Thiols as Mechanistic Probes for Catalysis by the Free Radical Enzyme Galactose Oxidase†

Rebekka M. Wachter and Bruce P. Branchaud*

Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

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ABSTRACT: Galactose oxidase, a mononuclear copper enzyme, oxidizes primary alcohols to aldehydes using molecular oxygen. A unique type of cross-link between tyrosine 272, an active-site copper ligand, and cysteine 228 provides a modified tyrosine radical site believed to act as a one-electron redox center. Substrate analogs incorporating a primary thiol group in place of the primary alcohol group in normal substrates (RCH_2OH) have been studied as active-site mechanistic probes. Thiol sulfur coordinates to the active-site copper, leading to enzyme inactivation in a time- and concentration-dependent manner. The mechanism of inactivation involves redox chemistry related to the active-site redox centers, though inactivation does not proceed through the rate-determining hydrogen atom abstraction step that occurs in alcohol oxidation. Thiols are therefore classified as active-site-directed redox inactivators. The thiol analog of galactose, 6-Thio-Me-Gal, is also turned over by the enzyme, albeit at a much reduced rate, indicating that the energetics of turnover is changed significantly. Thiols constitute a particularly good model of the *ground state* enzyme−substrate complex. The Michaelis complex for thiol substrate analogs is stabilized at least 200-fold compared to the analogous alcohol substrates, whereas the transition state of H atom abstraction is destabilized, presumably due to a slight increase in distances of reacting atoms and weakening of hydrogen-bonding interactions due to the larger atomic radius of sulfur compared to that of oxygen.

In recent years, evidence has been accumulating that many enzymes proceed through radical mechanisms (Pederson & Finazzi-Agrò, 1993; Marsh, 1995). Most of these reactions involve redox cofactors for which radical intermediates are quite plausible on the basis of model studies of cofactor chemistry. New types of mechanisms have been proposed recently involving protein radicals, mostly on amino acid side chains (Stubbe, 1989; Prince, 1988), but even on the protein backbone as in pyruvate formate-lyase (Parast et al., 1995a). H atom abstraction from a substrate by a protein-derived radical is likely to occur in the mechanisms of several of these protein radical enzymes, including the various types of ribonucleotide reductases [binuclear iron + tyrosyl radical, adenosylcobalamin (B_{12}) + protein radical, binuclear manganese + protein radical] (Licht et al., 1996), several B_{12} enzymes (Harkins & Grissom, 1994), and prostaglandin-H synthase (mononuclear iron + tyrosyl radical) in prostaglandin biosynthesis (Tsai et al., 1995).

GOase[1] from the filamentous wheat-rot fungus *Fusarium* spp. (Ögel et al., 1994) catalyzes the oxidation of primary alcohols with O_2, producing aldehydes and H_2O_2 ($RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$) (Kosman, 1984). It is a single polypeptide with a molecular mass of 68 500 Da (Ito et al., 1994). It is now established 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) (Babcock et al., 1992; Ito et al., 1991). The unusual Tyr 272 is one of the equatorial ligands of the square-pyramidal copper center. GOase can exist in three distinct, stable oxidation states (Whittaker & Whittaker, 1988). 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. Spectroscopic evidence strongly indicates that a tyrosine radical is the radical center, that the tyrosine radical is directly coordinated to the copper center, and that the highest oxidation state of GOase is the catalytically active form of the enzyme (Babcock et al., 1992; Whittaker & Whittaker, 1988).

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 (Whittaker & Whittaker, 1988). Taking advantage of the structural data from X-ray crystallography (Ito et al., 1991) and kinetic evidence with radical-probing substrates (Montague-Smith et al., unpublished experiments), we proposed a more detailed mechanistic scheme (Branchaud et al., 1993). 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 galactose oxidase led Whittaker to propose that tyrosine 495 could act as the

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* Author to whom correspondence should be addressed. Phone: 541-346-4627. Fax: 541-346-4645. E-mail: bbranch@oregon.uoregon.edu.

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[1] Abbreviations: GOase, galactose oxidase; Me-Gal, methyl-β-D-galactopyranoside; 6-Thio-Me-Gal, methyl 6-deoxy-6-thio-β-D-galactopyranoside; rt, room temperature; TEA, triethylamine; DMAP, 4-(dimethylamino)pyridine; TsCl, *p*-toluenesulfonyl chloride; Ac_2O, acetic anhydride; KSAc, potassium thioacetate; NHE, normal hydrogen electrode.

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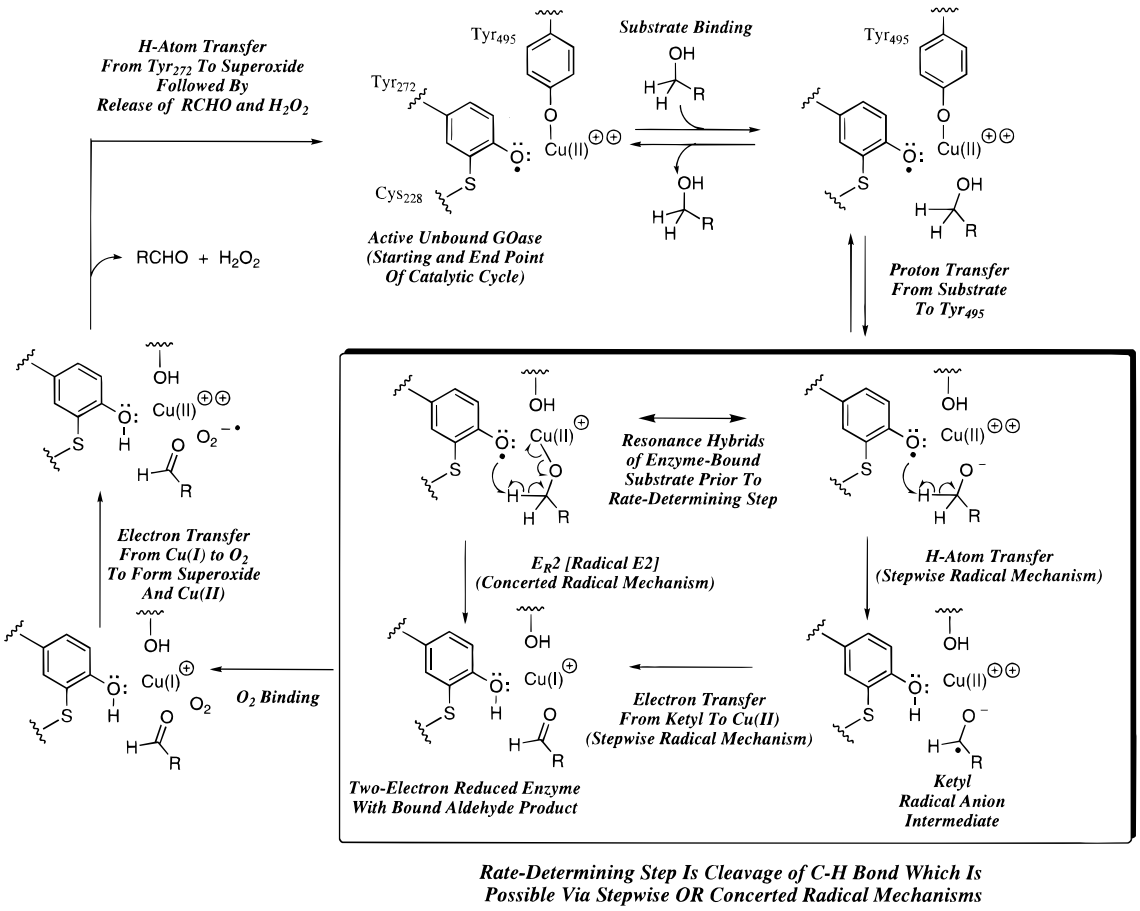
Scheme 1: Two Possible Radical Mechanisms for Galactose Oxidase: Stepwise Catalysis via a Ketyl Radical Intermediate versus Concerted E2-like Oxidation of the Alcohol, Either Mechanism Using Tyrosine 272 and Copper as One-Electron Redox Centers

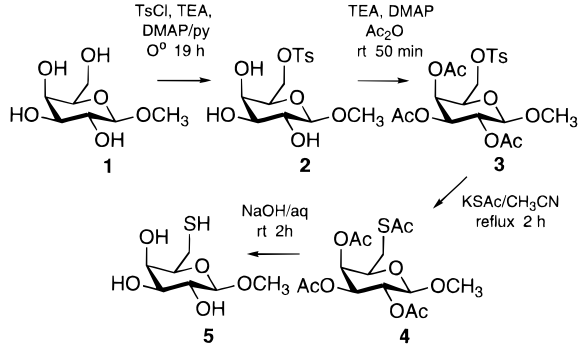


base (Whittaker & Whittaker, 1993). Although there are some minor differences in the proposed mechanisms, the central feature of them all is that enzymic catalysis is proposed to proceed by a stepwise radical mechanism with a substrate-derived ketyl radical as a key intermediate (Scheme 1). Using β-substituted ethanols as radical-probing substrates, we were able to give evidence toward the existence of the radical intermediate and estimate that its lifetime is in the nanosecond range or shorter (Montague-Smith et al., unpublished experiments). This led us to consider the possibility of a concerted mechanism (Scheme 1), where H atom abstraction and electron transfer to copper occur essentially simultaneously. Note that 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) (Maradufu et al., 1971).

Among carbohydrate hexoses, GOase turns over galactose-containing substrates rapidly ($k_{cat} = 400-800\ s^{-1}$) but does not even bind glucose up to a 1 M concentration (Wachter & Branchaud, 1996). Nevertheless, it is able to oxidize a broad variety of non-carbohydrate alcohols at rates that vary over several orders of magnitude, depending on the particular alcohol substituent. The enzyme therefore constitutes an ideal system for studying the relative strength of ground state and transition state binding of substrates and substrate analogs. We are interested in appropriate model systems of the Michaelis complex and mechanistic probes that will allow study of the rate-limiting transition state and the putative ketyl radical anion intermediate. The choice of analogs for the studies reported herein was based on two ideas. First, a good inhibitor useful for characterization of binding interactions would incorporate a minimum amount of structural changes to the substrate yet exhibit improved affinity to the active site. Second, a good mechanism-based inactivator would exhibit transition state stabilization analogous to that of the substrate to facilitate the rate-limiting catalytic event but then effect discontinuation of turnover some steps into the catalytic cycle, resulting in a dead-end complex or inactivated enzyme (Silverman, 1995).

We replaced the primary alcohol of a series of substrates with a thiol, arguing that affinity to the active site would be improved due to the strong sulfur−copper coordination bond. We anticipated that the hydrogen atom transfer from the α-carbon of the thiol to the enzymic tyrosine phenoxy radical would occur just as with alcohol substrates (Scheme 1) and that the thioketyl radical anion intermediate would be stabilized by overlap and delocalization with the adjacent sulfur orbitals. We further anticipated that electron transfer to the Cu(II) ion would become less favorable for thiols compared to alcohols due to poor overlap (weak bonding) of the sulfur−carbon π-bond in the presumed thioaldehyde product. It was anticipated that, when thiols were processed,

Scheme 2: Synthesis of Methyl 6-Deoxy-6-thio-β-D-galactopyranoside (6-Thio-Me-Gal, **5**)



GOase would become stalled in a dead-end, one-electron-reduced catalytically inactive state.

In the present work, we compare ground and transition state binding of alcohol substrates and their thiol analogs and show that the corresponding thiols constitute an excellent model of the Michaelis complex. We further show that redox-active substrates such as thiols may undergo electron transfer reactions in the active site of a radical enzyme that follow pathways separate from the normal type of turnover reaction seen with alcohol substrates.

MATERIALS AND METHODS

Synthesis of 6-Thio-Me-Gal, Compound 5

General. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Reactions involving reagents sensitive to moisture were conducted under an atmosphere of nitrogen with freshly distilled dry reagents and solvents. Analytical TLC was performed using aluminum-backed TLC plates (Whatman #4420222 with a UV indicator) and a hexanes/ethyl acetate solvent system (1:1). Compounds were visualized after dipping the TLC plates in an anisaldehyde dip (25 mL anisaldehyde, 25 mL of concentrated H_2SO_4, 450 mL of 95% EtOH, and 0.5 mL of HOAc), blotting them dry, and then heating with a heat gun. Column chromatography was performed according to Still et al. (1978), using silica gel 60 (E. Merck, Darmstadt, Germany). Spectral and analytical data for compounds **3**−**5** are available as Supporting Information. The synthetic approach is summarized in Scheme 2.

*Methyl 6-O-(p-Toluenesulfonyl)-β-D-galactopyranoside (**2**).* *p*-Toluenesulfonyl chloride in ethyl ether was washed with 10% NaOH, dried over Na_2SO_4 in the presence of charcoal, filtered, and recrystallized over dry ice from ethyl ether. A three-neck, 250 mL round-bottomed flask was charged with dry pyridine freshly distilled over KOH under N_2 (100 mL) and cooled to 0 °C. Methyl β-D-galactopyranoside **1** (2.50 g, 12.9 mmol), 2 equiv of TEA (3.59 mL), 1.2 equiv of DMAP (1.89 g), and 1.1 equiv of *p*-toluenesulfonyl chloride (2.70 g, 14.2 mmol) were added in that order. The solution was allowed to stir under nitrogen at 0 °C for 19 h, and another 0.1 equiv of *p*-toluenesulfonyl chloride (0.245 g) was added and stirring continued for 1.5 h at rt. The deep orange-red material was carried on without any isolation or purification and without transfer to another flask.

*Methyl 6-O-(p-Toluenesulfonyl)-2,3,4-tri-O-acetyl-β-D-galactopyranoside (**3**).* Nine equivalents of TEA (16.15 mL), 0.92 equiv of DMAP (1.44 g), and 15 equiv of acetic anhydride (18.2 mL, 193 mmol) were added to the tosylated product **2**, and the mixture was allowed to stir under N_2 at rt for 50 min. The product was extracted once into ether by addition of diethyl ether and water, quickly set on ice, neutralized by addition of aqueous sodium bicarbonate, and washed once with water. The ether solution was then dried over $MgSO_4$, filtered, and evaporated under high vacuum overnight, yielding a yellow liquid **3**. This material was also used without further purification.

*Methyl 6-Deoxy-6-(thioacetyl)-2,3,4-tri-O-acetyl-β-D-galactopyranoside (**4**).* For the following additions, the stoichiometry of reagents used was based on the assumption that the conversion of the 12.9 mmol of **1** to **3** had occurred quantitatively. A 250 mL round-bottomed flask was charged with all of crude compound **3**, and acetonitrile (120 mL) freshly distilled over CaH_2 under N_2 was added under N_2. The solution was cooled to near 0 °C with an air/water bath. After complete solubilization, 3.2 equiv of potassium thioacetate (4.67 g, 41.0 mmol) was added without further purification and the suspension refluxed under N_2 for 2 h. After cooling, the material was diluted with $CHCl_3$ (150 mL) and extracted first with saturated NaCl/H_2O (100 mL) and then with H_2O (80 mL). The aqueous layer was washed three times with $CHCl_3$. All organic fractions were pooled, evaporated to 300 mL, and washed with H_2O (3 × 200 mL) and then saturated NaCl/H_2O (200 mL), and all solvents were removed at reduced pressure using a rotary evaporator. The crude yield of the brown solid was 3.24 g. The material was purified by flash chromatography (1:1 Hexane/EtOAc) to give the thioacetylated compound **4** (633 mg, 13.4% from **1**).

*Methyl 6-Deoxy-6-thio-β-D-galactopyranoside (**5**).* The acetylated galactopyranoside **4** was routinely deprotected by weighing out 40−50 mg of **4**, adding 250 μL of 2 M NaOH under nitrogen, and allowing the solution to stir under N_2 for roughly 2 h. The solution was then neutralized by anaerobic addition of 250 μL of 2 M HCl. Four hundred microliters was withdrawn and immediately diluted 10-fold with 100 mM phosphate buffer (pH 3.5) to prevent disulfide formation which can occur under basic conditions. Aliquots (50 μL) of the deprotected thiogalactopyranoside **5** were then frozen at −80 °C.

Enzymology

Enzyme Preparations. An overexpressing clone of GOase in *Aspergillus nidulans* was a generous gift from Dr. M. J. McPherson (Department of Biochemistry and Molecular Biology, University of Leeds, U.K.). The vector pGOF101, containing the structural gene for GOase, was transformed into *A. nidulans* strain G191, leading to high-level heterologous expression of GOase from the inducible glucoamylase promoter (Baron et al., 1994). Purification of the secreted enzyme from the culture medium was performed essentially as described (Baron et al., 1994), with minor modifications. Briefly, the culture medium was filtered through a nylon-mesh cloth and the protein precipitated by ammonium sulfate addition to 80% saturation. The pellet was then dialyzed and subjected to anion exchange chromatography on DEAE-cellulose (Tressel & Kosman, 1980a), followed by affinity chromatography on Sepharose 6B (Hatton & Regoeczi, 1982).

Enzyme Activity Assays and Determination of Turnover Numbers. Two direct assay systems were employed. In the first assay, activity was measured spectrally by monitoring the conversion of 3-methoxybenzyl alcohol to aldehyde at 314 nm as described previously (Tressel & Kosman, 1980b). Routinely, the purified enzyme preparation was activated by oxidation using a ferricyanide-bound QAE Sephadex resin (Montague-Smith et al., 1992). GOase (7×10^{-5} nmol, 10 μL) was then injected into a cuvette holding 80 mM 3-methoxybenzyl alcohol in 0.9 mL of 150 mM PIPES (pH 6.8) and 0.1 mL of THF (freshly distilled), and the turnover rate was determined from the slope of the absorbance time scan during the first 5 s.

In the second 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). For routine assays, the enzyme was activated as above, and 0.12 nmol of GOase was injected into 3 mL of air-saturated (0.27 mM O_2) 400 mM galactose in 100 mM sodium phosphate buffer (pH 6.8) at 25 °C. For determination of turnover numbers (k_{cat}) of Me-Gal, benzyl alcohol, and ethanol, the amount of injected enzyme ranged from 0.12 to 0.95 nmol depending on the response of the substrate. Rates of oxygen consumption were measured at concentrations from 10 to 100 mM for Me-Gal and benzyl alcohol and 60 to 300 mM for ethanol. Due to solubility problems, turnover of benzyl alcohol had to be determined in 30% THF (freshly distilled). Turnover of Me-Gal was therefore measured in both 30% THF and aqueous solution. The extracted kinetic constants, k_{cat} and K_m, did not differ significantly in 30% THF/H_2O vs aqueous solution. Direct computer fitting of measured rates to the Michaelis−Menten equation (MacCurveFit, Kevin Raner Software, Mt. Waverley, Australia) was used to determine kinetic constants and their standard deviations. The amount of oxygen consumed under saturating conditions was converted to turnover numbers by assuming that 1 mol of oxygen is consumed per mole of enzyme with each turnover (Kosman, 1984).

Turnover of thiols was also assayed polarographically at concentrations from 50 to 600 μM. Although saturation behavior was observed up to approximately 400 μM in the case of 6-Thio-Me-Gal (**5**), a good fit to the Michaelis−Menten equation was not obtained since observed rates were inconsistent and unreasonably large at concentrations above roughly 400 μM. H_2S, a presumed product of turnover (see below), is known to interfere with the oxygen electrode at higher concentrations, resulting in inconsistent readings. Therefore, the turnover number (k_{cat}) for 6-Thio-Me-Gal under saturating conditions was estimated by doubling the rate obtained at 100 μM, the concentration equal to the K_D determined by competition experiments (see below). We found this approach to be a reasonable approximation to k_{cat} since turnover is rather slow, so that $K_m \sim K_D$. Turnover of benzyl mercaptan and ethanethiol was not measurable polarographically since the response was difficult to distinguish from the background response at equal concentrations of hydrogen sulfide.

Visible Absorption Spectroscopy. Freshly prepared protein in 100 mM phosphate buffer (pH 6.8) was concentrated by Centricon (Amicon, Beverly, MA) to 5 mg/mL, ferricyanide-oxidized (Montague-Smith et al., 1992), and assayed for activity. The protein was then microcentrifuged for 5 min to remove any insolubles and used immediately for spectral analysis. The absorbance against a buffer blank was collected between 900 and 250 nm on a CARY 3E UV−VIS spectrophotometer (scan rate of 150 nm/min, data interval of 0.5 nm). The cuvette holder was thermostated at 25 °C. An aliquot of freshly thawed 6-Thio-Me-Gal (**5**) stock solution (see above) was added to both the sample and the blank cuvette (thiol:protein = 23 mol/mol) and allowed to equilibrate for 2 min. The scan was then repeated. Data were transferred into CA-Cricket Graph III (Computer Associates International, Inc.) for analysis and presentation.

Determination of Thiol Dissociation Constants. Dissociation constants were estimated in competition experiments using the spectral assay described above at concentrations between 10 and 80 mM 3-methoxybenzyl alcohol, varying thiol inhibitor concentrations between 0 and 250 μM. The data followed linear competitive inhibition. The dissociation constant, $K_D = ([E] + [I])/[EI]$, was estimated from a Dixon plot by visual inspection (Segel, 1993).

6-Thio-Me-Gal Inactivation Kinetics. Time-dependent inactivation studies were carried out under aerobic conditions at 25 °C. PIPES (150 mM) at pH 6.8 was swirled in air and allowed to equilibrate at 25 °C for 15 min. The same solution was used for each set of inactivations, including their controls without inactivator. A small aliquot of 6-Thio-Me-Gal (**5**) stock was added to an aliquot of PIPES buffer to give a final volume of 480 μL, varying the inactivator concentration between 4 and 600 μM. Activated GOase (40 μL) was added at time zero (588 nM enzyme) and the solution incubated for up to 10 min. At indicated time intervals, 100 μL aliquots were removed and assayed for residual GOase activity using the spectral assay described above. The concentration of the inactivator was diluted 10-fold when injecting the aliquot into a cuvette containing 80 mM 3-methoxybenzyl alcohol substrate in 10% THF (final volume of 1 mL). A control experiment without inactivator present was carried out at the beginning and end of each set of inactivations. No inactivation was observed in the control within the time frame of interest, and the average control activity at each time point was used as a 100% reference value for calculation of the percent of the activity remaining in the inactivation experiments. All kinetic runs were plotted in semilog form and fitted to the equation of a straight line using CA-Cricket Graph III (Fogure 3). Late data points that did not fit the initial linear region were discarded. At lower 6-Thio-Me-Gal concentrations, linearity was maintained for roughly two half-lives (75% inactivation). At higher concentrations, rates were fast enough so that only one time point could be collected within two half-lives, and pseudo-first-order behavior had to be inferred. Inactivation rates (k_{obs}) at each thiol concentration were calculated from the linear curve fit to the data assuming first-order kinetics (Walsh, 1977, 1982; Silverman, 1995). Individual calculated k_{obs} values were then computer-fitted to the equation $k_{obs} = (k_{inact}[thiol])/(K_i + [thiol])$ using MacCurveFit (Kevin Raner Software, Mt. Waverly, Australia). The equation is based on standard Michaelis−Menten saturation kinetics, substituting k_{inact} for k_{cat} and K_i for K_m. Therefore, $K_i = K_D + k_{inact}/k_{on}$. A best estimate of the limiting k_{inact} under saturation conditions, the K_i, and their respective standard errors are extracted by the program. 6-Thio-Me-Gal inactivation kinetics were also carried out in 30% THF as described

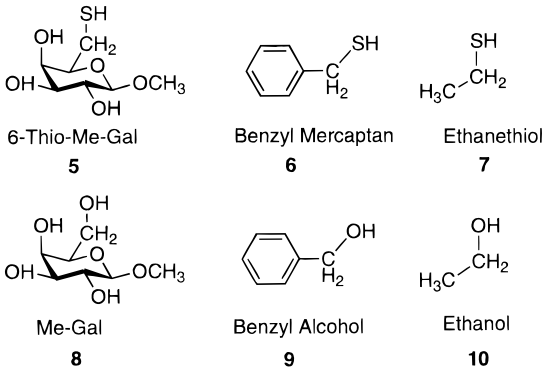


FIGURE 1: Thiol inactivators and corresponding substrate alcohols.

below. No change in rates in the presence of THF cosolvent was observed.

Ethanethiol and Benzyl Mercaptan Inactivation Kinetics. Ethanethiol and benzyl mercaptan (Aldrich, Milwaukee, WI) were diluted anaerobically into freshly distilled THF to prepare an inactivator stock solution. Due to the low aqueous solubility of these thiols, a 10 mL solution of 70% 150 mM PIPES (pH 6.8) and 30% freshly distilled THF (v/v) was prepared aerobically immediately before the experiment. A small aliquot of the inactivator stock was mixed in by swirling under air, resulting in the final thiol concentration used for the experiment. This solution was then allowed to equilibrate under air for 5 min before addition of protein to a 480 μL aliquot to give a total volume of 520 μL. Inactivation kinetic runs were then carried out exactly as described for 6-Thio-Me-Gal. The thiol concentration range was limited to 20−100 μM due to solubility problems at higher concentrations, resulting in a larger error on k_{inact} as compared to that of 6-Thio-Me-Gal. Data were routinely collected for at least two half-lives (roughly 4 min), though linearity was maintained up to almost complete loss of enzyme activity (roughly 10 min).

Hydrogen Sulfide Inactivation Kinetics. Sodium sulfide (Aldrich) was solubilized in 500 mM HCland then diluted 10-fold into 150 mM PIPES (pH 6.8) to give a 1 mM stock solution to be used immediately. Inactivation kinetics were then carried out exactly as described for 6-Thio-Me-Gal. Within a concentration range of 5−60 μM hydrogen sulfide, data followed pseudo-first-order behavior for roughly two half-lives (1.2 min). Only two time points could be collected before the response started leveling off.

RESULTS

Turnover Rates of Alcohols Exhibit a Strong Substituent Effect. We have determined turnover numbers and Michaelis constants for Me-Gal (**8**), benzyl alcohol (**9**), and ethanol (**10**) by fitting of measured rates to a Michaelis-Menten saturation curve (Figure 1 and Table 1). Turnover numbers are consistent with relative turnover rates published previously (Amaral et al., 1966; Kosman, 1984). The three alcohols differ in turnover rates by at least 1.5 orders of magnitude (Me-Gal and benzyl alcohol) and by more than 4 orders of magnitude (Me-Gal and ethanol) (Table 1). These large variations are thought to be directly related to differential stabilization of the transition state for rate-limiting breakage of the substrate H−C_α bond (Scheme 1). The enzyme active site is ideally suited to bind the transition state of galactose (Wachter & Branchaud, 1996), hence the much larger turnover rate of Me-Gal. Though the transition state of benzyl alcohol cannot be stabilized to the same degree by the enzyme itself, it has a built-in stabilization due to orbital overlap of the C−H bond undergoing cleavage on the substrate C_α with the π-orbital system of the aromatic ring. Ethanol on the other hand does not have such opportunities for specific transition state stabilization and so turns over very slowly.

Selection of a Series of Thiol Inactivators Based on Turnover Rates of Corresponding Alcohols. In order to show that a particular compound is a mechanism-based inactivator, it is essential to show that the inactivation proceeds through a catalytic step, i.e., that turnover and inactivation share a common step that is on the normal reaction pathway of the enzyme (Silverman, 1995). To this end, we investigated a series of thiols with structurally and chemically divergent substituents that were modeled on alcohol substrates with widely differing turnover rates (Figure 1). We investigated whether inactivation rates of 6-Thio-Me-Gal (**5**), benzyl mercaptan (**6**), and ethanethiol (**7**) follow the same trend as turnover rates of corresponding alcohols (Table 1). We also investigated inactivation by hydrogen sulfide as a negative control, since in this case any observed inactivation would be due to thiol chemistry alone, without direct relevance to the normal catalytic mechanism of GOase.

Sulfur Anion Directly Coordinates to the Enzymatic Copper Center. Absorption spectroscopy was used to probe for changes in the active site copper environment upon 6-Thio-Me-Gal binding. The spectrum of fully oxidized GOase (Figure 2) is consistent with previous studies (Whittaker & Whittaker, 1988), where the intense absorption features have been assigned to electronic transitions associated with the phenoxy radical of Tyr 272 (Whittaker et al., 1989). The 445 nm transition has tentatively been assigned to an intraradical transition within a stacked aromatic chromophore, i.e., the modified tyrosine and the stacked tryptophan composing the radical site. The broad red band around 800 nm was suggested to arise as a mixture of ligand-to-metal charge transfer (LMCT) and charge-resonance excitation between the aromatic π-systems involved in the charge transfer complex (Whittaker & Whittaker, 1993). Addition of 6-Thio-Me-Gal at a 23-fold molar excess over protein is thought to result in turnover until substrate depletion occurs (see below), indicating that the spectrum collected may be that of the product H_2S−copper adduct. The 445 nm transition and the broad band at 800 nm have disappeared completely, indicating that the tyrosine radical has been quenched. A new transition has appeared around 390 nm. This transition is interpreted as thiolate-to-copper LMCT involving a tetragonal copper geometry with a Cu(II) redox state (Lever, 1984). Clearly, the sulfur anion, either of 6-Thio-Me-Gal or of hydrogen sulfide, is directly coordinated to copper without changing the basic metal coordination geometry determined by X-ray crystallography (Ito et al., 1991). The copper redox state is also unaffected, though the tyrosyl radical is quenched in the presence of thiol.

Inactivation Rates of Thiols with Differing Substituents Do Not Vary Significantly. Incubation of active GOase with the four thiols listed in Table 2 resulted in time- and concentration-dependent enzyme inactivation. The kinetics of inactivation, shown for 6-Thio-Me-Gal (Figure 3), exhibited pseudo-first-order behavior for at least two half-lives.

Table 1: Michaelis Constants for Alcohol Turnover[a]

substrate	k_{cat} (s^{-1})	K_m (mM)	relative k_{cat}	relative K_m
Me-Gal (**8**)	271 (±1.7%)	21 (±5.4%)	1	1
benzyl alcohol (**9**)	7.9 (±18%)	70 (±13%)	0.029	3.3
ethanol (**10**)	0.02 (±26%)	4×10^2 (±4 × 10%)	0.000074	19

[a] Numbers in parentheses represent relative standard deviations. For procedures, see Materials and Methods.

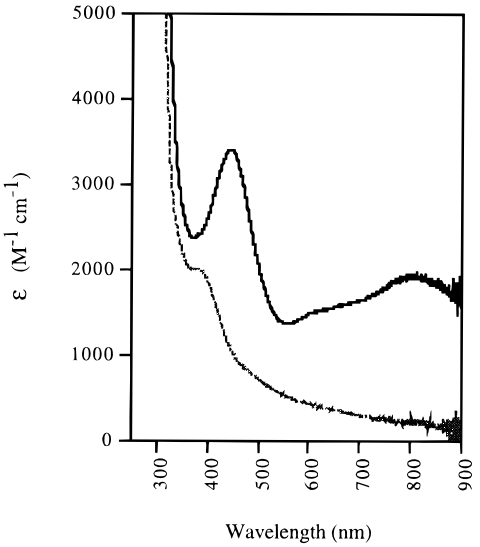


FIGURE 2: Absorbance spectrum of GOase (5 mg/mL), freshly activated (top, solid scan) and after addition of 6-Thio-Me-Gal (**5**) at 23-fold molar excess over protein (bottom, broken scan). For experimental details, see Materials and Methods.

Table 2: Thermodynamic (K_D) and Kinetic (k_{inact}, K_i) Constants for Thiol Inhibition and Inactivation[a]

inactivator	K_D (μM)	k_{inact} (s^{-1})	K_i (μM)
6-Thio-Me-Gal (**5**)	1.1×10^2	0.086 (±9.9%)	2.9×10^2 (±20%)
benzyl mercaptan (**6**)	90	0.014 (±29%)	1.0×10^2 (±49%)
ethanethiol (**7**)	89	0.011 (±27%)	89 (±44%)
hydrogen sulfide	20	0.040 (±12%)	33 (±25%)

[a] K_Ds were estimated by visual inspection only. Numbers in parentheses represent relative standard deviations. For procedures, see Materials and Methods.

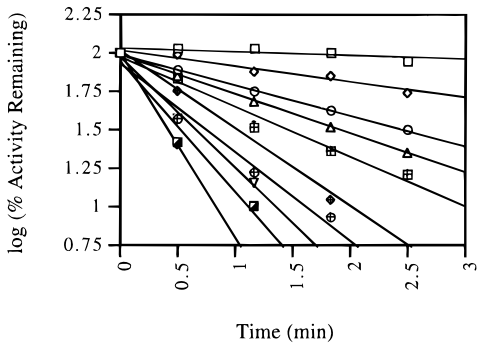


FIGURE 3: Time- and concentration-dependent inactivation of GOase by 6-Thio-Me-Gal. The inactivation rate, k_{obs}, at each inactivator concentration is calculated from the slope of each line. 6-Thio-Me-Gal concentrations are as follows (from top to bottom): 0, 4, 10, 25, 50, 100, 140, 200, 260, and 300 mM. For experimental details, see Materials and Methods.

Calculated rates of inactivation were fitted to a saturation plot, and the limiting k_{inact} and the K_i were extracted (Figure 4). The k_{inact} for benzyl mercaptan and ethanethiol, 0.01 s^{-1}, was essentially the same within the error of the experiment (Table 2). This is inconsistent with rate-limiting hydrogen

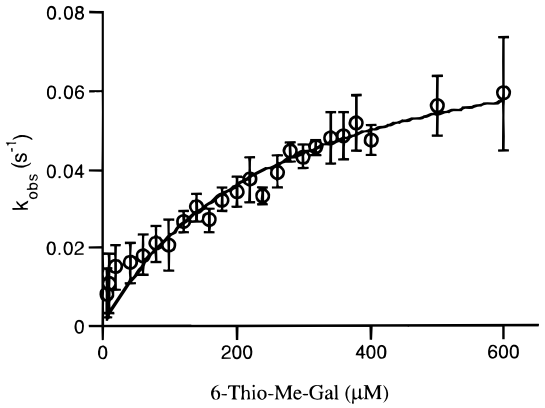


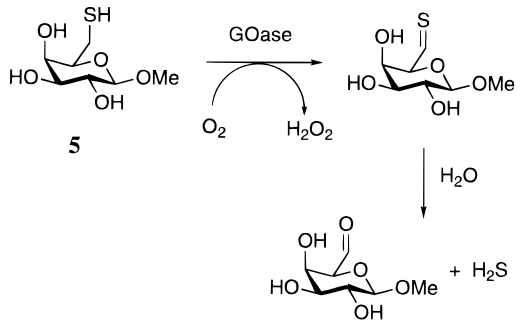
FIGURE 4: Saturation curve for inactivation by 6-Thio-Me-Gal. Average inactivation rates for each inactivator concentration are fitted to a Michaelis−Menten-type saturation curve. Error bars represent standard deviations ($n = 3-6$). For experimental details, see Materials and Methods.

atom abstraction as the mechanism of inactivation since these inactivation rates do not follow the same trend as turnover rates of the corresponding alcohols which differ by several orders of magnitude.

Unexpectedly, H_2S itself inactivated GOase at a rate 3−4 times faster than that of benzyl mercaptan and ethanethiol (Table 2). Inactivation rates measured at H_2S concentrations up to $3K_D$ saturate around 0.04 s^{-1}, with a K_i close to the thermodynamic K_D (see competition experiments below). This behavior indicates an active-site-directed process with specific binding followed by a chemical conversion leading to enzyme inactivation. Since this conversion cannot involve a hydrogen atom abstraction step, inactivation must proceed via a different mechanism, one that does not include the rate-limiting step of turnover. This is consistent with the conclusions reached above for the mechanism of inactivation by benzyl mercaptan and ethanethiol.

Hydrogen Sulfide May Inactivate GOase by a Mixed Mechanism. When H_2S concentrations were increased to $4K_D$ and above, observed rates of inactivation were faster than calculated on the basis of simple saturation, indicating a change in the predominant mechanism of inactivation (data not shown). We suggest that, under the experimental conditions, the redox properties of hydrogen sulfide may affect protein activity in a variety of ways. At a low hydrogen sulfide concentration, inactivation may predominantly proceed via the same pathway as inactivation by ethanethiol and benzyl mercaptan. At higher concentrations, the inactivation mechanism may proceed by a variety of pathways, some of which may be more specific to hydrogen sulfide. The standard reduction potential of HS^- against the NHE has been determined to be 1.15 V and that for the protonated species H_2S 1.5 V (Surdhar & Armstrong, 1986). The standard reduction potentials of ethyl and propyl thiolates have been determined to be 0.8 V, and that of the corresponding protonated thiols has been determined to be 1.4 V (Surdhar & Armstrong, 1986). Deprotonated thiolates are

Scheme 3: Production of Hydrogen Sulfide during Turnover of 6-Thio-Me-Gal (**5**)[a]



[a] The unstable thioaldehyde is hydrolyzed to the oxyaldehyde and hydrogen sulfide in the presence of water.

considerably more redox-active than the protonated species regardless of the substituent if any. For example, they would certainly be more capable of reducing the protein phenoxy radical ($E_{1/2}$ = 0.4 V vs NHE) (Hamilton et al., 1978; Johnson et al., 1985). The pK_a of hydrogen sulfide is 7.0 in phosphate buffers at 25 °C (Ellis & Golding, 1959), whereas the pK_a of ethanethiol is 10.6 and that of β-hydroxy ethanethiol 9.7 (Irving et al., 1964). At the experimental pH of 6.8, almost 50% of the hydrogen sulfide is deprotonated, whereas the alkyl thiols are nearly completely protonated. We suggest that deprotonated hydrogen sulfide is capable of carrying out redox chemistry that not only results in quenching of the phenoxy radical but may have a variety of other effects such as reduction of protein disulfides, binding of two hydrogen sulfide molecules to one copper center, or the removal of copper from the enzyme by precipitation of CuS. We have not investigated this point any further since it is not central to the issue at hand.

Hydrogen Sulfide May Be Produced during 6-Thio-Me-Gal Inactivation. If enzyme inactivation in the presence of thiols is a process unrelated to the particular thiol substituent, why then is the observed inactivation rate of 6-Thio-Me-Gal faster than that of benzyl mercaptan and ethanethiol by a factor of 7 (Table 2)? It is not fast enough to suggest a hydrogen atom abstraction step, i.e., not orders of magnitude faster as is observed with relative turnover rates of corresponding alcohols. It is possible to explain the increased inactivation rate by the production of the unstable thioaldehyde product during the thiol turnover reaction described below (Scheme 3). Thioaldehydes have extremely high reactivities associated with the poor overlap of the carbon 2p orbital and the sulfur 3p orbital (McGregor & Sherrington, 1993). Thiocarbonyl compounds degrade rapidly to aldehyde and hydrogen sulfide in aqueous solution (Duus, 1979; Campaigne, 1966). Since hydrogen sulfide itself inactivates the enzyme, the continuous production of yet another inactivator will result in an increased rate of activity loss so that the k_{obs} is a composite of k_{inact} for 6-Thio-Me-Gal and k_{inact} for hydrogen sulfide. In support of this hypothesis, we have observed that, at 1−3 μM 6-Thio-Me-Gal, inactivation rates are close to zero for the first 30 s and then increase with a concave slope, i.e., increased rate of inactivation as the reaction proceeds further toward completion (Figure 5). This phenomenon suggests that, as H_2S is produced by turnover, its solution concentration increases continuously until it is able to compete successfully for the active site and to contribute to the observed inactivation rate.

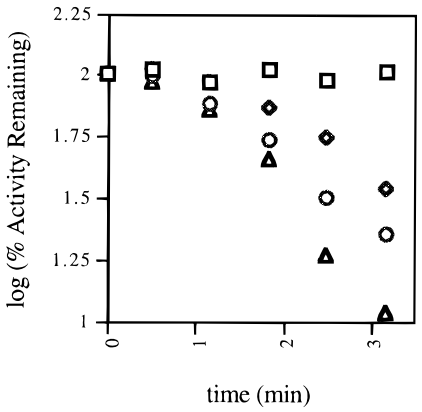
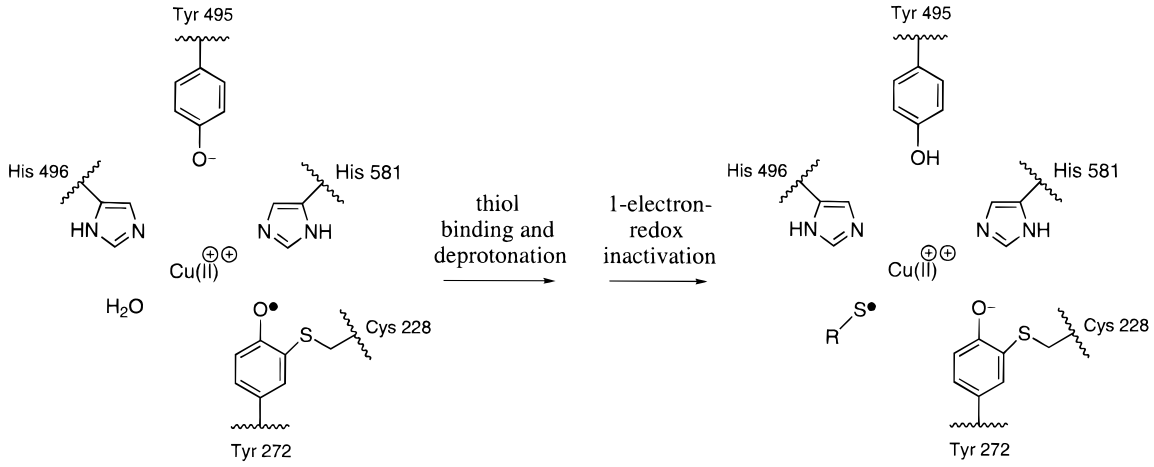


FIGURE 5: Inactivation of GOase in the presence of low concentrations of 6-Thio-Me-Gal. Concentrations are as follows: 0 μM = open squares, 1 μM = open diamonds, 2 μM = open circles, and 3 μM = open triangles. The increase in inactivation rates after an initial lag time is thought to be due to the production of hydrogen sulfide as a part of turnover of 6-Thio-Me-Gal.

Mechanism of Inactivation by Thiols. We have observed that bound thiol can be removed by dialysis, and the protein can be reactivated by the mild oxidizing agent ferricyanide. Our interpretation of these results is that thiols, after coordination to copper, reduce the protein phenoxy radical by effecting an electron transfer from sulfur to the oxy radical (Scheme 4). The resultant thiyl radical may then undergo redox disproportionation and oxidation reactions with solution components. For example, association with thiolates would produce the disulfide radical anion, a strong reductant that will transfer an electron to some other species such as molecular oxygen, resulting in the production of disulfides (Asmus, 1990). The proposed mechanism of enzyme inactivation by electron transfer from the thiolate to the tyrosyl radical is consistent with the observation that there is no significant substituent effect on thiol inactivation rates (Table 2).

GOase Turns Over 6-Thio-Me-Gal. We have estimated the turnover number of 6-Thio-Me-Gal to be 3.7 s^{-1} (±26%) under saturating conditions. This number was obtained by doubling the rate measured at the concentration equal to the K_D determined by competition experiments, since our assay is unreliable at higher thiol concentrations (see Materials and Methods; for 6-Thio-Me-Gal turnover data, see Supporting Information). Benzyl mercaptan and ethanethiol turnover could not be determined with our assay and are thought to proceed slowly if at all. The partition ratio k_{cat}/k_{inact} (Walsh, 1977) for 6-Thio-Me-Gal is estimated to be 43 if it is based on the observed 6-Thio-Me-Gal inactivation rate, i.e., 3.7 s^{-1}/0.086 s^{-1}. If the intrinsic inactivation rate by 6-Thio-Me-Gal was identical to the rate observed for ethanethiol inactivation, the partition ratio would be closer to 336, i.e., 3.7 s^{-1}/0.011 s^{-1}. We are unable to determine the intrinsic 6-Thio-Me-Gal inactivation rate since the observed inactivation rate of 6-Thio-Me-Gal appears to be a composite of inactivation by both 6-Thio-Me-Gal and hydrogen sulfide, with the hydrogen sulfide concentration continuously changing during the experiment. Partition ratios can also be estimated from turnover experiments by calculating the number of turnover events that have occurred at the time of complete enzyme inactivation. Using the polarographic assay, the partition ratio was estimated to be 200. This is consistent with an intrinsic inactivation rate of 0.01−0.02

Scheme 4: Phenoxy Radical of Tyr 272 Is Quenched upon Thiol Binding and Electron Transfer



Oxidized Active Form

1-Electron-Reduced Inactive Form with Bound Thiyl Radical

s^{-1} for 6-Thio-Me-Gal, very close to the rates measured for ethanethiol and benzyl mercaptan (Table 2). We infer that all thiols investigated inactivate at roughly the same intrinsic rate.

Michaelis Inactivation Constants (K_i) of Thiols Are Consistent with the Thermodynamic Dissociation Constants (K_D) and Do Not Vary Significantly. The thermodynamic dissociation constants determined by competition experiments (K_D), and the Michaelis−Menten-type inactivation constants determined by inactivation kinetics (K_i) are generally in close agreement with each other (Table 2). This is not surprising. Since inactivation rates are very slow, the k_{inact}/k_{on} term in $K_i = K_D + k_{inact}/k_{on}$ will be small so that $K_i \sim K_D$. The only exception is 6-Thio-Me-Gal, where the K_i is 3 times larger than the K_D. We explain this phenomenon by the simultaneous production of H_2S during kinetic experiments, which competes for binding to the active site and so increases the observed K_i.

The binding and inactivation constants for H_2S are 3−5-fold lower than for the other thiols. Due to its small size, hydrogen sulfide has few interactions with the protein active site, so why do we see increased affinity? Hydrogen sulfide has a low pK_a of 7.0 (Ellis & Golding, 1959), whereas the pK_a of the other thiols is roughly 10 based on data on ethanethiol (pK_a = 10.6) and β-hydroxyethanethiol (pK_a = 9.7; Irving et al., 1964). Since the experiment is carried out at pH 6.8, hydrogen sulfide is almost 50% deprotonated whereas all other thiols investigated are more fully protonated and less of the enzyme inhibitory thiolate anion is available to bind to the enzyme. Thiolate anions bind more tightly to copper than their protonated counterparts because of the increased acidity of coordinated acids (Houghton, 1979). We suggest that all four thiols bind to the active site with about the same affinity when effects due to competition and proton equilibria are excluded.

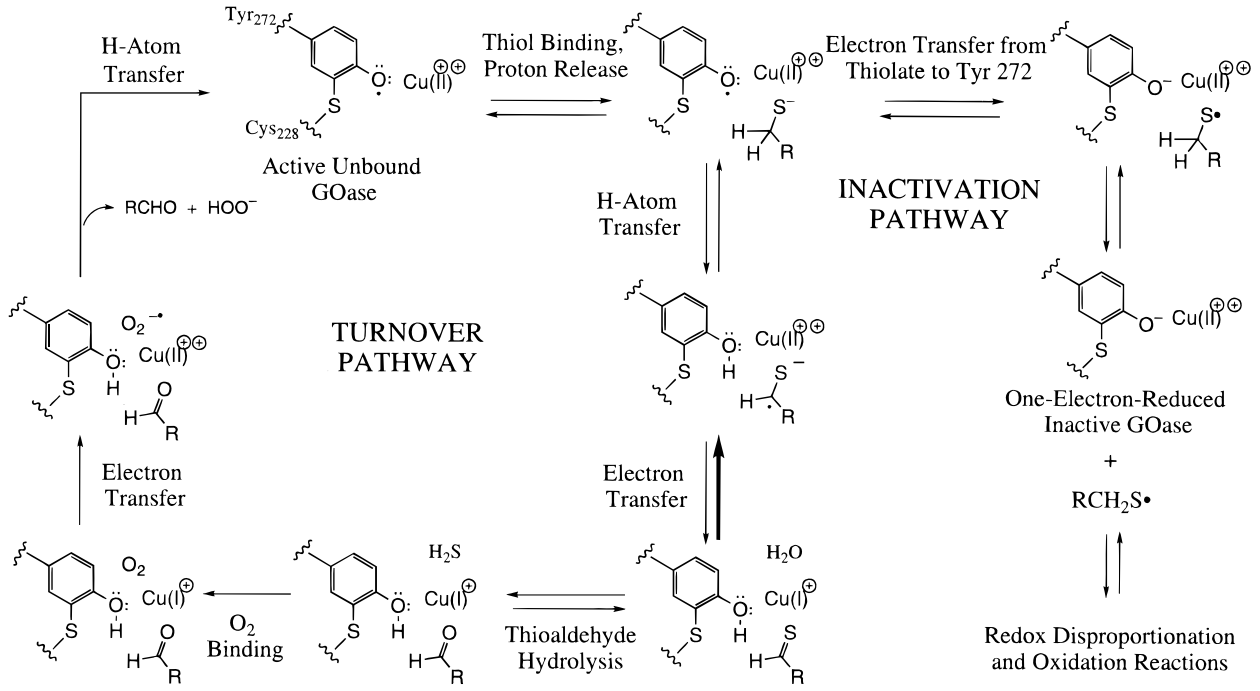
DISCUSSION

Thiols Can Be Classified as Active-Site-Directed Redox Inactivators of GOase. Thiols are specific in binding to the active site and coordinate directly to the copper center. 6-Thio-Me-Gal is turned over by the enzyme. Thiols are time-dependent inactivators of GOase, though during inactivation thiols do not proceed through the rate-limiting hydrogen atom abstraction step that occurs in alcohol oxidation. Since the inactivation pathway appears to branch away from the turnover pathway early in the catalytic cycle, the inactivation mechanism does not share the most essential steps of catalysis. We have postulated that the inactivation pathway involves an electron transfer from the substrate thiolate to the protein phenoxy radical, resulting in the formation of a thiyl radical (Scheme 4). Oxidation of a thiolate within the redox-active site of the enzyme is perhaps not entirely surprising. Since GOase contains both a protein-based radical and a metal center, it is ideally suited to carrying out redox chemistry. Thiyl radicals have recently been implicated as reaction intermediates in enzyme catalysis, specifically with protein radicals such as pyruvate formate-lyase (Parast et al., 1995b), some ribonucleotide reductases (Licht et al., 1996), and others (Mason & Rao, 1990). It has also been shown that various heme peroxidases can catalyze the direct enzymatic oxidation of thiols to the corresponding thiyl radical (Svensson et al., 1993).

From an energetic point of view, the proposed pathway of inactivation is entirely reasonable. There is precedent for the chemical oxidation of thiolates by phenoxy radicals (Surdhar & Armstrong, 1987). The standard redox potential of alkyl thiolates is 0.8 V (Surdhar & Armstrong, 1986), and that of phenols is 1.34 V vs NHE (Surdhar & Armstrong, 1987). Electron transfer from a thiolate to a phenoxy radical is therefore exothermic and very rapid in solution. Why, if a similar process is at work in the enzyme active site, is the enzyme inactivation rate so slow? The midpoint redox potential of a tyrosine in solution at pH 7 has been shown to be 1.05 V (DeFelippis et al., 1989), whereas the midpoint potential of the cross-linked protein Tyr 272 has been shown to be 0.4 V, also at pH 7 (Hamilton et al., 1978; Johnson et al., 1985). Since the potential of Tyr 272 is about 0.6 V lower, electron transfer from the thiolate to the Tyr 272 radical in the protein might be expected to be slightly endothermic, although binding of thiolates to the copper should perturb the GOase redox potential from the 0.4 V value for active GOase.

6-Thio-Me-Gal Is Turned Over by the Enzyme. 6-Thio-Me-Gal turnover is only 70−80-fold slower than turnover

Scheme 5: Summary of GOase Reaction with 6-Thio-Me-Gal



of the corresponding alcohol (3.7 vs 271 s^{-1}). We found this observation rather surprising since the expected thioaldehyde product should be a very unstable compound and should be of high energy due to poor π-orbital overlap between C and S (McGregor & Sherrington, 1993). We initially argued that rate-limiting H atom abstraction from a thiol substrate such as 6-Thio-Me-Gal would occur just as with alcohols, that the thioketyl radical anion intermediate would be stabilized by resonance with sulfur, and that electron transfer to copper to give the thioaldehyde product would be slow or absent. If electron transfer to the metal is indeed endothermic as predicted, the turnover reaction may be driven by the immediate degradation of the thioaldehyde to the stable oxyaldehyde in the presence of water (Duus, 1979; Campaigne, 1966). Thus, the oxidation of thiol to thioaldehyde may be a slightly endothermic equilibrium which is driven to completion by hydrolysis of the thioaldehyde. If the hydrolysis occurs at the active site, it would provide a means of directly delivering H_2S to the active site.

Alternatively, electron transfer from the thioketyl radical to Cu(II) may be exothermic, just less so than with alcohol substrates. We have not examined this point in detail since it was not the main focus of our study and since the assays at hand have proven to be inadequate for detailed quantitative thiol turnover studies. The proposed turnover and inactivation mechanisms of thiol interaction with the GOase active site are summarized in Scheme 5.

Transition State Stabilization Leads to Rate Enhancement. The outstanding characteristic of enzymes is that they specifically bind their substrates and that this binding energy may be used to lower the activation energy of the chemical steps (Fersht, 1985). It is catalytically advantageous for an enzyme to be complementary to the structure of the transition state of the substrate rather than to the structure of the ground state. This general concept was originally introduced by Haldane (1930) and implies that enzymes have evolved to bind the transition state more strongly than the substrates themselves, resulting in tremendous rate enhancement. A corollary of this idea implies that it is catalytically advantageous to bind substrates weakly (Fersht, 1985), thus avoiding the formation of a thermodynamic energy minimum of the enzyme−substrate complex.

We found that GOase is an enzyme that is particularly well-suited to study of these ideas since it accepts substrates with widely differing substituent groups. It has long been noted that the K_ms of known substrates all fall within the millimolar range. For example, we found that the K_m of the worst known substrate, ethanol, is only 19-fold larger than the K_m of one of the best known substrates, Me-Gal (Table 1). This indicates that, in the ground state, binding of Me-Gal is only slightly more favorable then binding of ethanol, though Me-Gal can form numerous hydrogen bonds in the active site and exhibits excellent shape and electrostatic complementarity (Wachter & Branchaud, 1996). On the other hand, transition state binding of Me-Gal is considerably more favorable than transition state binding of ethanol, as judged by a relative turnover rate 14 000-fold greater than that of ethanol. The data indicate that specific interactions in the transition state with a good substrate lead to tremendous rate enhancement.

Thiols Constitute a Particularly Good Example of Ground State Binding. In ground state binding, the individual energetic contributions change the overall binding energy very little (see above), consistent with the idea that enzymes have evolved to bind the ground state more weakly than the transition state. The ground state binding energy of thiol substrate analogs appears to be rather similar and independent of substituent group (Table 2). This is intriguing since 6-Thio-Me-Gal has the same hydrogen-bonding potential as galactose; on the other hand, benzyl mercaptan and ethanethiol have almost no hydrogen-bonding potential, and hydrogen sulfide does not even have shape or van der Waals interaction energies. Why do the binding energies of alcohols vary more than the binding energies of corresponding thiols? We explain this observation by the longer bond length of a sulfur atom vs that of an oxygen atom. The

copper−oxygen coordination distance upon binding of an alcohol may be equal to that of other equatorial oxy ligands in the crystal structure, 2.0−2.3 Å (Ito et al., 1994). When sulfur is substituted for oxygen, this bond may become longer since both short (2.3 Å) and long (2.9 Å) Cu−S distances occur in small-molecule metal complexes (Hathaway, 1987). A S−C bond is 0.4 Å longer than the corresponding O−C bond, resulting in a further displacement of 6-Thio-Me-Gal relative to galactose in the active site. A small amount of "sliding out" of the active site would be enough to weaken hydrogen-bonding interactions yet not enough to change the binding mode extensively. The overall fit of thiols in the active site should be roughly the same as that of alcohols.

Thiol analogs are particularly good ground state models as the direct coordination to copper is maintained, with the positioning of the analog in the active site similar to the positioning of the substrate, whereas other interactions are weakened, in particular the hydrogen bonds which are thought to lead to increased stabilization in the transition state (Wachter & Branchaud, 1996). The very strong Cu−S interaction results in tighter ground state binding by 2−3 orders of magnitude in relation to that of alcohols (Tables 1 and 2).

6-Thio-Me-Gal May Be Useful in Trapping the Michaelis Complex for Structural Studies. The enzyme−6-Thio-Me-Gal complex may be investigated for specific binding interactions and any enzyme structural changes upon sugar binding. We have postulated numerous hydrogen-bonding interactions between galactose and protein (Wachter & Branchaud, 1996). Whittaker has postulated that the axial Tyr 495 dissociates from the copper coordination sphere upon substrate binding (Whittaker & Whittaker, 1993). In a study aimed at investigation of the enzyme-substrate complex, inactive one-electron-reduced GOase could be employed to avoid turnover and anaerobic conditions to avoid enzyme reactivation in the presence of substrate and oxygen (Montague-Smith et al., unpublished experiments). Regardless of the specific technique used, the stronger binding of the sulfur analog, by 2.5 orders of magnitude, should be a great advantage. For example, 6-Thio-Me-Gal may be used successfully in cocrystallography, since the strong sulfur−copper coordination bond may allow for successful competition of the analog with other copper ligands present in the mother liquor, such as high concentrations of acetate (Ito et al., 1994).

Conclusions. We have characterized thiol compounds as active-site-directed redox inactivators of GOase. During inactivation, they do not partition through rate-limiting H atom abstraction of alcohol turnover, though the galactose analog 6-Thio-Me-Gal is also turned over by the enzyme. Thiols constitute excellent ground state models of the enzyme−substrate complex. Due to a large increase in active-site binding affinity, they may prove useful in trapping the Michaelis complex for biophysical and structural studies.

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SUPPORTING INFORMATION AVAILABLE

Spectral data for compounds **3**−**5** and turnover data for 6-Thio-Me-Gal (2 pages). Ordering information is given on any current masthead page.

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