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Dissecting an Enzyme—Model Compounds for the Galactose Oxidase Radical Site

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ABSTRACT: *The active site of the enzyme galactose oxidase (GOase) contains a square-pyramidal monocopper site, one of whose ligands is a tyrosinate sidechain that is oxidized to an unusually stable radical in the active enzyme. The structure of this non-innocent tyrosinate is unique in two ways. First, the tyrosine ring is crosslinked to a neighboring cysteine residue, affording an orthoalkylsulfanyl-substituted phenoxide ligand. Second, this assembly is protected by a π–π interaction to a tryptophan indole group. We describe here a series of compounds designed to model various aspects of the structure of this unusual cofactor. Our studies have shown that the thermodynamic stability of the GOase radical can be attributed almost exclusively to its thioether substituent, that the π–π interaction contributes little to this stability, and that the assignment of the optical spectrum of the GOase radical is more complex than had been previously suggested.* © 2002 Wiley Periodicals, Inc. Heteroatom Chem 13:494–500, 2002; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/hc.10091

INTRODUCTION

Galactose oxidase (GOase) is the prototype, and by far the most studied example, of a class of enzymes termed the "radical-copper oxidases" [1,2]. These are extracellular enzymes that are secreted by wood-rot fungi, which may form part of the machinery of lignin degradation by these organisms. The reaction catalyzed by GOase is the aerobic oxidation of primary alcohols with the concomitant generation of hydrogen peroxide (Eq. (1)).

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$$

A wide variety of alcohols can be oxidized by GOase, to the extent that it is uncertain whether or not galactose is the preferred substrate in vivo. It has been known for some time that GOase contains 1 mol equiv. copper, and that, remarkably, the as-isolated Cu(II) form of the enzyme is catalytically inactive but can be activated by one-electron oxidation with cyanoferrate(III). This behavior has been explained by resonance Raman experiments, which showed that activated GOase contains a tyrosyl radical [3]. The non-innocent tyrosine was identified when the crystal structure of GOase was published in 1991 [4], and showed a tetragonal $[Cu(His)_2(Tyr)_2(OH_2)]$ complex with a long, apical Cu—Tyr bond (Fig. 1). This apparently simple structure is in fact remarkable, in that the basal tyrosinate ligand has been chemically modified by an oxidative crosslinking to a cysteine residue, yielding an *ortho*alkylsulfanyl substituent at the coordinated phenoxide ring. The TyrCys group is protected by a π–π interaction to an indole ring from a neighboring tryptophan side-chain. It is this chemically modified tyrosinate residue that is oxidized to a radical in active GOase.

Treatment of GOase with cyanoferrate(III) results in oxidation of the TyrCys cofactor to a phenoxyl radical [4]. The oxidation potential of this

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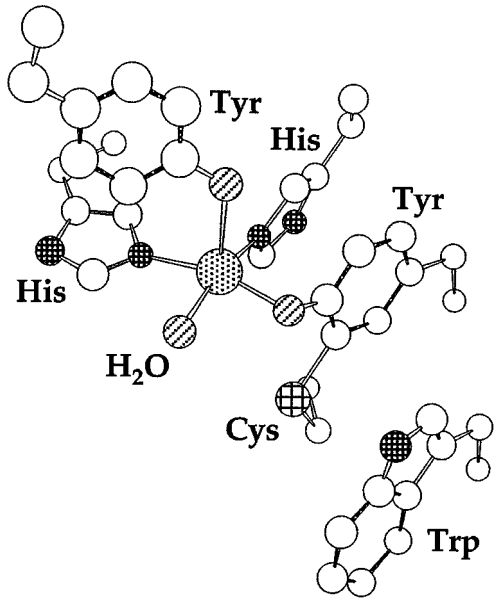


FIGURE 1 Molecular structure of the GOase copper center [4]. Amino acid abbreviations: Cys = cysteine, His = histidine, Tyr = tyrosine.

residue is ca. +0.45 V vs. *n*He, about half a volt less positive than for a "normal" tyrosine side-chain [5]. The resultant radical has a very unusual absorption spectrum, with an intense near-IR absorption between 600 and 1,200 nm, which has been suggested to arise from a Tyr → TyrCys· interligand charge-transfer (ILCT) process [6]. Once formed, the TyrCys· radical is very stable kinetically, with a half-life of up to a week under the correct conditions [6]. Alcohol oxidation by GOase proceeds by a free radical mechanism, for which abstraction of an H atom from the substrate by the TyrCys· radical appears to be rate-determining [7]. Thus, a mononuclear copper complex in GOase, which would normally be thought to be a one-electron acceptor, can effect a two-electron oxidation reaction. Although the radical-copper oxidases are something of a biochemical curiosity, the structure of the active site Cu complex with its modified phenoxide ligand is a challenge that has interested many people in the inorganic chemistry community. Hence, a large amount of model chemistry for the GOase active site has been published by others, as well as by us [8].

There have been two particularly important development that have come out of the synthetic GOase model chemistry. First has been the development of synthetic Cu(II)/phenoxyl radical chemistry, which did not exist before 1996. The first three of such compounds were published almost simultaneously by Tolman [9,10], Pierre [11], and Wieghardt [12], all of whom described sterically protected Cu(II)/phenoxyls generated in situ by oxidation of a phenoxide complex precursor. None of these early examples bore a thioether group at the phenoxyl center and, although they exhibited absorption spectra that were characteristic of phenoxyl radicals, these did not resemble the spectrum of the GOase radical very closely. This ambiguity was resolved by us [13,14] and by Itoh [15–17], who prepared Cu complexes of 2-alkylsulfanylphenoxyls that now showed an absorption spectrum similar to that of active GOase. Our system is described in more detail later. Finally, the first isolable example of a Cu(II)/phenoxyl has very recently been crystallized by Garner [18]. Almost all of these compounds exhibit antiferromagnetic superexchange between the Cu(II) and phenoxyl spins, although, Wieghardt has characterized two examples where this coupling is ferromagnetic [19]. This difference is related to the geometric orientation of the coordinated radical with respect to the $d_{x^2-y^2}$ magnetic orbital at Cu.

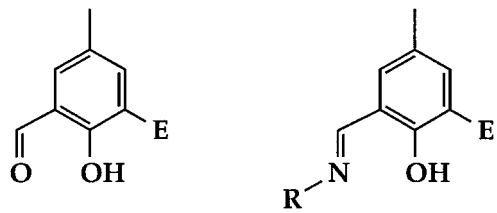
Secondly, genuinely biomimetic aerobic alcohol oxidation catalysts based on the GOase architecture have been prepared. That is, copper/phenoxyl complexes that catalyze the reaction depicted in Eq. (1) by a mechanism that is essentially identical to that adopted by the enzyme. The first of these to be published was an elegant series of molecules prepared by Stack, based on a heavily modified version of the classic "salen" type of Schiff base ligand [20,21]. Although catalytic activity was low (≤400 turnovers in MeCN solution after 20 h at 295 K), the intermediacy of Cu(II)/phenoxyl species in the catalytic cycle was proven by EXAFS measurements [21]. More efficient systems have come from Wieghardt's group, which has published three generations of GOase-mimetic catalysts based on derivatized bis(2-hydroxyphenyl)sulfide [22], bis(2-hydroxyphenyl)amide [23], and *N,N′*-bis(2-hydroxyphenyl)-1,2,-diaminobenzene [24] ligands. The latter catalysts oxidize ethanol through 4,500 turnovers after 45 h at 295 K in THF. A Cu/phenoxide electrocatalyst for Eq. (1) has also been communicated by Pierre [25], while stoichiometric alcohol oxidation by Cu(II)/phenoxyl species has been reported by Itoh [15,26].

We embarked on our own program of GOase model chemistry in early 1997, at the time when the first studies on Cu(II)/phenoxyl species were beginning to be published [9,11,12]. While this was clearly an important advance, we felt that these early reports did not address the question of how the structure of the GOase phenoxyl radical contributes to its unique chemistry. If these principles were understood, then they could be applied to the design of stable synthetic free radicals that might show useful reaction

chemistry or molecule-based magnetism. We therefore set out to determine how alkylsulfanylation, and/or the presence of a π–π interaction, would influence the properties of a phenoxyl or other aryl radical. We first addressed these two problems separately, in two series of compounds that we will now describe in turn.

Modeling the TyrCys Crosslink

As models for the 2-alkylsulfanylphenoxide motifs, we synthesized the salicylaldehyde derivatives HL^1-HL^3, and their Schiff bases HL^4R and HL^5R [14]. In order to cleanly obtain mononuclear five-coordinate complexes of these ligands, we selected tris-(3-phenylpyrazolyl)borate ($[Tp^{Ph}]^-$) to be the protecting group in our compounds, since a square-pyramidal $[CuL(Tp^{Ph})]$ (HL = a bidentate ligand) complex had been previously reported by others [27]. We were able to obtain $[CuL(Tp^{Ph})]$ ($HL = HL^1$, **1**; $HL = HL^2$, **2**; $HL = HL^3$, **3**; $HL = HL^4Me$, **4**; $HL = HL^4Ph$, **5**) in moderate yields by a one-pot complexation of $Cu(O_2CMe)_2 \cdot H_2O$ or $Cu(BF_4)_2 \cdot 6H_2O$ with KTp^{Ph} and the appropriate bidentate ligand. However, similar reactions of HL^5R led to the isolation of **2**, and/or of complexes arising from the degradation of the $[Tp^{Ph}]^-$ ligand [28]. The crystal structures of both **1** and **2** show near-regular square–pyramidal geometries, with the $[L^2]^-$ ligand in **2** coordinating through its phenoxido and carbonyl O-donors [13,14]. In contrast, **4** and **5** show much more twisted stereochemistries owing to steric repulsion between the $[L^4R]^-$ "R" substituent and an arm of the $[Tp^{Ph}]^-$ ligand [14]. This explains our inability to prepare $[Cu(L^5R)(Tp^{Ph})]$, since in **4** and **5** the H atom at the 3-position of $[L^4R]^-$ is pointing directly into a $[Tp^{Ph}]^-$ phenyl group. Accommodation of a methylsulfanyl substituent at that position, as in $[Cu(L^5R)(Tp^{Ph})]$, is therefore sterically impossible.



E =H, HL^1
E = SMe, HL^2
E = SeMe, HL^3

E =H, HL^{4}R
E = SMe, HL^{5}R
R = Me, Ph

1

Voltammetric studies of HL^1-HL^5R and of **1–5** in CH_2Cl_2/0.5 M $NBu^n_4PF_6$ showed a one-electron oxidation, which was chemically reversible for **2** but irreversible for all the other compounds. Comparison of the potential of this oxidation for HL^1 and HL^2, for HL^4R and HL^5R, and for **1** and **2**, showed that methylsulfanylation of a phenol ring reduces its oxidation potential by up to 0.55 V. This is a larger value than has been found by others [10,16,29,30], which can account entirely for the 0.5 V-stabilization of the GOase TyrCys$^{\cdot}$ radical compared to a "normal" tyrosyl. Interestingly, the ligand-based oxidation of **3** was irreversible under the conditions examined, which contrasted with the reversibility shown by **2**. This was rationalized using EHMO calculations, which showed that the Se atom in $L^{3\cdot}$ was less efficient at accepting unpaired spin-density from the phenoxyl ring than the S atom in $L^{2\cdot}$. Hence, the phenoxyl ligand in $\mathbf{3}^+$ should be more reactive towards coupling or recombination reactions than that in $\mathbf{2}^+$ [14]. Controlled potential electrolysis of **2** at a potential corresponding to the $\mathbf{2}/\mathbf{2}^+$ couple yielded an EPR-silent species, consistent with anti-ferromagnetic coupling of Cu(II) and $L^{2\cdot}$ spins in $\mathbf{2}^+$. Moreover, the absorption spectrum of $\mathbf{2}^+$ strongly resembled that of active GOase, with a broad, structured vis/NIR absorption centered near λ_{max} 850 nm (ϵ_{max} 1,200 M^{-1} cm^{-1}) (Fig. 2) [13]. This was the first time that the spectrum of GOase has been replicated in a synthetic species.

Modeling the TyrTrp π–π Interaction

Concurrently with the above work we prepared L^6, which is a bidentate ligand containing an

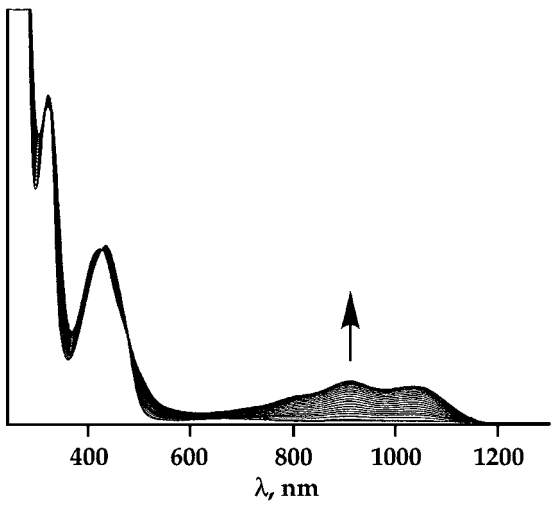
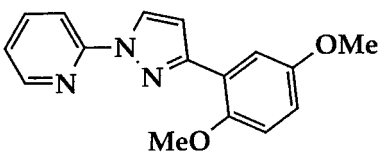


FIGURE 2 Conversion of **2** to $\mathbf{2}^+$ by controlled potential electrolysis in CH_2Cl_2/0.5 M $NBu^n_4PF_6$ at 243 K.

oxidisable dimethoxyphenyl substituent [31,32]. The complex $[Cu(L^6)(Tp^{Ph})]BF_4$ (**6.BF$_4$**) contains a square-pyramidal Cu center, in which the L^6 dimethoxyphenyl group takes part in a π–π interaction with a phenyl ring from the $[Tp^{Ph}]^-$ coligand (Fig. 3) [31]. Although this feature cannot be probed by NMR spectroscopy, a combination of UV–vis, EPR, and ^{1}H NMR measurements did establish that the coordination geometry at Cu in **6.BF$_4$** in CH_2Cl_2 solution is the same as in the solid state. Hence, the π–π stacking to L^6 is also probably still present in solution. Cyclic voltammetry of **6.BF$_4$** in the same solvent showed a fully reversible L^6-based oxidation, which contrasted with the more usual irreversible oxidation shown by uncomplexed L^6, and by $[Cu(L^6)_2](BF_4)_2$, under the same conditions. This led us to suggest that the π–π stack in **6.BF$_4$** may lead to a kinetic stabilization of the coordinated $[L^{6\bullet}]^+$ radical cation [31]. However, the complex $[Cu(L^6)(Tp^{Cy})]BF_4$ ($[Tp^{Cy}]^-$ = tris(3-cyclohexylpyrazolyl)borate, **7.BF$_4$**), which lacks a π–π interaction to L^6 but is otherwise identical to **6.BF$_4$**, also exhibits a reversible $L^6/[L^{6\bullet}]^+$ couple at a potential ca. 0.1 V more positive than that of **6.BF$_4$** [33]. Deconvolution of the voltammograms showed that the half-lives of $\mathbf{6}^{2+}$ and $\mathbf{7}^{2+}$ at 295 K were identical to each other within experimental error. Hence, the π–π interaction in **6.BF$_4$** has no bearing on the stability of the $[L^{6\bullet}]^+$ radical, beyond affording it some steric protection.



L^6

Controlled potential electrolysis of **6.BF$_4$** at a potential corresponding to the $\mathbf{6}^+/\mathbf{6}^{2+}$ couple proceeds isosbestically. The absorption spectrum of $\mathbf{6}^{2+}$ contains UV peaks that closely resemble those shown by $[C_6H_4(OMe)_2\text{-}1,4^{\cdot}]^+$, together with a near-IR peak at λ_{max} 805 nm (ϵ_{max} 1,000 M^{-1} cm^{-1}) [33]. The latter can be assigned to a $[L^{6\bullet}]^+ \rightarrow [Tp^{Ph}]^-$ ILCT absorption, and provides conclusive proof that the π–π interaction in **6.BF$_4$** is also present in $\mathbf{6}^{2+}$. This product is a highly unusual example of a monocyclic aryl radical cation, which is stable enough to characterize by standard solution methods.

As an aid to the spectroscopic characterization of the $[L^{6\bullet}]^+/[Tp^{Ph}]^-$ moiety, we prepared the analogue

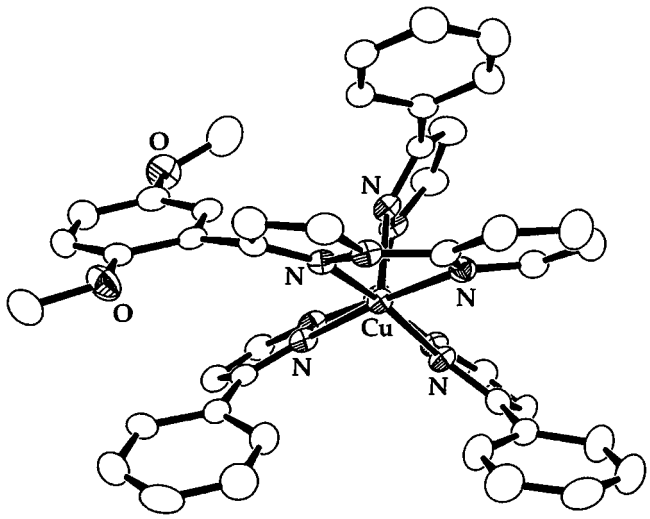
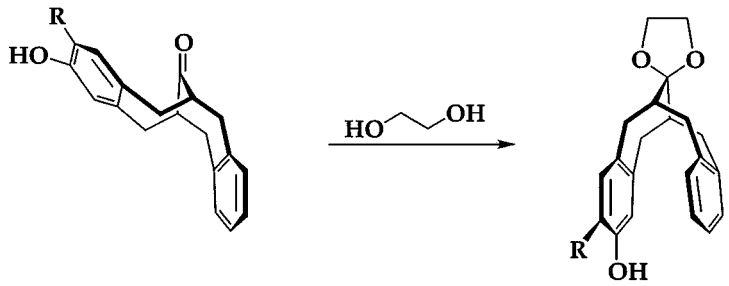


FIGURE 3 Crystal structure of the complex cation in **6.BF$_4$**.^{1}/2H_2O, emphasising the intramolecular π–π interaction [31]. All H atoms have been omitted for clarity. Thermal ellipsoids are at the 50% probability level.

$[Zn(L^6)(Tp^{Ph})]BF_4$ (**8.BF$_4$**), containing the spectroscopically inert metal ion Zn(II) [34]. Unfortunately, the Zn ion in **8.BF$_4$** has a more trigonal structure, which twists the L^6 ligand away from the overlying $[Tp^{Ph}]^-$ phenyl group. Whether for this reason, or because Zn(II) is a more labile metal center than Cu(II) in this ligand environment, the $\mathbf{8}^+/\mathbf{8}^{2+}$ oxidation is irreversible under our conditions.

Modeling the Complete GOase Radical Cofactor

To be certain that the conclusions from our two systems could be generalized, we combined the two unusual structural features of the GOase copper complex into a single molecule. Since alkylsulfanylation of a phenol is not difficult synthetically, the main challenge was to engineer a π–π interaction into our compounds. We initially studied systems containing two arene moieties tethered by three- or four-atom linkers, but soon found that these were too conformationally flexible to force the arene rings to stack upon each other [35]. Therefore, we next approached the bicyclic [3.3]*ortho*cyclophanes in Fig. 4, whose synthesis was pioneered by Mataka [36]. The parent ketones adopt fluxional boat, chair conformations, in which the annelated benzo groups are far apart (Fig. 4). However, acetalation of the carbonyl group causes a change to a rigid twin-chair conformation, in which the benzo rings are stacked above each other. Hence, by preparing and comparing the compounds in Fig. 4, we could dissect away the different structural elements of the GOase radical site in turn.



Boat:chair **Twin-chair**

9
R = H: no thioether group, no π-π stack

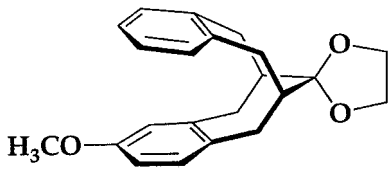
11
R = H: π-π stack but no thioether group

10
R = SMe: thioether group but no π-π stack

12
R = SMe: thioether group and π-π stack

FIGURE 4 Conformational switching in [3.3]*ortho*cyclophanes [36,37].

We achieved the synthesis of **9–13** following the Mataka methodology [37]. The structures of these compounds in solution and (for **9, 10**, and **13**) in the solid state are as shown in Fig. 4 (Fig. 5) [37,38]. Intriguingly, the cyclic voltamogram of **12** shows a quasi reversible voltammetric oxidation, in this case to a phenoxonium radical cation; oxidation of **9–11** occurs irreversibly [37]. However, all the Cu(II) complexes of **9–12** we have made have been highly labile in solution and the solid state, and/or have been unstable to an internal redox decomposition reaction [39]. This has precluded their forming stable oxidation products. We are presently preparing chelating derivatives of **9–12**, in order to overcome this problem.



13

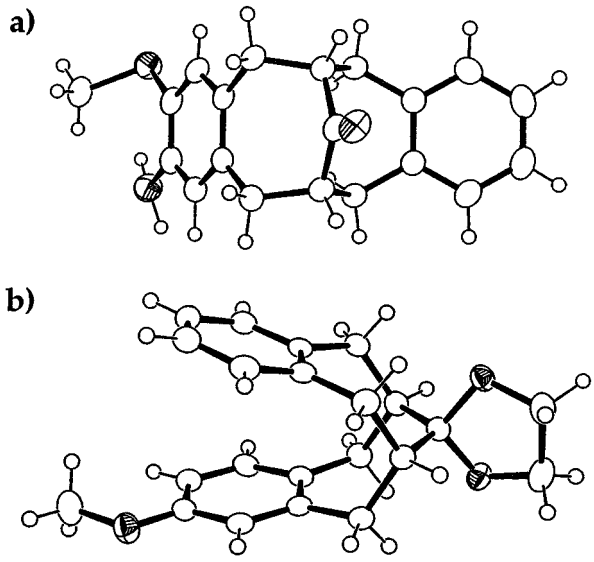


FIGURE 5 Single crystal X-ray structures of: (a) **10** and (b) **13** [37,38]. The O—H proton in **10** is disordered over two orientations. Thermal ellipsoids are at the 50% probability level.

CONCLUSIONS AND OUTLOOK

Our results to date have shown that most, if not all, of the unusual thermodynamic stability of the TyrCys· radical in GOase can be attributed to the thioether substituent at the phenoxide ring. The π–π interaction to this group, which is also present, probably serves only to protect the radical from attack by exogenous solvent. The relative properties of $\mathbf{2}^{+}$ and $\mathbf{3}^{+}$ suggest that if the crosslinking cysteine residue were mutated to selenocysteine, affording an *ortho*(alkylselenyl)tyrosyl cofactor, then the resultant mutant enzyme should form a much less stable active site radical. The absorption spectra of both $\mathbf{2}^{+}$ and $\mathbf{6}^{2+}$ contain strong near-IR peaks. This strongly implies that the vis/NIR absorption of GOase must contain local excitations from the TyrCys· radical and/or Cu → TyrCys· metal-ligand charge transfer (MLCT)

band(s), as well as a Trp → TyrCys· ILCT peak, in addition to the Tyr → TyrCys· ILCT that had been proposed previously [6].

Through this work and that by others [8], the properties of the TyrCys· radical are now quite well understood. However, this is only one of a series of oxidatively modified amino acids that have been discovered, mostly in copper proteins [40] although the first example from an iron enzyme has appeared very recently [41]. Most of these modified amino acid residues appear to have a redox function, which in some cases is still poorly defined. Attention is now also turning to elucidation of how these unusual amino acids are formed in vivo. In GOase, biosynthesis of the TyrCys cofactor is a self-processing reaction involving both Cu and O_2 [42], which involves substantial changes in the structure of the active site [43]. Both biochemistry and model chemistry will have a role in determining how nature has evolved a way to couple a nucleophilic phenol with a nucleophilic thiol under aerobic ambient conditions.

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