Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 3341–3346

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

PROBING THE RADICAL MECHANISM OF GALACTOSE OXIDASE USING AN ULTRAFAST RADICAL PROBE

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Received 29 September 1999; accepted 26 October 1999

Abstract: Processing of *trans*-2-phenylcyclopropylmethanols **5** and **6** by the monocopper/tyrosine radical enzyme galactose oxidase led to mechanism-based inactivation with a partition ratio, $(k_{cat} + k_{inact})/k_{inact}$, of approximately 1 and a primary deuterium isotope effect, $k_{inact(H)}/k_{inact(D)}$, of 3.2. The data are consistent with a radical mechanism for galactose oxidase with a short lived ketyl radical anion intermediate.

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 ($RCH_2OH + O_2 \Rightarrow RCHO + H_2O_2$).[2-4] GOase contains two one-electron redox centers in the active site; a copper that can redox shuttle between reduced Cu(I) and oxidized Cu(II) and a tyrosine (Tyr272) that can redox shuttle between reduced tyrosine and oxidized tyrosine radical.[5,6] Tyr272 is crosslinked to Cys228 with a covalent C–S bond as shown in Schemes 1 and 2.[6,7] GOase can exist in three distinct oxidation states.[6] Catalytically active enzyme is fully oxidized, with oxidized Cu(II) and oxidized Tyr272 radical. One-electron-reduced enzyme is catalytically inactive. Two-electron-reduced enzyme can be formed by interaction of an alcohol substrate with the enzyme under anaerobic conditions and spontaneously oxidizes to the fully oxidized enzyme in the presence of O_2.[6] Fully oxidized and one-electron reduced forms are readily interconvertible under aerobic conditions using one-electron redox reagents.[8]

Based primarily on work from Whittaker's group[6,9,10] and from our group[11-14] the radical reaction mechanism shown in Scheme 1 is now accepted as the most plausible mechanism for GOase.[15] Significant information on the mechanism has been provided by studies with radical-probing substrates. In 1993 our group reported a novel type of reversible mechanism-based inactivation in which processing of a radical-probing quadricyclane substrate by GOase led to the formation of one-electron-reduced catalytically inactive enzyme.[11] The inactivation process was efficient with a partition ratio, $(k_{cat} + k_{inact})/k_{inact}$, of approximately 1. There was a large primary deuterium isotope effect, $k_{inact(H)}/k_{inact(D)}$ of 6.3 when the CH_2OH group was replaced with the CD_2OH group. The inactive enzyme could be fully reactivated by treatment with the one-electron oxidant $K_3Fe(CN)_6$. The data indicated that a ketyl radical anion radical intermediate had been formed, as shown in the mechanism in Scheme 1. In 1997 we reported studies of the GOase mechanism using β-haloethanols as substrates and mechanism-based inactivators.[13] Mechanism-based inactivation of GOase by β-haloethanols exhibited a primary deuterium isotope effect and a small halide leaving group effect, leading to the conclusion that the GOase reaction proceeds through a short-lived ketyl radical anion intermediate (Scheme 1) or it proceeds through a closely related concerted E_2R mechanism with considerable ketyl radical anion character in the transition state.[13]

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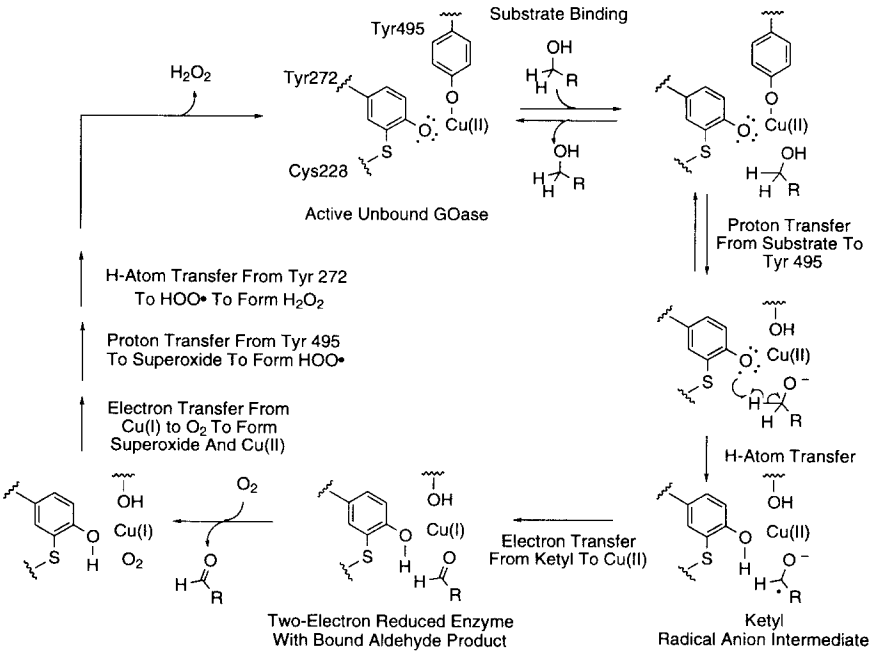
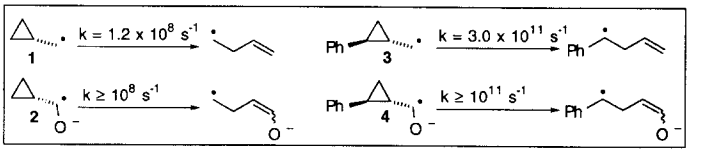


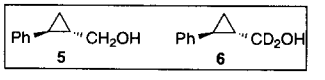
Scheme 1.

To gather additional definitive evidence on the radical mechanism of GOase we decided to use an ultrafast radical probe. An ultrafast radical probe should allow very efficient detection of transient radical intermediates.

The cyclopropylcarbinyl radical **1** rearranges by cyclopropane ring cleavage with a rate constant of 1.2×10^8 s^{-1} at 25 °C.[16] Ketyl radical anion **2** rearranges by cyclopropane ring cleavage with a rate constant that is at least that fast and probably faster.[17] The *trans*-2-phenylcyclopropylcarbinyl radical **3** rearranges with a rate constant of 3.0×10^{11} s^{-1} at 25 °C,[18] 2,500 times faster than **1**. Since **1** and **2** have similar rates of rearrangement, it is reasonable to assume that **4** will rearranged approximately as fast as **3**, at about 10^{11} s^{-1}.



GOase will only accept primary alcohols as substrates. We chose to examine primary alcohols *trans*-2-phenylcyclopropylmethanols **5** and **6** as ultrafast radical probes for the GOase mechanism.



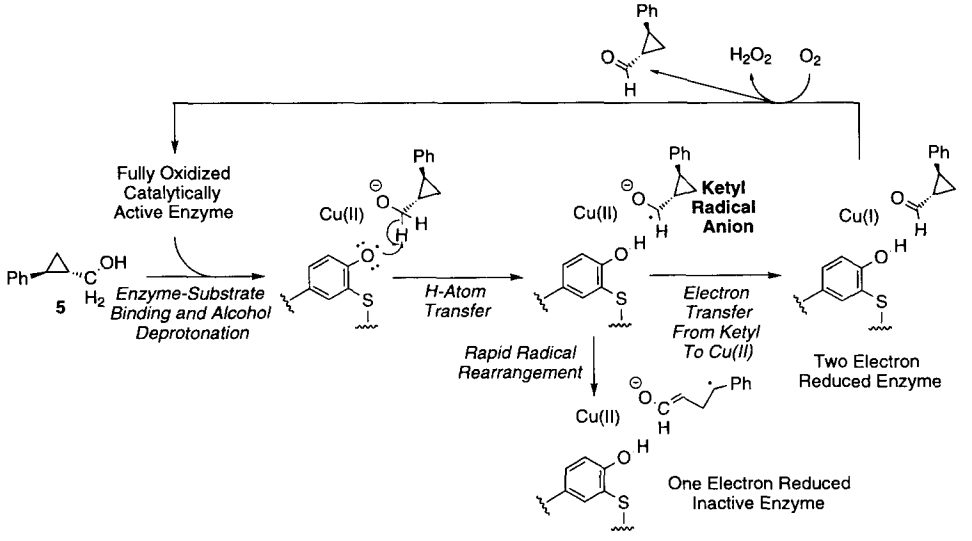
Radical probing substrates **5** and **6** were found to be concentration and time dependent inactivators of GOase. The inactivation rate and binding constants for **5** are k_{inact} = 0.0026 s^{-1} (±25%) and K_I = 5.6 mM (±63%). The inactivation rate and binding constants for **6** are k_{inact} = 0.00082 s^{-1} (±13%) and K_I = 5.1 mM (±42%).

The inactivations were fully reversible; addition of $K_3Fe(CN)_6$ to the incubation mixture led to complete recovery of initial activity. Addition of galactose to **5**/GOase incubations reduced the rate of inactivation, indicating competition for the active site.

Catalytic turnover of **5** and **6** was not observed using a Clark-type oxygen electrode in a standard assay as described previously.[14] Efficient inactivation by **5** and **6** with no observable turnover indicates that the partition ratio, $(k_{cat} + k_{inact})/k_{inact}$, is approximately 1 for both substrate/inactivators.

Inactivation of GOase by [α,α-$^2H_2$]-**6** compared to inactivation of GOase by [α,α-H_2]-**5** displayed a primary deuterium isotope effect, $k_{inact(H)}/k_{inact(D)}$, of 3.2. An isotope effect on turnover of **5** versus **6** could not be determined because turnover was not detectable.

The results for **5** and **6** display all of the key requirements for mechanism-based inactivation: (i) saturable, pseudo-first order kinetics, (ii) time-dependent loss of enzyme activity, (iii) inactivation slowed by addition of substrate (protection of the active site), (iv) involvement of catalytic step (primary deuterium kinetic isotope effect[19] on inactivation).[20,21] The satisfaction of the above criteria implies that enzyme inactivation is a result of an enzyme catalyzed reaction and that **5** and **6** are mechanism-based inactivators of GOase. The inactivation by **5** can be reversed with one-electron oxidants. The formation of one-electron reduced enzyme is consistent with the inactivation mechanism shown in Scheme 2. The ketyl radical anion intermediate, formed by H-atom transfer from substrate to Tyr272, partitions between two pathways, one leading to turnover and the other to inactivation. The extraordinarily rapid rate of rearrangement of ketyl radical anion **4** ensures that most of the reaction flux partitions into the inactivation pathway and little or none partitions into the turnover pathway. In conclusion, the results reported in this paper indicate that GOase proceeds via a radical mechanism with a short-lived ketyl radical anion as a key intermediate.



Scheme 2.

Materials and Methods

Isolation and Purification of Galactose Oxidase. Galactose oxidase (EC 1.1.3.9) was purified as reported[22] from the *A. Nidulans* transformant, pGOF101, a generous gift from Professor M. McPherson. A pool of enzyme from a single preparation was used for all kinetic experiments. Immediately prior to use the enzyme was activated using ferricyanide-bound QAE Sephadex resin as previously reported.[8]

Enzyme Activity Assay. Activity was measured spectrally by monitoring the conversion of 3-methoxybenzyl alcohol to aldehyde at 314 nm as described previously.[23] Routinely, 0.043 to 0.094 nmol activated GOase was injected into a cuvette with 80 mM 3-methoxybenzylalcohol in 69 mM PIPES pH 6.8/30% THF, to make a total volume of 1 mL. The k_{obs} was determined from the slope of the absorbance timescan during the first 2.5 s.

Inactivation Kinetics. Inactivator concentrations between 1 and 20 mM for **5** and **6** were used. The inactivator solutions contained 10% THF to solubilize **5** and **6**. The inactivator solutions were incubated at 25 °C with active enzyme. 50-μL aliquots were removed at specified time intervals (usually 1-minute intervals) and assayed for residual GOase activity using the spectrophotometric assay described above. The concentration of the inactivator was diluted 20-fold when injecting the aliquot into a cuvette containing the assay mixture described above. All experiments were replicated at least three times. In every case inactivation occurred by a saturable, pseudo-first-order process, and k_{obs} values at various concentrations of inactivator were obtained from plots of log percent residual activity vs. time. The kinetic constants k_{inact} and K_I were determined by computer-fitting the observed rate constants at various inactivator concentrations to a Michaelis–Menten type of equation.[14]

Reactivation of One-Electron-Reduced GOase. Active GOase was incubated with 40 mM **5** in 10% THF/buffer. Aliquots were removed at specified times and the residual activity was measured using Clark-type oxygen electrode in a standard assay as described previously.[14] When the residual activity stabilized (at about 20%) 20 μL of 100 mM $K_3Fe(CN)_6$ was added to the incubation and the activity was found to be fully recovered. A standard for 100% activity was determined from a control incubation containing no inactivator.

Synthesis of *trans*-(2)-Phenylcyclopropanemethanol (5). *trans*-2-Phenylcyclopropanecarboxylic acid was reduced to *trans*-2-phenylcyclopropanemethanol (79981-48-9) using $LiAlH_4$. by the method of Sneen et al.[24,25] The product was obtained as a colorless oil in a yield of 84.5%. ^1H NMR (300 Hz, $CDCl_3$): δ 0.97 (m, 2H, ring-CH_2), 1.45 (s, m, 2H, OH, ring-CH in position 1), 1.82 (m, 1H, ring-CH in position 2), 3.63 (m, 2H, CH_2OH), 7.06–7.29 (m, 5H, Ph) in agreement with previously reported NMR data.[26,27]

Synthesis of [α,α-$^2H_2$]-*trans*-(2)-Phenylcyclopropanemethanol (6). (α,α-$^2H_2$)-*trans*-2-Phenylcyclopropane-methanol (122566-27-2) was obtained as above, substituting $LiAlD_4$ for $LiAlH_4$. The product was recovered in 87.4% yield with an isotopic enrichment of 98% ^2H as determined by low-resolution mass spectroscopy. The ^1H NMR was in agreement with previously reported data[26,27] except that the CH_2OH was absent.

Acknowledgment: This research was supported by NSF Grant MCB-9311514. We thank Brian Arbogast at Oregon State University for determining the isotopic composition of deuterium-labeled **6**.

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