β-Haloethanol Substrates as Probes for Radical Mechanisms for Galactose Oxidase

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Abstract: Ketyl radical anions with a halogen substitutent on the carbon adjacent to ketyl are known to rapidly rearrange by halide anion ejection. Such a rearrangement is an ideal probe for possible ketyl radical anion intermediates in the catalytic mechanism of the monocopper/tyrosine radical enzyme galactose oxidase (GOase). Turnover of β-fluoro-, β-chloro-, β-bromo-, and β-iodoethanol by GOase leads to mechanism-based inactivation of the enzyme by trapping the enzyme in a catalytically inactive one-electron-reduced form. Presuming that mechanism-based inactivation and turnover proceed through the same reactive intermediates, the data reported here narrow down the possible mechanisms for the substrate oxidation step (the two electron transfer from substrate to enzyme) to two similar possibilities. Either the reaction proceeds through a short-lived ketyl radical anion intermediate or it proceeds through a closely related concerted E_2R mechanism with considerable ketyl radical anion character in the transition state.

Introduction

Galactose oxidase (GOase) from the filamentous wheat-rot fungus *Fusarium* spp.[1] catalyzes the oxidation of primary alcohols with O_2, producing aldehydes and H_2O_2 ($RCH_2OH + O_2 = RCHO + H_2O_2$). GOase is a single polypeptide with a molecular mass of 68 500.[2] The mechanism of GOase has been an active research topic for three decades.[3] A breakthrough in studies of GOase came in 1991 when a three-dimensional model of the structure of GOase was obtained from X-ray crystallographic studies.[2,4] The structure revealed that GOase contains two one-electron redox centers, the well-known mononuclear copper center, and a tyrosine center covalently cross-linked (at the ortho position to the −OH) to a cysteine (i.e.; Tyr 272 and Cys 228 cross-link). The unusual Tyr 272 is one of the equatorial ligands of the square-pyramidal copper center. Tyr 272 is believed to be the organic free radical in GOase.[5,6] GOase can exist in three distinct, stable oxidation states. These can be assigned as highest oxidation state—Cu(II) and tyrosine radical, intermediate oxidation state —Cu(II) and tyrosine (in equilibrium with Cu(I) and tyrosine radical), and lowest oxidation state —Cu(I) and tyrosine, with the highest oxidation state (Cu(II) and tyrosine radical) as the catalytically active form of the enzyme.[7]

Before a structural model of GOase became available from X-ray crystallographic data, Whittaker proposed a new type of radical mechanism utilizing the tyrosine-like protein radical that he had detected on the basis of extensive spectroscopic evidence.[7] By taking advantage of the structural data from X-ray crystallography and kinetic evidence with radical-probing substrates, we proposed a more detailed mechanistic scheme.[8] Whittaker subsequently refined the mechanism further—we proposed that the alcohol is deprotonated by a nearby histidine acting as a base, but spectroscopic studies of anion binding to GOase led Whittaker to propose that tyrosine 495 could act as the base.[9] Although there are some minor differences in the proposed mechanisms, the central feature of them all is that enzymic catalysis was proposed to proceed by a stepwise radical mechanism with a substrate-derived ketyl radical as a key intermediate. The possibility that the substrate oxidation step in the GOase reaction mechanism might proceed by either a stepwise or a concerted E_R2 mechanism as shown in Scheme 1 was recently discussed in a paper on molecular modeling of transition-state structures for the GOase reaction.[10,11] The concerted mechanism may at first appear to be unusual but it is simply a radical analog of an E2 elimination reaction, and thus might be called an E_R2 reaction. Both the stepwise and concerted mechanisms are consistent with mechanistic evidence from kinetic studies including (1) an ordered binding mechanism with substrate bindings and product releases occurring in the order shown and (2) cleavage of the α C−H bond as the fully rate-determining step (known from the nearly full primary deuterium isotope effect when the α position is substituted with deuterium).[12]

A ketyl radical anion is a possible key intermediate in the GOase reaction mechanism. Previous work from our group using a quadricyclane-containing round-trip radical-probing substrate provided evidence for the existence of a radical intermediate in the GOase reaction.[8] In that work we uncovered a novel type of mechanism-based inactivation in which processing of the radical-probing substrate diverted from the normal reaction pathway and led to the formation of the one-electron-

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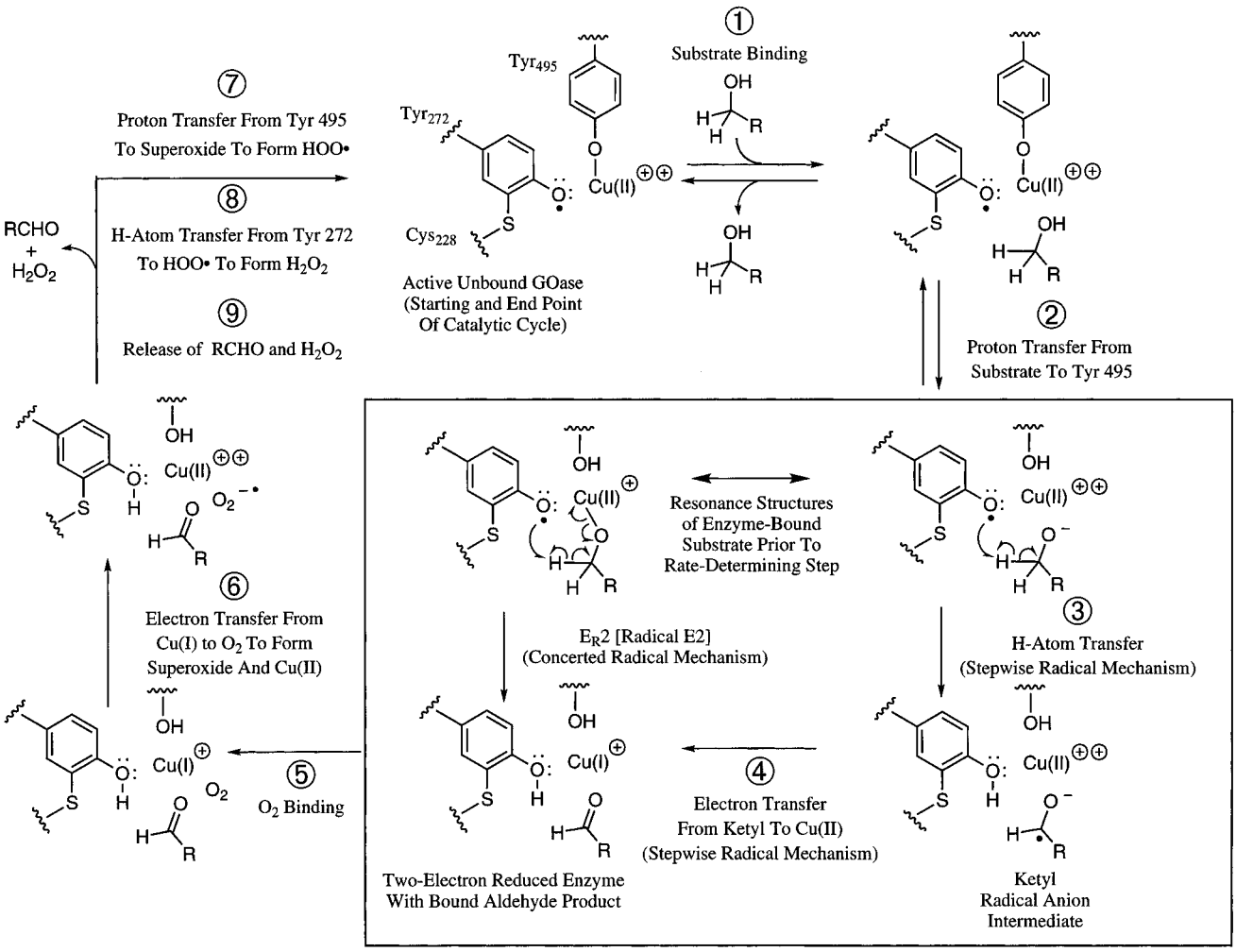
Scheme 1. Two Possible Radical Mechanisms for Galactose Oxidase-Stepwise Catalysis via a Ketyl Radical Intermediate vs Concerted E2-Like Oxidation of the Alcohol, Either Mechanism Using Tyrosine-272 and Copper as One-Electron Redox Centers



reduced catalytically inactive form of the enzyme. The inactivation process had a large primary deuterium isotope effect when the CH_2OH group was replaced with the CD_2OH group. Those two key pieces of data, (1) mechanism-based formation of a one-electron-reduced form of the enzyme and (2) a large primary deuterium isotope effect on the formation of the one-electron-reduced form of the enzyme, led us to conclude that a radical intermediate had been formed.

Although the evidence was solid from the previous work, we did not feel it was conclusive because it used only a single radical-probing substrate. We therefore devised a more comprehensive study in which several related radical-probing substrates were used to explore whether there was a correlation of the radical properties of the radical-probing substrates with their ability to inactivate the enzyme.

Tanner and co-workers have shown that α-halo ketyl radical anions rearrange rapidly by halide anion ejection as shown in Scheme 2.[13] The reaction for the fluoro ketyl radical anion **1** was found to have a rate constant of $5.2 \times 10^9\ s^{-1}$ at room temperature (Scheme 2). The reactions for the chloro ketyl radical anion **2** and the bromo ketyl radical anion **3** could not be measured precisely but both could be estimated to be greater than $10^9\ s^{-1}$ at room temperature. The iodo ketyl radical anion was not studied. On the basis of this work one would expect that a radical probe using this type of ketyl radical anion

(11) The possibility of a Cu(III)-mediated mechanism for GOase continues to be raised in discussions that B.P.B. has had with various colleagues. This possibility was proposed many years ago to account for the two electron oxidation of the substrate by the only redox center known at that time to be in GOase, the Cu (see ref 24). Now that Tyr 272 has been identified as a second redox center in GOase, the mechanisms using Cu(II) and tyrosine radical shown in Scheme 1 have been accepted as the most reasonable. Although many Cu(III) complexes are known, they are usually stabilized by several anionic ligands such as amide anions (see references at end of this footnote). It is highly unlikely that a similar stabilization of Cu(III) is possible by the copper ligands in GOase (two histidines, one normal tyrosine, and the tyrosine cross-linked to cysteine). The only possibility that might be plausible is if at least one, and preferably both, histidines were deprotonated and the histidine anions then acted as strongly electron-donating ligands to stabilize the Cu(III). Deprotonation of the histidines might seem unresonable since the pK_a of the imidazole functional group is about 14−15. However, since metal complexation is known to lower the pK_a of complexed ligands by as much as several pK_a units, complexation of the histidines with Cu might make deprotonation of the histidine imidazoles feasible. The available evidence on GOase, including the structural data from X-ray crystallographic studies, cannot determine the protonation state of the imidazoles. If the imidazoles are deprotonated then Cu(III) becomes a more viable possibility. At this time, with the available evidence the Cu(II) mechanism seems more likely. Recent references on stable Cu(III) complexes: (a) Ruiz, R.; Surville-Barland, C.; Aukauloo, A.; Anxolabehere-Mallart, E.; Journaux, Y.; Cano, J.; Munos, C. *J. Chem. Soc., Dalton Trans.* **1997**, 745−751. (b) Kou, F.; Zhu, S.; Lin, H.; Ma, K.; Chen, Y. *Polyhedron* **1997**, *16*, 741−747. (c) Hans, J.; Kruger, H.-J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2827−2830. (d) McDonald, M. R.; Scheper, W. M.; Lee, H. D.; Margerum, D. W. *Inorg. Chem.* **1995**, *34*, 229−237.

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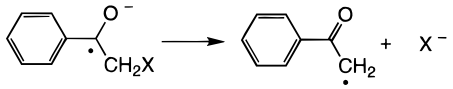
Scheme 2. Rearrangement of α-Halo Ketyl Radical Anions by Halide Anion Ejection[a]



1, X = F, $k = 5.2 \times 10^9$ s^{-1}
2, X = Cl, $k > 10^9$ s^{-1}
3, X = Br, $k > 10^9$ s^{-1}

[a] Rates were determined by Tanner et al.[13]

elimination of halide anion should be fast for all four of the common halides but that there should be some small differences in the rate of reaction due to the leaving group ability of the halide anion (fastest = I > Br > Cl > F = slowest).

GOase will only accept primary alcohols as substrates. Thus we chose to examine β-haloethanols **4**−**7** as probes for radical mechanisms in GOase. These radical probes should detect either stepwise or concerted radical mechanisms, as described in the following two paragraphs.

If a ketyl radical anion intermediate is formed during catalysis of alcohol oxidation by GOase, that ketyl radical anion intermediate should partition between normal turnover and radical rearrangement (Scheme 3). The radical rearrangement should lead to inactivation because it should trap the enzyme in the catalytically inactive one-electron-reduced form. The rearranged radical, an aldehyde radical, will be incapable of donating an electron to the enzyme since that would generate an extremely unstable carbocation α to the carbonyl. The aldehyde radical could accept an electron from the enzyme in the form of a hydrogen atom transferred from Tyr 272 to the aldehyde radical to produce acetaldehyde. Such a reaction would regenerate fully oxidized, catalytically active enzyme. That reaction should occur with the same frequency regardless of which haloethanol is used since the same aldehyde radical intermediate would be formed in all cases. It is also likely that the aldehyde radical could easily diffuse out of the active site since it should not be a good ligand for Cu(II) and there should be no other significant bonding interactions between it and the active site. In any event, if ketyl radical anions are formed during turnover of β-haloethanols, all four β-haloethanols would be expected to be inactivators, with some small differences in the rate of reaction due to the leaving group ability of the halide anion as found in the work of Tanner and co-workers (Scheme 2).[13]

The β-haloethanols could also provide information about the concerted E_R2 mechanism if it is the reaction pathway. In that case there would be a partitioning between an E_R2 turnover pathway and an E_R2 halide elimination pathway (Scheme 4). If E_R2 halide elimination occurs during turnover of β-haloethanols all four β-haloethanols would be expected to be inactivators. The effect of the leaving group on inactivation rates can provide insight into how synchronous or asynchronous the C−H and C−X bond cleavages are in E_R2 transition states. This point is developed further in the Discussion section.

Results

Each compound shown in Table 1 was used to inactivate GOase. Aliquots of activated GOase[14] were incubated with inactivator. At selected time points, samples of enzyme were withdrawn and assayed for residual activity using 3-methoxybenzyl alcohol in a standard UV−visible assay for GOase activity.[15] Rate data for each time point were divided by the rate obtained for a control reaction run in the absence of inactivator to give % relative residual activity. Inactivations were carried out until about 50% of initial activity was obtained (1 half-life). Plots of log (% relative residual activity) vs time were linear except for ethanol (see below), indicating that the inactivations exhibited pseudo-first-order kinetics for all of the halo inactivators in Table 1. All inactivations exhibited concentration-dependent saturation kinetics with faster rates of inactivation at higher concentrations of inactivator. The slopes of the lines from the semilog plots provided k_{obs} values for the inactivation of GOase at a particular concentration of inactivator. It is often common practice to perform a Kitz−Wilson double reciprocal plot[16] of $1/k_{obs}$ vs 1/[I] to obtain values of k_{inact} and K_I.[17] Instead of using Kitz−Wilson plots the observed rates at varying inactivator concentrations were computer fitted to a saturation curve, $k_{obs} = k_{inact}[I]/(K_I + [I])$ and kinetic constants k_{inact} and K_I were extracted. This method provided more consistent results and also provided a direct calculation of errors.

Inactivation by ethanol was included as a control to estimate the rate of background inactivation in the absence of halogens. Pseudo-first-order behavior could not be observed in plots of log (% relative residual activity) vs time. However, the response was saturatable and computer fitting a saturation curve, $k_{obs} = k_{inact}[I]/(K_I + [I])$, provided the kinetic constants k_{inact} and K_I shown in Table 1. We did not attempt to characterize the mechanism(s) of the inactivation by ethanol and did not use the ethanol inactivation rate to correct any of the halogen-dependent rates presented in Table 1.

In this study it was relatively easy to get good measurements of k_{inact} and K_I since a sensitive UV−visible assay was used to monitor residual enzyme activity as a function of time in incubations of GOase with the radical-probing substrate/inactivators. In contrast, it proved impossible to obtain meaningful values of k_{cat} and K_M for turnover since it was difficult to measure low levels of turnover activity with the noisy and somewhat insensitive oxygen electrode assay, the only assay available for nonchromogenic β-haloethanol substrates. This problem has been encountered by others examining aliphatic primary alcohols as substrates for GOase. One report lists the following relative activities of primary alcohol substrates for GOase: galactose, 1; ethanol, propanol, butanol, <0.0001; 2-chloroethanol, 0.0007; and 2-nitroethanol, 0.0013.[18] Another report lists a somewhat different set of relative activities: galactose, 100; ethanol, 0.003; propanol, 0.002; 2-choloroethanol, 0.06; 2-nitroethanol, 1.6.[19] Converting the data from the first study to a scale of galactose = 100, the following comparisons can be made: galactose, 100; ethanol, <0.01 or 0.003; propanol, <0.01 or 0.002; 2-chloroethanol, 0.07 or 0.06; and 2-nitroethanol, 0.13 or 1.6. These data from separate studies from the same research group illustrate two points. First, primary aliphatic alcohols are turned over by GOase at a slow rate. Second, the rates do not differ greatly from one aliphatic alcohol to another, with the possible exception of 2-nitroethanol. In our experience we obtained similar results. The data are not adequate to rigorously calculate k_{cat} or K_m values but it is

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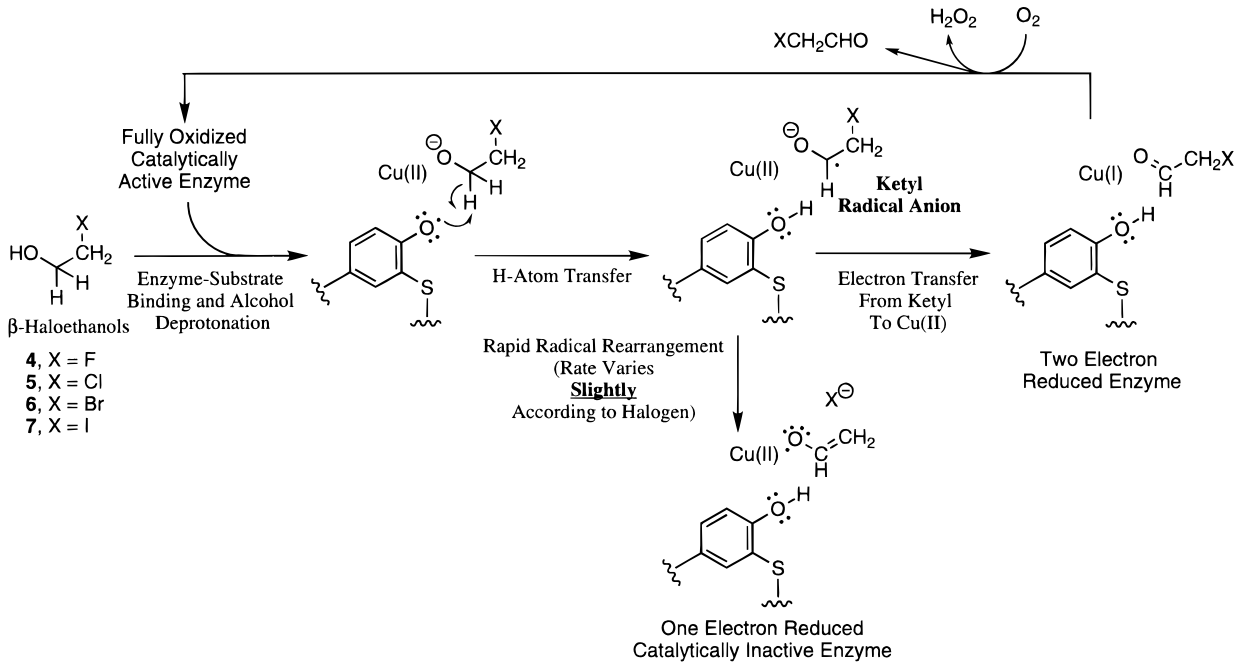
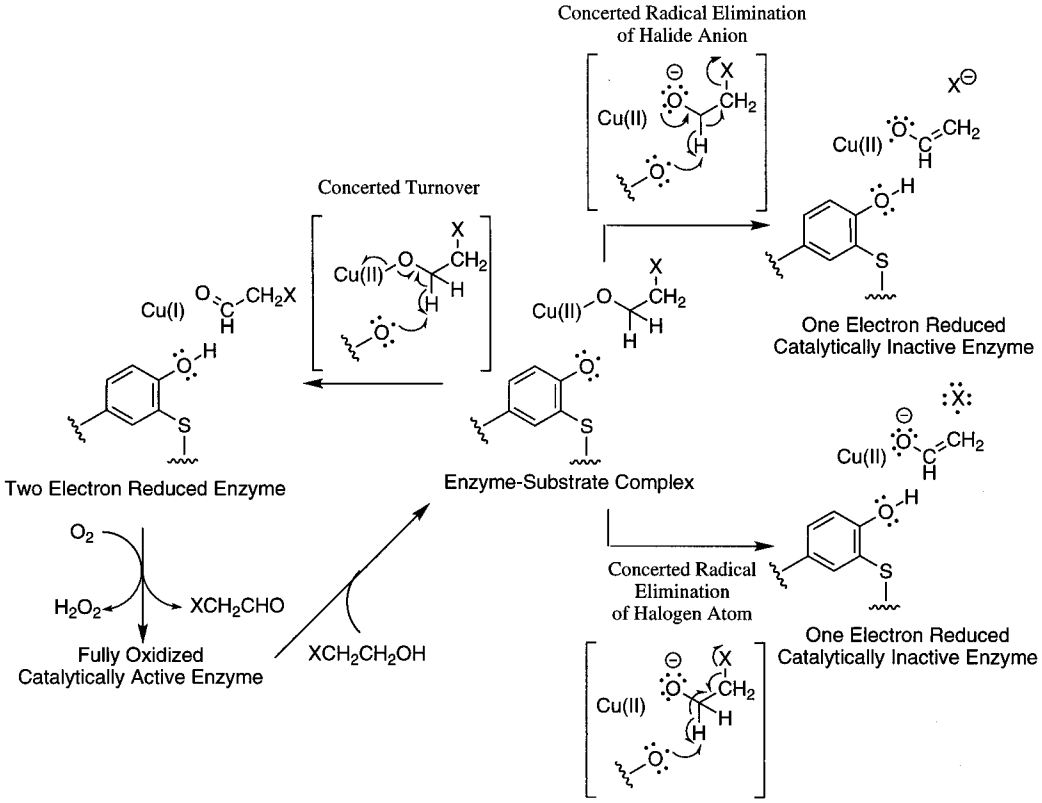
Scheme 3. Possible Stepwise Ketyl Radical Anion Mechanisms for Turnover and Inactivation



Scheme 4. Possible Concerted Mechanisms for Turnover and Inactivation



possible to estimate that the weak turnover that we see for 1,1-diprotioethanol and the 1,1-diprotio-β-haloethanols is consistent with the previously reported values, with turnover numbers in the vicinity of 0.02 s^{-1}. There is considerable error in this estimate but it is certain that all of the 1,1-diprotioalcohols, ethanol, and the β-haloethanols have roughly similar turnover numbers. Because the turnover data is so crude it has not been used to calculate partition ratios, k_{cat}/k_{inact}. Nevertheless, direct and meaningful comparisons can be made between the k_{inact} data in Table 1 since turnover numbers for aliphatic alcohol substrates are known to be similar. Even if the turnover numbers varied by as much as 1 order of magnitude, which is unlikely, it would still not change the fact that there is a small leaving group effect and the conclusions that can be drawn from that fact (see Discussion).

GOase inactivated by all of the substrates in Table 1 can be essentially completely reactivated by treatment with the one-electron oxidant ferricyanide. This fact strongly suggests that the inactivations produce the one-electron-reduced form of GOase but they do not prove it. To examine this point definitively, a redox titration of substrate-inactivated enzyme was carried out.

Table 1. Kinetic Constants for Inactivation of Galactose Oxidase by β-Haloethanols

substrate	k_{inact} (s^{-1})	rel. k_{inact}	k_H/k_D	K_I (mM)
$HOCH_2CH_2F$	0.000 13 (±17%)	2.0		10 (±44%)
$HOCH_2CH_2Cl$	0.000 43 (±44%)	6.6		16 (±94%)
$HOCH_2CH_2Br$	0.001 3 (±31%)	20		20 (±60%)
$HOCH_2CH_2I$	0.000 54 (±16%)	8.3		10 (±41%)
$HOCD_2CH_2F$	0.000 10 (±37%)	1.5	—[a]	15 (±82%)
$HOCD_2CH_2Cl$	0.000 14 (±23%)	2.1	3.1	10 (±60%)
$HOCD_2CH_2Br$	0.000 33 (±17%)	5.1	3.9	15 (±37%)
$HOCH_2CH_3$	0.000 065 (±18%)	1.0		5.0 (64%)

[a] The deuterium isotope effect for β-fluoroethanol cannot be determined with certainty due to overlapping errors at one standard deviation.

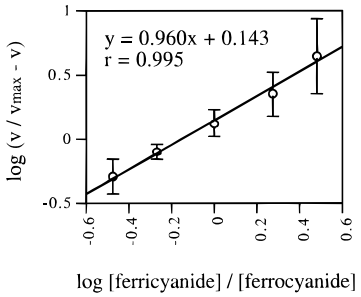


Figure 1. Effects of different molar ratios of ferricyanide to ferrocyanide on the rate of the GOase reaction after complete inactivation by bromoethanol. Plotted points are the average of eight individual measurements carried out on two different days, with error bars representing the standard deviation. A least-squares fit to a straight line gave a correlation coefficient R of 0.995, and a slope of 0.96.

To prepare substrate-inactivated enzyme, GOase was fully activated,[14] then combined with 2-bromoethanol *under anaerobic conditions* (see Discussion on this point), and extensively dialyzed to remove the inactivator. The dialysis was carried out anaerobically until remaining activity was well below 5% of initial activity. The enzyme was then equilibrated in a series of redox buffers to achieve partial reactivation (reoxidation) as a function of buffer potential, and the ratio of oxidized to reduced enzyme was determined with the oxygen electrode assay of activity. The molar ratio of ferricyanide to protein was kept below 1.0. This provides specific information about the substrate-inactivated form since the electron acceptor is limiting, so that some fraction of enzyme has to remain inactive *because it was inactive to begin with due to treatment with bromoethanol*. According to the Nernst equation, the slope of the line plotted in Figure 1 must equal the number of electrons transferred to reactivate the enzyme. The fit to a straight line was excellent (correlation coefficient $R = 0.995$), and the slope was determined to be 0.96 (±15%, standard deviation), demonstrating that bromoethanol inactivated GOase is in the one-electron-reduced form.[20] This is the first time that turnover-inactivated GOase has been shown, unequivocally, to be in the one-electron-reduced form. This experiment is superior to previous experiments using excess ferricyanide to completely reactivate GOase that had been inactivated with a quadricyclane-containing round-trip radical-probing substrate.[8] In those studies complete reactivation by ferricyanide was used to infer that inactivated enzyme was in the one-electron-reduced form since ferricyanide is well known to convert the one-electron-reduced form into the fully oxidized catalytically active form. Those experiments did not provide the direct evidence found here that proves that turnover-inactivated enzyme is in the one-electron-reduced form. This experiment demonstrates that inactivation of GOase with 2-bromoethanol produces the one-electron-reduced enzyme. Although this type of experiment was not done with the other inactivators in Table 1 it is reasonable to presume that their inactivations are proceeding by the same mechanism as 2-bromoethanol.

Product identification would be desirable in this study but it was not carried out for the following reason. Small amounts of products are produced due to low turnover and relatively efficient inactivation. It might be possible to isolate small amounts of aldehyde product(s) as derivatives, such as 2,4-dinitrophenylhydrazones. However, it would not be possible to know whether aldehyde products were formed by an enzymatic reaction or by nonenzymic alcohol autooxidation which could easily be initiated by stray radicals produced by galactose oxidase.

Discussion

Two mechanistically significant conclusions can be drawn from the data summarized in Table 1. First, there is a significant primary deuterium isotope effect on the rate of inactivation. Second, the effect of the leaving group on the rate of inactivation is small.

The characteristic features of the mechanisms of ionic elimination reactions, which have been studied in great detail, provide a framework for interpreting the data in Table 1. Ionic elimination mechanisms cover a wide spectrum of mechanistic possibilities ranging from a stepwise mechanism with a long-lived anion intermediate, the (E1cB)R mechanism, to various types of synchronous or asynchronous concerted E2 mechanisms.[21] Two important criteria of mechanism are the primary deuterium isotope effect and the effect of the leaving group. Of all of the possible elimination mechanisms only two exhibit the large primary deuterium isotope effect and the small effect of the leaving group that is found in Table 1. These are the stepwise anionic 1,2 $(E1cB)_{irr}$ mechanism and the closely related concerted but asynchronous E2 mechanisms with considerable C−H bond cleavage and very little C−X bond cleavage in the transition state.

In the $(E1cB)_{irr}$ mechanism a carbanion intermediate is irreversibly formed in the first step by deprotonation at the 1 position followed by elimination of the leaving group at the 2 position in a rapid second step. The large primary deuterium isotope effect, typically $k_H/k_D = 2-8$, is reasonable since irreversible deprotonation is rate determining. The small effect of the leaving group is reasonable since the C−X bond is intact in the transition state, so that the leaving group should affect the rate only through the polarity of the C−X bond.

In the spectrum of possible elimination mechanisms the $(E1cB)_{irr}$ mechanism is the stepwise mechanism which is closest to the stepwise/concerted borderline. The next mechanism in the spectrum, after crossing over from stepwise to concerted, is the asynchronous E2 mechanism with considerable C−H bond cleavage and very little C−X bond cleavage in the transition state. This mechanism is unique among concerted E2 eliminations in having a large primary deuterium isotope effect and a small effect of the leaving group. In contrast, most standard E2 eliminations, in which both C−H and C−X bond cleavage have progressed to some degree in the transition state, will have large primary deuterium isotope effects and large leaving group

(20) This experiment has been used previously to establish that the catalytically inactive form of GOase is one-electron-reduced: Hamilton, G. A.; Adolf, P. K.; deJersey, J.; DuBois, G. C.; Dyrkacz, G. R.; Libby, R. D. *J. Am. Chem. Soc.* **1978**, *100*, 1899−1912.

(21) Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*, 3rd ed.; Harper & Row Harper Collins Publishers: New York, 1987; pp 591−608, especially table on p 592.

effects.[22] For example, for eliminations from 2-phenylethyl halides k_H/k_D values range from 2.3 to 8.0 and the leaving group effect of F:Cl:Br:I is 1:67:4020:26067 for the eliminations using ethoxide in ethanol at 30 °C. For the analogous eliminations from 2-(2,4-dinitrophenyl)ethyl halides the leaving group effect of F:Cl:Br:I is 1:2:9:14.[23] For the second case the electron-withdrawing nitro groups in the phenyl ring make the C−H bond cleavage by deprotonation proceed ahead of C−X bond cleavage in the transition state. In this particular example deuterium isotope effects were not measured but they are expected to be large for this type of reaction in general.[21]

A similar analysis of C−H and C−X bond breaking can be done for possible mechanisms for inactivation of GOase by β-haloethanols. Only two closely related mechanisms, one stepwise and one concerted, are consistent with the data in Table 1.

One possible inactivation mechanism is stepwise, proceeding through a ketyl radical anion intermediate. In this inactivation mechanism the first step of H-atom transfer from substrate to Tyr 272 should be largely rate-determining and should have a significant primary deuterium isotope effect. Halide elimination from the ketyl radical anion intermediate should be rapid with a small effect of the leaving group if the reaction is analogous to the $(E1cB)_{irr}$ mechanism. Consistent with this idea, a small effect of the leaving group for halide elimination from ketyl radical anions has been observed in the studies of Tanner and co-workers discussed in the Introduction.[13]

The other possible inactivation mechanism is an asynchronous concerted E_R2 mechanism through a transition state with considerable C−H bond cleavage and much less C−X bond cleavage, in other words an asynchronous E_R2 elimination with considerable ketyl radical anion character in the transition state. In this inactivation mechanism the significant cleavage of the C−H bond in the transition state should result in a significant primary deuterium isotope effect, whereas the smaller amount of C−X cleavage in the transition state should lead to a small effect of the leaving group.

If one assumes that the β-haloethanol-induced inactivations are probing the mechanism of the normal substrate oxidation process by directly competing with it then there are two possible mechanisms for that substrate oxidation process. One is the stepwise mechanism via a short-lived ketyl radical anion intermediate (Scheme 3). The other is a concerted E_R2 mechanism with considerable ketyl radical anion character in the transition state (Scheme 3). The possibility that the substrate oxidation step in the GOase reaction mechanism might proceed by a concerted E_R2 mechanism as shown in Scheme 1 was recently discussed in a paper on molecular modeling of transition state structures for the GOase reaction.[10] That work showed that either stepwise or concerted mechanisms could be accommodated by the topology of the GOase active site and its binding interactions with galactose as a substrate. This work goes beyond that work by defining the details of possible mechanisms, intermediates, and transition-state structures.

One possible factor in favor of the concerted mechanism is that if a ketyl radical anion intermediate was formed in the oxidation of galactose it would be expected to break the C5 C−O (the C−O bond in the pyran ring) in the same manner that C−X bonds are cleaved when a ketyl radical anion is placed adjacent to them. This is an interesting possibility that might account for some of the turnover-dependent inactivation that is seen with galactose, although other mechanisms such as superoxide leakage later in the pathway have been proposed for such turnover-induced inactivation.[24] An argument against this possibility can be made by considering our recent molecular modeling studies.[10] When a radical transition state for H-atom transfer from galactose to Tyr 272 is docked into the active site and energy minimized, the C−H bond that is cleaved and the C−O bond in the pyranose ring are gauche to each other and thus at roughly a 60° angle to each other. If one then envisions C−H bond cleavage to form a ketyl radical anion intermediate, the ketyl and the rest of the galactose should be held by the enzyme so that the ketyl radical anion π system and the potentially cleavable C−O bond in the pyranose ring are held at at least 60° and probably closer to 90° due to rehybridization and structural reorganization at C6 as it converts from sp^3 in the substrate to sp^2 in the ketyl radical anion. In order for the C5 C−O bond to be cleaved efficiently stereoelectronic factors mandate that the ketyl π system and the C5 C−O bond should be aligned at nearly 0°, not the nearly orthogonal enzyme-imposed alignment indicated by the molecular modeling studies. Thus, one could argue that the enzyme may have evolved to allow the formation of a highly reactive ketyl radical on the galactose substrate by holding galactose in a conformation which prevents the destruction of the galactose ring by a ketyl radical anion-induced ring cleavage. Such arguments would also hold if the substrate oxidation mechanism were the concerted radical E_R2 mechanism with considerable ketyl radical anion character in the transition state since a concerted radical E_R2 cleavage of the galactose ring could be competitive with substrate oxidation.

Conclusions

Mechanism-based inactivations using radical-probing β-haloethanols provide strong evidence for a radical mechanism for GOase. Presuming that mechanism-based inactivation and turnover proceed through the same reactive intermediates, the data reported here narrow down the possible mechanisms for the substrate oxidation step (the two electron transfer from substrate to enzyme) to two similar possibilities. Either the reaction proceeds through a short-lived ketyl radical anion intermediate or it proceeds through a closely related concerted E_2R mechanism with considerable ketyl radical anion character in the transition state. Since distinguishing between such closely related types of mechanisms for ionic elimination reactions has proven to be very difficult,[22] it may be impossible to distinguish between these two possible mechanisms for GOase.

Experimental Section

General Methods. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. THF was distilled under N_2 from Na/benzophenone immediately prior to use. Absolute ethanol was purchased from Quantum Chemical Corporation (Tuscola, IL). 2-Fluoroethanol, 2-chloroethanol, 2-bromoethanol, 2-iodoethanol, 3-methoxybenzyl alcohol, and fluoroacetic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). 1,1-Dideuterio-2-chloroethanol and 1,1-dideuterio-2-bromoethanol were purchased from Cambridge Isotope Laboratories (Woburn, MA) and were 98% isotopically enriched. 2-Bromoethanol and 3-methoxybenzyl alcohol were distilled prior to use.

^{1}H NMR data were collected on a General Electric QE-300 NMR (300 MHz) locked on deuterium in the solvent. ^{19}F NMR data were collected on a General Electric QE-360 NMR instrument (470 MHz) using α,α,α-trifluorotoluene as a standard. Low-resolution mass spectra

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were obtained on a Varian VG 12-250 or VG ZAB 2FHF. Ions were generated by electron impact.

Synthesis of 1,1-Dideuterio-2-fluoroethanol. Fluoroacetic acid (Na salt, 6.0 g, 60 mmol) was suspended in 75 mL of anhydrous diethyl ether in an oven-dried 250-mL round-bottom flask under positive nitrogen pressure. To this solution was added 1 equiv of lithium aluminum deuteride (3.0 g, 60 mmol) over 15 min. Considerable gas evolution accompanied the addition. The reaction was allowed to proceed for 48 h at room temperature and then was quenched with 2 mL water, followed by 2 mL 50% NaOH, and then 6 mL water. The reaction mixture was filtered, and the retained salts were washed 2× with 10 mL of diethyl ether. The combined filtrates were neutralized to pH 7 with H_2SO_4 and then saturated with NaCl. The organic layer was separated from the aqueous layer. The aqueous layer was extracted 6 times with 10 mL of diethyl ether each time. All organic extracts were combined, dried 2 h over Na_2SO_4, and then filtered to remove solids. The ethereal solution was fractionally distilled to remove solvent. The undistilled residue was washed into a 50-mL flask with dichloromethane. Solvent was removed by rotary evaporation. The deuterated product was obtained in poor yield as a clear liquid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.45 (s), 4.60 (s); ^{19}F NMR δ 16.4 (triplet of triplets); low-resolution MS (*m/e*) = 66.2; calculated 66.2; isotopic enrichment, 98.5% ^{2}H.

Enzyme Preparations. Galactose oxidase (EC 1.1.3.9) was isolated from the culture media of *Fusarium* spp. *(Dactylium) dendroides* (NRRL 2903, ATCC 46032) according to published procedures.[7] GOase purified to homogeneity was quick-frozen in aliquots in liquid nitrogen and stored at −80 °C. To minimize variations in GOase activity, all kinetic experiments were carried out using the same enzyme pool. Once thawed, enzyme aliquots were used for kinetic experiments the same day only.

Enzyme Activity Assays and Determination of Turnover Numbers. Two direct assay systems were employed. In the first assay, activity was measured polarographically by monitoring oxygen consumption using a Clark type oxygen electrode (YSI Model 5300 Biological Oxygen Monitor, Yellow Springs Instrument Co., Inc., Yellow Springs, OH) essentially as described previously.[24] Routinely, the purified enzyme preparation was activated by oxidation using a ferricyanide-bound QAE Sephadex resin.[14] An aliquot of the enzyme pool, usually 0.12 nmol, was then injected into 3 mL of air-saturated (0.27 mM O_2) 200 mM galactose in 100 mM sodium phosphate buffer pH 7.0 thermostated at 30 °C.

In the second assay, activity was measured spectrally by monitoring the conversion of 3-methoxybenzyl alcohol to aldehyde at 314 nm as described previously.[15] Routinely, 10 μL of activated GOase[14] (7×10^{-5} nmol) was injected into a cuvette holding 150 mM 3-methoxybenzyl alcohol in 0.75 mL of 100 mM phosphate buffer pH 7.0 and 0.25 mL THF (freshly distilled). The turnover rate was determined from the slope of the absorbance timescan during the first 2 s.

Inactivation Kinetics. Time-dependent inactivation studies were carried out under aerobic conditions at 30 °C. A series of inactivator stock solutions between 5 and 150 mM were prepared. Activated GOase stock solution (100 μL, 0.46 mg/mL) was added to an aliquot of phosphate buffer, and 25 μL of inactivator stock solution was added at time zero to give final inactivator concentrations between 1 and 30 mM. The mixture was then incubated at 30 °C for 8 min. Aliquots (25 μL) were removed at specified time intervals and assayed for residual GOase activity using the spectral assay described above. The concentration of the inactivator was diluted 40-fold when injecting the aliquot into a cuvette containing 150 mM 3-methoxybenzyl alcohol substrate in 75% 100 mM phosphate buffer pH 7.0, and 25% THF (final volume 1 mL). This stops the inactivation process due to dilution of the inactivator and due to competition from the higher, saturating concentration of 3-methoxybenzyl alcohol, a better substrate than any β-haloethanol. A control experiment without any inactivator present was carried out at the beginning and end of each set of inactivations. No inactivation was observed in the control within the timeframe of interest, and the control activity at each time point was used as a 100% reference value for calculation of % activity remaining in the inactivation experiments. All kinetic runs were plotted in semilog form, i.e., log(% activity remaining) vs inactivator concentration.[17] Consistent with pseudo-first-order behavior, linearity was maintained in all cases (except for ethanol) for at least 1 half-live (50% inactivation). Inactivation rates (k_{obs}) at each inactivator concentration were calculated from the slope of the straight line computer-fitted to the data using CA-Cricket Graph III.[17] To extract kinetic constants, the individual calculated k_{obs} were analyzed according to the equation $k_{obs} = k_{inact}[I]/(K_I + [I])$. Kinetic constants k_{inact} and K_I and their standard deviations were extracted by direct computer fitting of measured rates (k_{obs}) to the equation for saturation behavior using MacCurveFit (Kevin Raner Software, Mt. Waverley, Australia).

Preparation of Bromoethanol-Inactivated Enzyme. GOase was fully activated with ferricyanide-bound QAE Sephadex resin,[14] the resin was removed by filtration through glass wool, and the activity determined polarographically. A total of 5000 equiv of bromoethanol was added under air and the solution transferred into dialysis tubing. Anaerobic dialysis was carried out in 200 mL of well-deoxygenated phosphate buffer (100 mM pH 7.0) under nitrogen at 4 °C, with the buffer being changed six times over a period of two days. Residual activity was determined to be near zero.

Redox Titration of Bromoethanol-Inactivated Enzyme. Redox buffers containing various ratios of ferri- to ferrocyanide were freshly prepared immediately before use, and an aliquot of each was added to enzyme at a total ferro- + ferricyanide to protein molar ratio of 1.3, with the ferricyanide to protein ratio below 1.0 in each case. All solutions were kept under air on ice and contained 15.4 μM GOase and 20.0 μM total cyanide, with no more than 15 μM ferricyanide, in 80 mM phosphate pH 7.0. The samples were incubated on ice for 10 min. Aliquots (25 μL) were withdrawn and assayed for initial turnover velocity (V) polarographically using the oxygen electrode assay. To determine maximum velocity (V_{max}) achievable under these conditions, an aliquot of bromoethanol-inactivated enzyme was incubated with 1.3 equiv of oxidizing agent (ferricyanide) alone, and tested as above.

For each redox buffer, log[ferricyanide]/[ferrocyanide] was then plotted against the expression relating oxidized to reduced enzyme concentration, $\log[v/(v_{max} - v)]$. The slope of the line, n, is equal to the number of electrons transferred upon enzyme reactivation, i.e., oxidation of bromoethanol-inactivated enzyme. This is true since at equilibrium the redox potential of the cyanide couple is equal that of the protein couple. From the Nernst equation, it follows that $\epsilon^{\circ\prime} + 0.059/n \log[v/(v_{max} - v)] = 0.425 + 0.059/1 \log[\text{ferricyanide}]/[\text{ferrocyanide}]$. The formal redox potential for the cyanide couple at pH 7 in phospate buffer has been shown to be $\epsilon^{\circ\prime} = 0.425$,[25] with number of electrons transferred between oxidized and reduced species equal to one.

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