reagents to an aerobic solution of 23 μM enzyme resulted in rapid bleaching of the flavin followed by slow recovery of the flavin absorbance (Figure 2A). These results indicated that the glucose-reduced enzyme slowly reduced $CH_3CBr_2NO_2$. The products of the reduction of 5 mM $CH_3CBr_2NO_2$ by 10 mM glucose and 23 μM glucose oxidase were 1.0 equiv of Br^- (based on the initial concentration of $CH_3CBr_2NO_2$) and 0.9 equiv of $CH_3CHBrNO_2$ (quantified as the anion after dilution of a sample of the product into 100 mM KOH). After the reductive debromination of 5000 μM $CH_3CBr_2NO_2$ by 23 μM enzyme, the enzyme retained more than 80% of its activity.

The fact that $CH_3CBr{=}NO_2^-$ is the immediate product of this reaction was shown as follows. $CH_3CBr{=}NO_2^-$ is distinguished from $CH_3CBr_2NO_2$ by its strong UV absorbance at 240 nm and by its buffer-dependent rate of protonation (descending traces in Figure 2B). Thus, addition of glucose and the oxidase to solutions of $CH_3CBr_2NO_2$ containing 20 or 100 mM acetate at pH 5.0 causes buffer-dependent absorbance increases at 240 nm (ascending traces in Figure 2B). This is expected if $CH_3CBr{=}NO_2^-$ is the immediate product of the enzymatic reductive debromination of $CH_3CBr_2NO_2$. On the other hand, if $CH_3CHBrNO_2$ were the immediate product generated by the enzyme, there would be little absorbance change at 240 nm because the pH of the reaction mixture (pH 5.0) is well below the p*K* value of 7.0 (*7*) for the UV-absorbing $CH_3CBr{=}NO_2^-$. Because the enzymatic rate of $CH_3CBr{=}NO_2^-$ formation is the same in these two buffer systems (initial velocities of the two ascending traces in Figure 2B) and the rate of protonation of $CH_3CBr{=}NO_2^-$ is dependent upon the concentration of buffer (descending traces of Figure 2B), the extent of $CH_3CBr{=}NO_2^-$ accumulation in the enzymatic reaction is dependent upon buffer concentration. These results support

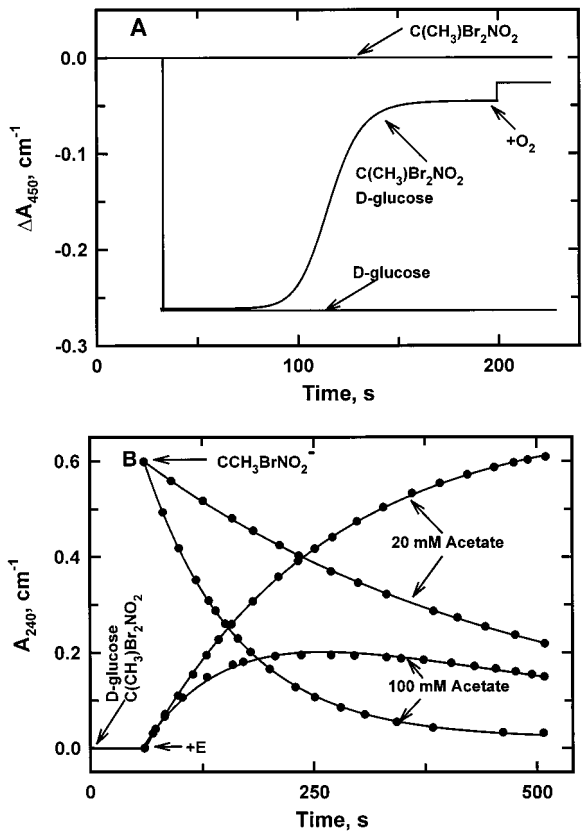
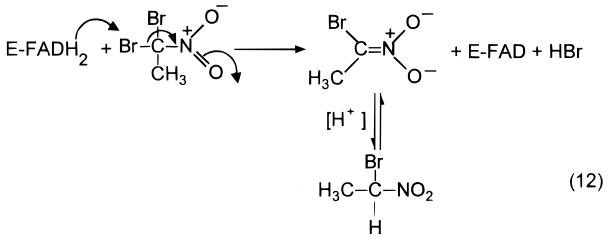


FIGURE 2: Glucose oxidase-catalyzed reduction of $CH_3CBr_2NO_2$ to $CH_3CBr{=}NO_2^-$. (A) Spectral changes associated with glucose oxidase during the enzyme-catalyzed oxidation of glucose by $CH_3CBr_2NO_2$. The absorbance of 23 μM glucose oxidase was monitored at 450 nm after addition (t = 30 s) of 5 mM D-glucose and 5 mM $CH_3CBr_2NO_2$ in the indicated combinations. Additional O_2 was added to the cuvette at the indicated time by mixing air into the solution. (B) Formation and protonation of $CH_3CBr{=}NO_2^-$ was monitored spectrophotometrically at 240 nm. The time courses for protonation of 0.1 mM $CH_3CBr{=}NO_2^-$ were determined at two different concentrations of acetate buffer (descending traces). The values of the pseudo-first-order rate constants for 20 and 100 mM acetate were 0.00245(1) and 0.0098(1) s^{-1}, respectively (eq 4a). The time courses for $CH_3CBrNO_2^-$ formation from 25 mM glucose and 2 mM $CH_3CBr_2NO_2$ were followed after initiation of the reactions with 0.46 μM glucose oxidase. The solutions initially contained 240 μM O_2, 0.1 mM EDTA, and either 100 or 20 mM acetate at pH 5.0. The formation of nitronate (A_{240}) in 20 mM acetate was described by eq 4b with a k_1 of 0.0014(1) s^{-1} and a k_2 fixed at 0.0024 s^{-1}. The time course for nitronate formation in 100 mM acetate was described with a k_1 of 0.0020(1) s^{-1} and a k_2 fixed at 0.0098 s^{-1}.

the conclusion that reduced glucose oxidase reductively debrominated $CH_3CBr_2NO_2$ to $CH_3CBr{=}NO_2^-$, which is then protonated to $CH_3CHBrNO_2$ (eq 12) in a buffer-dependent reaction.



(12)

Because the partition ratio for inactivation of glucose oxidase by $CH_3CBr_2NO_2$ was estimated to be greater that 1000,

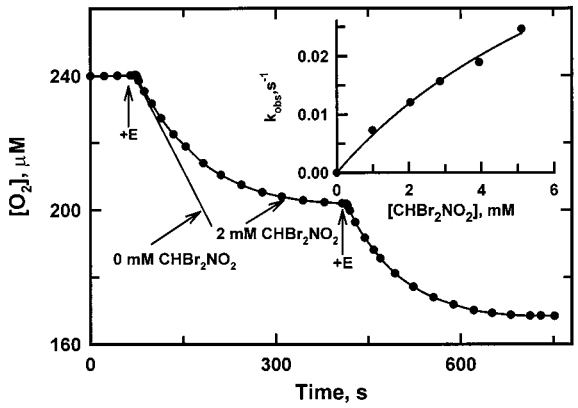


FIGURE 3: Kinetics for inactivation of glucose oxidase by $CHBr_2NO_2$. Oxidation of 100 mM glucose catalyzed by 2.3 nM glucose oxidase was inhibited in a first-order process by 2 mM $CHBr_2NO_2$ (lower trace). The value of the pseudo-first-order rate constant describing this process (eq 5) was 0.0098(1) s^{-1}. Addition of the enzyme to the spent reaction mixture caused a similar burst in O_2 uptake described by a pseudo-first-order rate constant of 0.0118-(2) s^{-1}. The time course for reduction of O_2 by D-glucose was linear in the absence of $CHBr_2NO_2$. The value of the pseudo-first-order rate constant for inhibition of glucose oxidase was hyperbolically dependent on $CHBr_2NO_2$ concentration (inset). The values of k and K (eq 6) were 0.07(1) s^{-1} and 9(3) mM, respectively. The oxygen concentration was initially 240 μM.

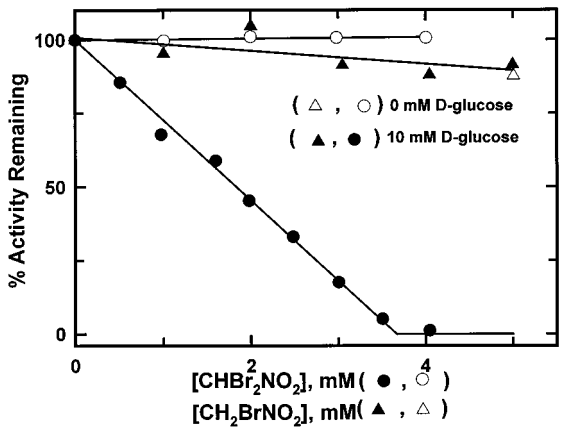
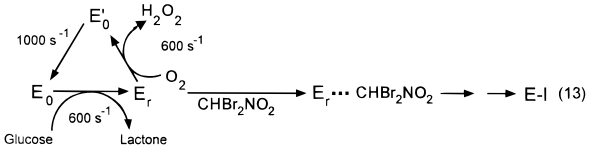


FIGURE 4: Stoichiometry for inactivation of glucose oxidase by $CHBr_2NO_2$ or CH_2BrNO_2. Glucose oxidase (23 μM) was reacted with $CHBr_2NO_2$ or CH_2BrNO_2 in the presence or absence of 10 mM glucose for 10 min. The oxidase activity of the treated enzyme was assayed polarographically with 20 mM glucose and 240 μM O_2. The reference activity (100%) was determined for samples of enzyme treated similarly except that $CHBr_2NO_2$ and CH_2BrNO_2 were omitted from the incubation mixtures. Equation 7 was fitted to the inhibition data with $CHBr_2NO_2$ and 10 mM D-glucose to give 3.67(4) mM $CHBr_2NO_2$ for complete inhibition.



inactivation of glucose oxidase by $CH_3CBr_2NO_2$ was not studied further.

Dibromonitromethane Rapidly Inactivates Glucose Oxidase in the Presence of Glucose. The demonstration above that reduced glucose oxidase catalyzes the reductive debromination of $CH_3CBr_2NO_2$ to $CH_3CBr{=}NO_2^-$ encouraged us to attempt to generate $CHBr{=}NO_2^-$ at the active site and thus inactivate the enzyme in a glucose-dependent manner. This is what we observed (Figure 3). Addition of 2.3 nM glucose oxidase to a solution of 100 mM D-glucose and 240 μM O_2 at pH 5.0 causes a constant rate of O_2 uptake, whereas inclusion of 2 mM $CHBr_2NO_2$ causes a first-order decay in the rate of O_2 uptake that is reproduced by a second addition of glucose oxidase to the medium (Figure 3). The enzyme is not inactivated by $CHBr_2NO_2$ in the absence of glucose (Figure 4), so reduction of the enzyme by D-glucose is a prerequisite for inactivation by $CHBr_2NO_2$.

The pseudo-first-order rate constant describing the decrease in the rate of O_2 uptake during the inactivation of glucose oxidase by $CHBr_2NO_2$ depends hyperbolically on the concentration of $CHBr_2NO_2$ (inset of Figure 3). At a saturating concentration of $CHBr_2NO_2$, therefore, inactivation is controlled by a first-order intramolecular process. Because the pseudo-first-order rate constant for reduction of glucose oxidase by 100 mM D-glucose (600 s^{-1}) is 15000-fold greater than the limiting rate constant for inactivation of the enzyme at pH 5.0 and 25 °C (0.07 s^{-1}, inset of Figure 3), the rate-limiting intramolecular process does not involve reduction of the enzyme. Thus, a complex between the reduced enzyme and $CHBr_2NO_2$ is an obligate intermediate, and the limiting first-order rate constant in the inactivation reaction at infinite concentrations of $CHBr_2NO_2$ and glucose is some intramolecular process (or processes) lying between formation of the $E_r{-}CHBr_2NO_2$ intermediate and the inactivation event. This model is summarized in eq 13 for the case of 100 mM D-glucose and 240 μM O_2 with the three relevant intermediates in the glucose turnover cycle (*10*).

In the presence of excess glucose, 3.67(4) mM $CHBr_2NO_2$ was required to titrate the activity of 23 μM glucose oxidase (Figure 4). Thus, the partition ratio is 160. Analogous titration curves were generated and similar partition ratios calculated when glucose (limiting enzyme and excess $CHBr_2NO_2$) or enzyme (limiting $CHBr_2NO_2$ and excess glucose) was the titrant (data not shown). In the absence of glucose, 2 mM CH_2BrNO_2 or $CHBr_2NO_2$ had no effect on the enzyme during the course of these experiments (Figure 4). However, as demonstrated previously, high concentrations of CH_2BrNO_2 or prolonged equilibration of the enzyme with low concentrations of CH_2BrNO_2 inactivates the enzyme slowly in the absence of glucose (Figure 1).

The bimolecular rate constant for oxidation of reduced glucose oxidase by $CHBr_2NO_2$ was estimated from these data as follows. The kinetic parameters for inactivation of glucose oxidase by $CHBr_2NO_2$ in the presence of 100 mM glucose and 240 μM O_2 are as follows: K_m = 9 mM and k_{inact} = 0.07 s^{-1} (Figure 3). Because $CHBr_2NO_2$ is reduced by E_r, the value of 8 M^{-1} s^{-1} calculated for k_{inact}/K_m from these kinetics must be corrected for the fraction of enzyme that is in the reduced form during turnover. From the rate constants given in eq 13, the fraction of enzyme in the reduced state during turnover is 0.4. Thus, the corrected value for k_{inact}/K_m is 20 M^{-1} s^{-1}. Since 160 turnovers (the partition ratio) are required for inactivation, the bimolecular rate constant for reduction of $CHBr_2NO_2$ is 3.2 × 10^3 M^{-1} s^{-1}. The bimolecular rate constants for reduction of O_2 and $CH_3CBr_2NO_2$ by E_r are 2.5 × 10^6 (*10*) and 5.1 × 10^2 M^{-1} s^{-1}, respectively, at pH 5.0.

A lower estimate for the first-order rate constant for modification of the enzyme-bound flavin in the $HE_0^+{-}CHBr{=}NO_2^-$ intermediate can also be estimated from these

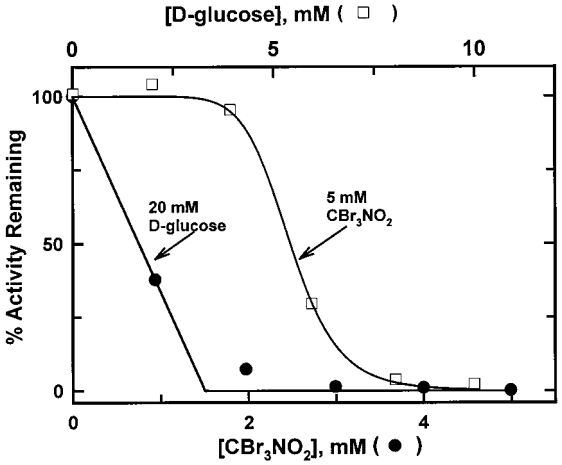


FIGURE 5: Titration of glucose oxidase activity with CBr_3NO_2. Glucose oxidase (23 μM) was treated with excess glucose (20 mM) and varying concentrations of CBr_3NO_2 for 10 min. Equation 7 was fitted to these data to give a value of 1.50(8) mM for the value of nitroalkane for complete inhibition. Alternatively, the enzyme was treated with excess CBr_3NO_2 (5 mM) and varying concentrations of glucose for 10 min. Equation 8 was fitted to these data to give values for K and n of 5.40(9) mM and 9(1), respectively. The enzyme was assayed for oxidative activity as described in the legend of Figure 4. The reference activity (100%) was determined on enzyme that was reacted with 20 mM glucose or 5 mM CBr_3NO_2 separately. These activities were similar to those for the untreated enzyme.

data. Because the oxidized enzyme does not accumulate during the inactivation reaction, the first-order rate constant for formation of the N-5 adduct must be at least 1 order of magnitude larger that the apparent rate constant for inactivation (0.07 s^{-1}). The analogous rate constant for inactivation of D-amino acid oxidase by $CH_3CCl{=}NO_2^-$ is 0.02 s^{-1} (*2*).

In addition to $CHBr_2NO_2$, CBr_3NO_2 inactivates glucose oxidase in a glucose-dependent reaction (Figure 5). The enzymatic activity was titrated with CBr_3NO_2 in the presence of excess glucose. However, titration of the oxidase activity with glucose in the presence of 5 mM CBr_3NO_2 resulted in a decrease in enzymatic activity only after addition of approximately 2 mM glucose. This suggests that the enzyme reduces CBr_3NO_2 to $CHBr_2NO_2$ prior to inactivation. Thus, if CBr_3NO_2 were a much better oxidative substrate than $CHBr_2NO_2$, the "lag" in activity loss with limiting glucose would correspond to the buildup of $CHBr_2NO_2$ to a sufficiently high concentration that this species could then compete effectively with CBr_3NO_2 for enzymatic reducing equivalents. These processes are outlined in eq 14.

$$CBr_3NO_2 + \text{glucose} \xrightarrow{HE_0^+} CHBr_2NO_2 \quad (14a)$$

$$CHBr_2NO_3 + \text{glucose} \xrightarrow{HE_0^+} HE_0^+{-}FAD{-}CHBr{=}NO_2^- \rightarrow \text{E-1} \quad (14b)$$

This scheme predicts that the ratio of the number of moles of CBr_3NO_2 consumed to the number of moles of enzyme inactivated would be similar to the ratio with $CHBr_2NO_2$ as the inactivator (i.e., 160). Consequently, 23 μM glucose oxidase should be inactivated by 3.7 mM CBr_3NO_2. Complete titration of 23 μM enzyme required 1.50(8) mM CBr_3NO_2 (Figure 5).

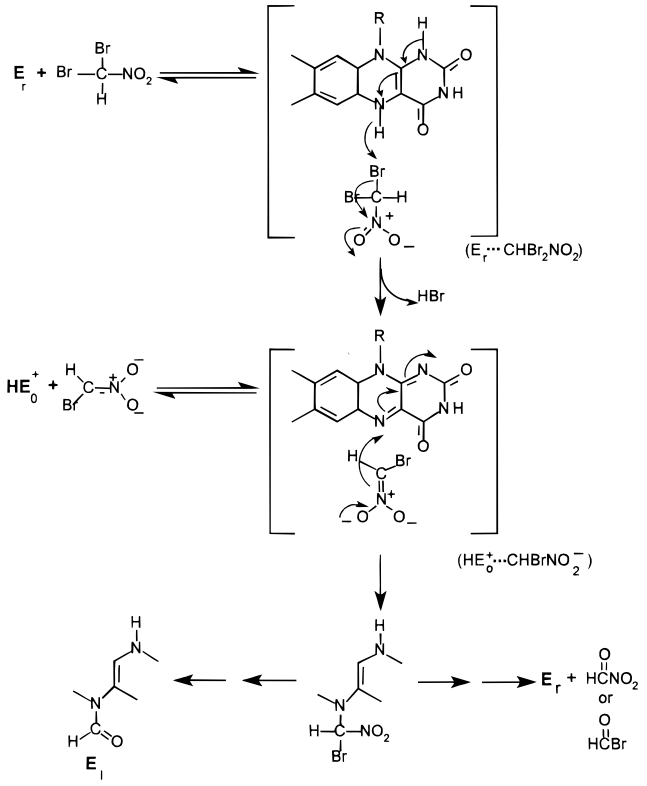
It has been shown above that glucose oxidase reduces dibromonitroalkanes to the corresponding monobromonitroalkane anions (Figure 2) and that the oxidized form of the enzyme is inactivated by $CHBr{=}NO_2^-$ with a partition ratio[3] of <0.1 (Figure 1). These data, however, do not establish whether the $CHBr{=}NO_2^-$ generated from $CHBr_2NO_2$ inactivates the enzyme by consecutive intramolecular steps without obligatory dissociation of $CHBr{=}NO_2^-$ prior to inactivation. Nonetheless, dissociation of the enzymatically generated anion prior to inactivation is unlikely for two reasons. First, the rate of inactivation is similar with 10 and 100 mM glucose. If dissociation of the anion occurs prior to inactivation (i.e., the enzymatically generated anion is not initially bound correctly for inactivation), the rate of inactivation would be predicted to decrease as the concentration of glucose is increased because D-glucose and $CHBr{=}NO_2^-$ compete for HE_0^+. Second, the rate of inactivation and the rate of reductive debromination are similar with 20 and 100 mM potassium acetate buffer. If free anion were the inactivating species, the rate of inactivation would be predicted to decrease as the buffer concentration is increased because the buffer-catalyzed protonation of $CHBr{=}NO_2^-$ would decrease its steady state concentration. Thus, inactivation of glucose oxidase does not require obligatory dissociation of enzymatically generated $CHBr{=}NO_2^-$.

Finally, the possibility that CH_2BrNO_2, $CHBr_2NO_2$, and CBr_3NO_2 might inactivate reduced glucose oxidase through a trivial alkylation reaction was ruled out by the following experiments. Reduced enzyme was not inactivated, nor was the visible spectrum of the enzyme modified by treatment of the enzyme with 4 mM iodoacetamide or 10 mM bromoacetone for 100 min at room temperature in the presence of 20 mM D-glucose.

The Semiquinone of Glucose Oxidase Is Not Involved in the Reductive Debromination of $CHBr_2NO_2$. The question as to whether the semiquinone state of the enzyme is an obligatory intermediate in the glucose-dependent inactivation of glucose oxidase by $CHBr_2NO_2$ was addressed by spectrophotometric experiments. Because the extinction coefficient for the inactivated enzyme at 400 nm is much less than that for the reduced or oxidized enzyme, the anaerobic inactivation process is conveniently monitored by the decrease in the absorbance of the enzyme at 400 nm. $CHBr_2NO_2$ (5 mM) (K_m = 9 mM) and 100 mM glucose modify the enzyme-bound flavin with a $t_{1/2}$ of 10 s (monitored by the absorbance decrease at 400 nm), close to the $t_{1/2}$ of ~17 s for the loss of enzymatic activity in an aerobic turnover experiment [from the inset of Figure 3 after an approximate correction for the fraction of enzyme (0.4) in the fully reduced state].

There was no absorbance change at 450 nm during the course of the inactivation experiment. Because oxidized forms of the enzyme absorb intensely at this wavelength, this lack of absorbance increase indicates that the enzyme remains predominantly in the reduced state during the inactivation reaction, and intermediates such as $HE_0^+{-}CHBr{=}NO_2^-$ must not accumulate significantly during the reaction. Therefore, the rate-determining first-order process in the inactivation reaction occurs after formation of the $E_r{-}CHBr_2NO_2$ intermediate and before, or coincident with, oxidation of the enzyme. If oxidation of the enzyme were to proceed through discrete single-electron transfer steps,

Scheme 2



rather than the ionic mechanism of Scheme 2 (see the Discussion), then the glucose oxidase semiquinone would be generated transiently during the inactivation reaction and would be able to be detected spectrophotometrically if the second electron transfer step were not much faster than the first.[4] The neutral semiquinone is conveniently monitored by its absorbance at 570 nm [$\epsilon_{575} = 4140\ M^{-1}\ cm^{-1}$ (*5*)]. Addition of 5 mM $CHBr_2NO_2$ and 100 D-glucose to the enzyme does not result in a transient absorbance increase at 570 nm (data not shown), indicating that the glucose oxidase semiquinone is not a significant intermediate during the inactivation reaction.

Structure and Properties of the Inactivated Enzyme. Our best preparations of $CHBr_2NO_2$-modified glucose oxidase retain approximately 1% of the glucose oxidizing activity of native enzyme. The visible spectrum of E_I resembles that of the enzyme reduced by glucose, but has a much lower absorbance at 400 nm and a distinctive shoulder at 315 nm (Figure 6). Inactivation of D-amino acid oxidase by $CH_3CHClNO_2$ causes a similar spectral shoulder (*2*).

Treatment of glucose oxidase with $CHBr_2NO_2$ and glucose causes covalent modification of more than 90% of the enzyme-bound flavin. The spectrum of inactivated enzyme has a small, but distinctive, peak in the 450 nm region with 10% of the 450 nm absorbance of native oxidase (Figure 6). This absorbance is bleached slowly by D-glucose. Thus, a small fraction of the enzyme is modified by $CHBr_2NO_2$ at

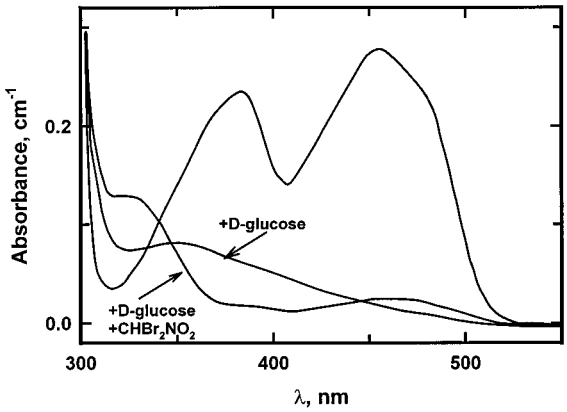


FIGURE 6: Optical spectrum of glucose oxidase inactivated by $CHBr_2NO_2$. Glucose oxidase (5 mL, 23 μM) was inactivated with 20 mM D-glucose and 5 mM $CHBr_2NO_2$. Excess glucose and $CHBr_2NO_2$ were separated from the enzyme on a column of Sephadex G-25 resin (2.5 cm × 25 cm). Two milliliter fractions were collected. The spectrum of the fraction with maximum absorbance was taken to be the spectrum of the inactivated enzyme. The spectrum of the oxidized enzyme (upper trace) was recorded with an enzyme solution that had been treated similarly except that this solution had not been exposed to $CHBr_2NO_2$ or glucose. The spectrum of reduced enzyme (middle trace) was recorded after addition of 10 mM D-glucose to the enzyme sample used to record the spectrum of the oxidized enzyme.

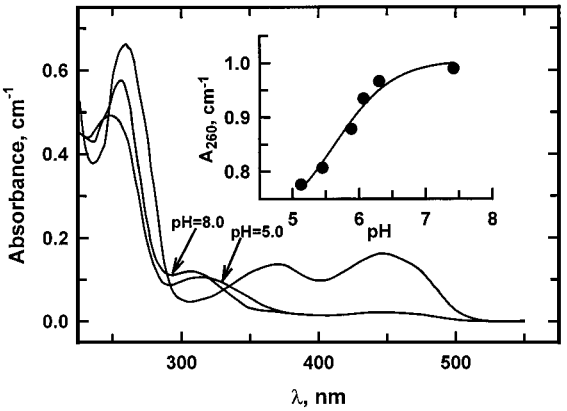


FIGURE 7: Optical spectra of the covalent flavin adduct resolved from $CHBr_2NO_2$-inactivated glucose oxidase. Inactivated glucose oxidase was prepared as described in the legend of Figure 6, and was resolved into the coenzyme and precipitated apoenzyme as described in Experimental Procedures. The spectrum of the modified flavin was recorded at pH 5.0 and 8.0. The adduct was converted to FAD (upper trace) by photolysis for 100 min at pH 5.0 as described in Experimental Procedures. Spectrophotometric titration of the adduct was monitored at 260 nm after adjusting the pH of the solution to the desired value with either 1 M acetic acid or 1 M tris base (inset). The p*K* value was 5.6(2).

an apoenzyme site such that the flavin can still be slowly reduced by glucose. Formyl bromide (O═CHBr) and formyl nitrite (O═$CHNO_2$) are likely products. If either of these reactive products acylates a group on the protein that contributes to catalysis, a fraction of the enzyme could be partially inactivated by reaction at a site other than the flavin.

The modified flavin of CH_2BrNO_2-inactivated glucose oxidase was resolved from the protein by heat treatment (Experimental Procedures). The visible spectrum of the resolved flavin is similar to that of the inactivated enzyme (Figures 6 and 7) with an absorbance peak at 317 nm at pH 5.0 that corresponds to the shoulder at 315 nm in the spectrum of the modified enzyme. Titration of the modified

[4] These data do not unequivocally eliminate the possibility of transient formation of glucose oxidase semiquinone. If the oxidation of the E_r to the semiquinone were much slower than the oxidation of the semiquinone to HE_0^+, the semiquinone would not accumulate during the reaction. Because this sequence of events would be kinetically equivalent to a two-electron transfer process, the reactions presented in Schemes 1 and 2 are written as two-electron transfers.

flavin causes the peak to shift from 317 to 307 nm with a p*K* of 5.6(2) (Figure 7).

The spectral characteristics of the modified flavin and the presence of an ionizable group with a spectrophotometric p*K* of 5.6 (corresponding to N-1 of the reduced isoalloxazine nucleus) are typical of N-5 adducts in general and are characteristic of the 5-acyl-1,5-dihydroflavin structure in particular (*1*, *2*). While 5-formyl-1,5-dihydro-FAD (the adduct specified by Scheme 2 in the discussion) has not been described, the properties of the next higher homologue [5-acetyl-1,5-dihydro-FAD (*2*)] are almost identical to those we described here for the adduct obtained from $CHBr_2NO_2$-inactivated glucose oxidase.

The proposed structure of the adduct resolved from the $^{13}CH_2BrNO_2$-inactivated enzyme was confirmed by ^{13}C NMR spectroscopy. The ^{13}C NMR chemical shift of the proposed adduct was expected to be similar to that of [^{13}C-*carbonyl*]-*N*,*N*-dimethylformamide, which has a chemical shift of 165.2 ppm and a coupling constant of 196 Hz (methanol standard taken as 49.3 ppm).

The proton-decoupled ^{13}C NMR spectrum of the adduct in ^2H_2O has peaks at 164.2 and 165.3 ppm that have a ratio of intensities of approximately 1:4. While the observed chemical shifts are very close to that of the model compound (165.2 ppm), the occurrence of two resonances might seem to be incompatible with the proposed adduct. However, 5-acetyl-1,5-dihydroflavins are a mixture of slowly interconverting conformers that generate multiple resonances for a single ^{13}C center (*12*). The proton-coupled ^{13}C NMR spectrum of the glucose oxidase-derived adduct exhibits splitting of the larger peak at 164.2 ppm into a doublet with a coupling constant of 211 Hz. The small peak, presumably, is also split, but because of the loss of intensity in the coupled experiment, the resulting doublet is obscured by background noise. These data support the assignment of 5-formyl-1,5-dihydro-FAD to the flavin adduct resolved from CH_2BrNO_2-inactivated glucose oxidase.

The glucose oxidizing activity and the flavin absorbance of native enzyme were recovered ($t_{1/2} = 6$ min) by irradiation of E_I with 355 nm light as described in Experimental Procedures. The oxidized flavin was regenerated from the modified flavin by photolysis with 355 nm radiation or treatment with nitrous acid. Under experimental conditions identical to those used to reactivate the inactivated enzyme, the resolved flavin adduct is photolyzed at one-third the rate ($t_{1/2} = 20$ min) that E_I is photolyzed ($t_{1/2} = 6$ min).

DISCUSSION

Previously, we have shown that $CH_3CHClNO_2$ is a suicide substrate for renal D-amino acid oxidase, whereas it is neither a substrate nor an inactivator of glucose oxidase (*2*). We considered three possible reasons for this negative result. First, $CH_3CHClNO_2$ may be sterically restricted from access to the N-5 region of the enzyme-bound flavin. Thus, $(CH_3)_2C{=}NO_2^-$, which is isosteric with $CH_3CCl{=}NO_2^-$, is a poor reductive substrate for glucose oxidase, whereas $CH_3CH{=}NO_2^-$ is a better reductive substrate than glucose (*3*). Second, CN^- does not cause inactivation of the glucose oxidase during turnover of this substrate, whereas it inactivates D-amino acid oxidase during turnover of nitroethane (*1*, *3*). These results suggested quantitative and even qualitative differences between the reaction intermediates of the two enzymes. Third, and perhaps most significantly, the reactive species in the reductive half-reaction are protonated glucose oxidase (HE_0^+, p$K = 3.5$) and the nitroethane anion ($CH_3CH{=}NO_2^-$, p$K = 8.3$). If this requirement likewise is needed in the case of CH_3CClNO_2 (p$K = 7.0$), then the product $[HE_0^+][CH_3CCl{=}NO_2^-]$ (which together with the bimolecular rate constant governing the encounter of HE_0^+ and $CH_3CCl{=}NO_2^-$ determines the measured rate of inactivation of the enzyme) would always have a small numerical value when these species are at bulk protonic equilibrium.

We have now demonstrated that CH_2BrNO_2 inactivates glucose oxidase slowly at pH 5.0. The inactivation reaction has properties characteristic of a suicide substrate (*11*, *13*, *14*) with a partition ratio that is less than 0.1. The enzyme-bound flavin is covalently modified in 90% yield to 5-formyl-1,5-dihydro-FAD. The $EH_0^+{-}CHBr{=}NO_2^-$ intermediate is presumably the initial complex on the inactivation pathway. Formation of this species in protonic equilibrium with buffer is unfavorable because of the p*K* values of EH_0^+ (p$K = 3.5$) and $CHBr{=}NO_2^-$ (p$K = 8.3$). In an attempt to circumvent this problem, the enzyme was challenged with preformed anion. This protocol was unsuccessful because $CHBr{=}NO_2^-$ was reprontonated rapidly at pH values for which a significant fraction of the enzyme existed as HE_0^+. However, $CHBr_2NO_2$ is an efficient inactivator of the enzyme in the presence of glucose. Since $CH_3CBr_2NO_2$ is reductively debrominated to $CH_3CBr{=}NO_2^-$ in the presence of glucose, it was reasonable to posit that $CHBr{=}NO_2^-$ is generated from $CHBr_2NO_2$ and glucose at the active site of the enzyme (Scheme 2).

The enzyme-bound flavin was subsequently modified by $CHBr{=}NO_2^-$ by a mechanism analogous to that described for the inactivation of D-amino acid oxidase by $CH_3CHClNO_2$ (path C in Scheme 1).

The partition ratio for inactivation of glucose oxidase by $CHBr_2NO_2$ in the presence of glucose is 160, whereas the partition ratio for inactivation of the enzyme by CH_2BrNO_2 in the absence of glucose is less than 0.1. The difference in these values is attributed to the fact that the inactivating agent, $CHBr{=}NO_2^-$, is already present in the case of CH_2BrNO_2, but must be generated by reductive debromination of $CHBr_2NO_2$ by the reduced enzyme. The enzymatically generated anion in the $HE_0^+{-}CHBr{=}NO_2^-$ intermediate is partitioned between dissociation of this complex into HE_0^+ and $CHBr{=}NO_2^-$ and conversion of this complex to E_I in a ratio of 160:1. This means that the value of the rate constant for dissociation of the $HE_0^+{-}CHBr{=}NO_2^-$ intermediate is approximately 160-fold greater than the value of the rate constant for inactivation. Once dissociated from the enzyme, $CHBr{=}NO_2^-$ is effectively trapped on the time scale of these experiments by rapid protonation by acetate buffer.

There are many instances of enzymatic and nonenzymatic reduction of halonitroalkanes. For example, cytochrome P-450 catalyzes the one-electron reduction of halonitroalkanes. In particular, cytochrome P-450 catalyzes the reduction of chloropicrin (CCl_3NO_2) to nitromethane and chloride (*15*). Nonenzymatically, geminal bromonitroalkanes are reduced to the corresponding nitroalkanes by thiols (*8*) or by *N*-benzyl-1,4-dihydronicotinamide (*16*). Secondary bromonitroalkanes, such as $(HOCH_2)_2CBrNO_2$ (the preservative bronopol), have been proposed to be reduced nonenzymati-

cally by a radical anion mechanism (*9*, *17*). In contrast, oxidation of reduced glucose oxidase by $CHBr_2NO_2$ did not generate detectable amounts of the semiquinone, which suggests that radical intermediates are not involved in this reductive debromination reaction. Furthermore, inactivation of glucose oxidase by nucleophilic addition of $CHBr{=}NO_2^-$ to the flavin nucleus is remarkable in consideration of the lack of nucleophilicity of this anion as judged by its low reactivity with aldehydes or electrophilic alkenes (*8*). Thus, the apoprotein of glucose oxidase must play a crucial role in the addition of this anion to N-5 of enzyme-bound FAD.

Glucose oxidase and D-amino acid oxidase are reduced by nitroethane anion (*1*, *3*). During the reduction of D-amino acid oxidase by this anion, the enzyme is inactivated by cyanide without participation of the enzyme semiquinone. In contrast, glucose oxidase is not inactivated by $CH_3CH{=}NO_2^-$ in the presence of cyanide (*2*). Furthermore, D-amino acid oxidase is fully reduced by nitroalkane anions, whereas significant amounts of the semiquinone are produced during the reduction of glucose oxidase by $CH_3CH{=}NO_2^-$ (*3*). These results might suggest that $CH_3CH{=}NO_2^-$ reduces these flavoprotein oxidases via different mechanisms. However, the fraction of glucose oxidase reduced to the semiquinone by $CH_3CH{=}NO_2^-$ is dependent upon the source of enzyme. Thus, the semiquinone may not be an intrinsic intermediate in the reaction pathway but may be a side reaction that is dependent upon the past history of the enzyme. Because $(CH_3)_2C{=}NO_2^-$ is a good substrate for D-amino acid oxidase, but an ineffective substrate for glucose oxidase (*1*, *3*), steric restrictions are probably more stringent for the glucose oxidase-catalyzed oxidation of nitroalkanes. Thus, the lack of reactivity of cyanide with glucose oxidase during the reduction of the enzyme by $CH_3CH{=}NO_2^-$ could be the result of steric hindrance, and not the result of a different mechanism for reduction of these two enzymes by nitroalkanes. The finding that halonitroalkane anions inactivate glucose oxidase and D-amino acid oxidase by similar covalent modification of the flavin supports the proposal that these oxidases are reduced by nitroalkanes through similar mechanisms.

Finally, the mechanism, suggested by the data presented herein, whereby $CHBr_2NO_2$ inactivates glucose oxidase, is unusual in that this substrate is reduced by the enzyme to generate a reduced intermediate, $CHBr{=}NO_2^-$, that subsequently inactivates the enzyme. This property distinguishes $CHBr_2NO_2$ from other suicide inactivators of flavoprotein oxidases. Typically, other suicide inactivators of flavoprotein oxidase are oxidized by the enzyme prior to inactivating the enzyme. For example, propargylglycine, a typical acetylenic substrate analogue, is oxidized by D-amino acid oxidase prior to inactivation of the enzyme. The reduced enzyme is not inactivated by this substrate analogue (*18*). However, a recent example of an oxidoreductase that reduces an acetylenic substrate analogue prior to inactivation is uracil reductase. This enzyme requires NADPH for inactivation by 5-ethynyluracil. Presumably, the reduced enzyme reduces 5-ethynyluracil to 5,6-dihydro-5-ethynyluracil that subsequently inactivates the enzyme (*19*). The inactivation mechanism described herein is applicable to flavoprotein oxidases that preferentially accept the nitronate as a reducing substrate. Recently, a nitroalkane oxidase from *Fusarium oxysporum* has been shown to only oxidize the neutral nitroalkane (*20*, *21*). It has been demonstrated that this enzyme is inactivated by nitroethane nitronate during the oxidation of neutral nitroethane with formation of an N-5 flavin adduct (*21*), much like the inactivation of D-amino oxidase during the oxidation of nitroethane nitronate in the presence of cyanide (*1*). Because this enzyme utilizes the neutral nitroalkane, it would not be expected that the mechanism of inactivation described herein for glucose oxidase with $CHBr_2NO_2$ would be applicable to this nitroalkane-oxidizing enzyme.

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