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Minireview

The radical chemistry of galactose oxidase

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Abstract

Galactose oxidase is a free radical metalloenzyme containing a novel metalloradical complex, comprised of a protein radical coordinated to a copper ion in the active site. The unusually stable protein radical is formed from the redox-active side chain of a cross-linked tyrosine residue (Tyr–Cys). Biochemical studies on galactose oxidase have revealed a new class of oxidation mechanisms based on this free radical coupled-copper catalytic motif, defining an emerging family of enzymes, the radical–copper oxidases. Isotope kinetics and substrate reaction profiling have provided insight into the elementary steps of substrate oxidation in these enzymes, complementing structural studies on their active site. Galactose oxidase is remarkable in the extent to which free radicals are involved in all aspects of the enzyme function: serving as a key feature of the active site structure, defining the characteristic reactivity of the complex, and directing the biogenesis of the Tyr–Cys cofactor during protein maturation.

Keywords: Free radical; Metalloradical; Atom transfer; Oxidation–reduction; Copper; Biogenesis; Cofactor; Tyrosyl–cysteine; Oxidase, Self-processing

Free radicals have emerged as a fundamental feature of biochemical catalysis [1–7], associated with enzymes that have evolved strategies to take advantage of radical chemistry in bond activation and molecular rearrangements. The unusual chemical reactivity of free radicals can be traced to the presence of unpaired electrons in their electronic valence shell, making them in some ways the organic analog of transition metal ions [8,9]. Radicals are known to play important roles in biology, and although historically the initial focus was on their deleterious effects, there is now abundant evidence that radicals are involved in many essential life processes including DNA replication, respiration and photosynthesis. Free radicals have been recognized as key elements in the mechanisms of a wide range of enzymes, including ribonucleotide reductase [10–12], lysine-2,3-aminomutase [13,14], pyruvate-formate lyase [15], biotin synthase [16], prostaglandin H synthase [17], cytochrome *c* peroxidase [18], DNA photolyase [19], lipoyl synthase [20], and diol dehydrase [21], among others. Galactose oxidase [22–24], a fungal secretory enzyme widely used in bioanalytical and histological applications, is one of the best-characterized of these free radical enzymes.

The overall reaction catalyzed by galactose oxidase is the oxidation of a primary alcohol to the corresponding aldehyde, coupled to the reduction of dioxygen to hydrogen peroxide [25], the biologically important product:

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \quad (1)$$

Since both alcohol oxidation and O_2 reduction are two-electron processes, the catalytic reaction is conceptually equivalent to a transfer of the elements of dihydrogen between the two substrates. Biological hydrogen transfer generally involves specialized organic redox cofactors (for example, flavins, nicotinamide,

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quinones), with well-characterized reaction mechanisms. Galactose oxidase does not contain any of these conventional redox cofactors, and instead utilizes a very different type of active site, a free radical-coupled copper complex, to perform this chemistry [26]. The new type of active site structure implies that the reaction follows a novel biochemical redox mechanism based on free radicals and the two-electron reactivity of the metalloradical complex.

X-ray crystallographic studies on galactose oxidase have revealed the structural basis for the unusual reactivity of galactose oxidase: the protein contains a novel post-translational covalent modification, a cross-link between tyrosine and cysteine side chains, forming a tyrosyl–cysteine (Tyr–Cys)[1] dimeric amino acid [27]. Spectroscopic and biochemical studies have demonstrated that this Tyr–Cys site is the redox-active site in the protein, forming a stable free radical upon mild oxidation [28–30]. The structural data available from crystallography is complemented by extensive kinetic and spectroscopic studies [31–35], making possible a detailed structure-based analysis of the enzyme mechanism.

Active site structure

The active site of galactose oxidase is a shallow, exposed copper complex, in which the metal is bound by four amino acid side chains: two tyrosines (Tyr272 and Tyr495) and two histidines (His496 and His581) (Fig. 1) [27]. As indicated above, one of the two tyrosines (Tyr272) has been found crystallographically to be cross-linked at the Cε carbon of the phenolic side chain to the Sγ sulfur of Cys228, forming tyrosyl–cysteine (Tyr–Cys) (Scheme 1). The thioether bond that links the two residues affects both the structure and reactivity of the protein. Structurally, the cross-link contributes to the rigidity of the active site, similar to the effect a disulfide bond would have on the protein. However, unlike a disulfide bond, the thioether bond is formed irreversibly and is not susceptible to reductive cleavage. The cross-link forms spontaneously in the protein in the presence of reduced copper (Cu^{1+}) and dioxygen (vide infra) [37].

The presence of a thioether substituent on the aromatic ring system of the Tyr–Cys cross-link site also alters the chemical reactivity of the side chain, making it easier to oxidize. Studies on model compounds have provided valuable insight into the properties of the Tyr–Cys cofactor [38–40]. Photoelectron spectroscopy of model compounds indicates that the one-electron oxidized state of the thioether-substituted phenol is stabilized nearly 0.5 V relative to the corresponding unsubstituted phenol, based on differences in ionization thresholds for the molecules in vacuo [39,40]. Electronic structure calculations on Tyr and Tyr–Cys species also support a substantial stabilization of the one-electron oxidized (phenoxyl) form in the cross-linked structure [41]. The altered reactivity of thioether-substituted tyrosine is, in some ways, reminiscent of the behavior of quino-cofactors [42], a family of protein post-translational oxidative modifications that transform tyrosine and tryptophan side chains into analogs of *ortho*- or *para*-quinone. However, quino-cofactors typically exhibit two-electron reactivity and characteristically form nucleophilic adducts with substrates during turnover. In contrast, the thioether substitution of the Tyr–Cys group restricts it to one-electron oxidation processes, resulting in stabilization of a protein free radical in the active site of galactose oxidase, and the Tyr–Cys ring-system does not typically undergo nucleophilic addition.

Another distinction between the Tyr–Cys in galactose oxidase and the quino-cofactors is the involvement of the former in direct metal interactions. In galactose oxidase, the Tyr–Cys is a ligand to the active site metal ion (a redox-active copper center), forming a metalloradical complex in the oxidized, active form of the enzyme (Scheme 2A). This free radical-coupled copper complex is extremely stable, and in the absence of reducing

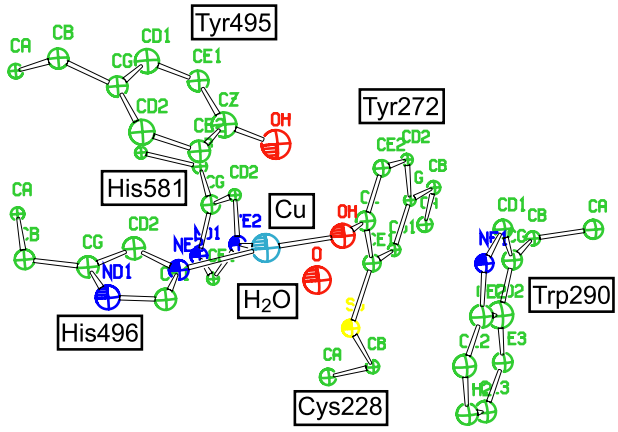
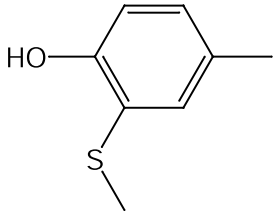
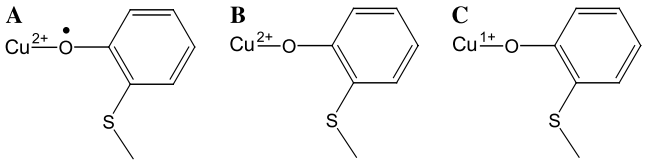


Fig. 1. The active site of galactose oxidase. The active site metal ion (Cu), inner coordination sphere (Tyr272, Tyr495, His496, His581, and coordinated solvent) and Trp 290 in the outer sphere of the complex. Based on PDB ID 1gog. Rendered using ORTEP-III [36].



Scheme 1.

[1] *Abbreviations used:* Tyr–Cys, tyrosyl–cysteine; SKIE, solvent deuterium kinetic isotope effect; SET, single-electron transfer; HAT, hydrogen atom transfer; PCET, proton-coupled electron transfer.



Scheme 2.

agents has been shown to persist for weeks at room temperature [34]. However, it reacts readily with a variety of electron donors, undergoing single-electron reduction to form a catalytically inactive non-radical–Cu^{2+} complex (Scheme 2B) [26]. Further reduction converts the Cu^{2+} center to Cu^{1+} (Scheme 2C), forming a fully reduced complex that is competent for reaction with O_2 and represents a catalytic intermediate in the turnover cycle (vide infra) [26].

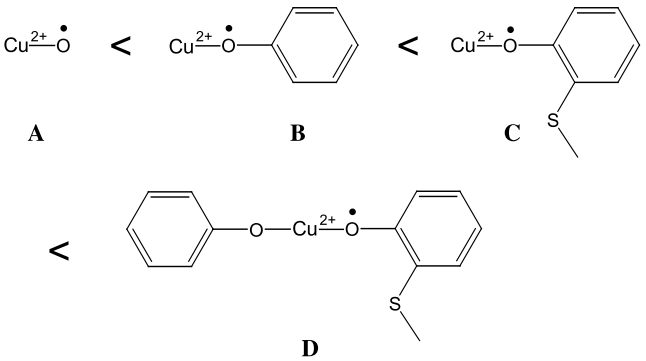
The coordination environment of the metal center is altered during redox and ligation changes in the active site. X-ray crystallographic data on galactose oxidase is restricted to states that are stable in the presence of crystallization reagents, effectively limiting structural data to the non-radical, inactive form (Scheme 2B). The metal environment in this form may be described as a distorted trigonal bipyramidal coordination polyhedron, with the long axis (the direction of weakest metal–ligand interaction) aligned with the coordinated solvent molecule. Acetate and azide ions displace the water and coordinate directly to the metal center [24], mimicking the ligand exchange reaction that has been proposed to occur during turnover, when primary alcohols bind to the metal ion in place of water [26]. The anionic ligands (acetate, azide) interact more strongly with the metal cation than neutral water molecule, and the increased bond strength is reflected in shorter bond distances to the exogenous ligands in crystal structures for the anion complexes. This increase in copper–exogenous ligand interaction occurs at the expense of the Cu–Tyr495 interactions, reflected in a stretch in the Cu–Tyr495 bond distance in the anion adducts, effectively lowering the coordination number of the metal ion to four in these complexes [43]. The reorganization of the inner sphere of the metal complex in response to changes in bonding strength is a characteristic of the plasticity of copper coordination chemistry [44].

Displacement of Tyr495 in the anion complexes is expected to affect the basicity of the phenolic side chain. Coordinated phenols are relatively acidic, exhibiting perturbed pK_as that are reflected in the higher metal binding affinity of the phenolate. As the metal interactions are decreased the basicity is expected to return to a more typical value ($pK_a = 10$). Studies on reductively inactivated galactose oxidase (Scheme 2B) have demonstrated that anion binding is coupled to uptake of a single proton by a group in the protein with a $pK_a > 9$, consistent with Tyr495 functioning as a general base in this context [45]. Mutagenesis experiments in which Tyr495 is replaced by Phe lead to a loss of this proton uptake behavior, supporting identification of Tyr495 as the site of proton uptake [46–48].

Reduction of the metal ion [forming the fully reduced state of the enzyme, (Scheme 2C)] leads to further changes in the active site structure. Although no X-ray crystallographic data is presently available for this form, X-ray absorption studies have shown that the metal ion becomes three-coordinate on reduction, with T-shaped coordination geometry, indicating that the Cu^{1+} complex most likely retains a planar (His_2,Tyr) ligand set involving His496, His581 and Tyr272 [49]. Three-coordinate structures are very favorable for Cu^{1+} [44], and the decrease in interaction with the exogenous solvent and Tyr495 in the reduced complex is likely to be mechanistically significant.

The active enzyme, comprising the Tyr–Cys free radical complexed to the Cu^{2+} metal ion, is too reactive for X-ray crystallographic analysis (due to sensitivity to reduction by precipitants, cryo-stabilizers, or photoelectrons generated by X-ray beam) although on the basis of X-ray absorption spectroscopy its structure is expected to be very similar to that reported for the inactive form [50]. What kind of chemistry might be expected of this complex? What aspects of its structure are likely to be important? Free radical–metal interactions are rare in biochemistry, so there is no real reference point to answer these questions. We can, however, view the active site structure in the context of related molecules for insight into its structure and reactivity.

The core of the catalytic complex may be idealized as a simple inorganic species, a cuproxyl complex (Scheme 3A) in which the metal center is associated with a monoatomic oxygen free radical. This is known to be an extremely "hot" oxidant ($E > 1$ V) that is involved in copper-based Fenton chemistry and is capable of oxidizing unactivated protein side chains. Substitution with a phenolic ring (Scheme 3B) provides a mechanism for electronic delocalization, stabilizing the free radical complex. Although there are no known biological complexes with precisely this structure, re-



Scheme 3.

lated inorganic complexes have been synthesized and characterized, demonstrating the degree of stabilization afforded by the phenolic ring system. Thioether substitution of the phenoxyl ligand (Scheme 3C), corresponding to the Tyr–Cys site in galactose oxidase, confers additional stability to the free radical complex.

Coordination by a second phenolic group (Scheme 3D) will further stabilize the metalloradical complex, accounting for the unusually low redox potential of the free radical site in galactose oxidase ($E_m = 0.45$ V) [26,31]. Stabilization in the *bis*-phenol complex has been attributed to the extended electronic delocalization that can occur via ligand-to-ligand charge transfer [51], allowing the unpaired electron to be delocalized over two (nearly) identical ligands. The superposition of two limiting resonance forms (Scheme 4) illustrates the delocalization pathway, mediated by covalent interactions with the metal ion. The situation is exactly analogous to resonance stabilization that occurs in mixed valent bridged binuclear metal complexes, but inverted so that the metal ion serves the role of the bridge in this case. The geometry of the metalloradical complex in galactose oxidase is very favorable for the electronic interactions required for resonance stabilization. In particular, the orientation of Tyr272 maximizes overlap between the redox orbital on the phenoxyl oxygen and the half-occupied d-orbital on Cu, reflected in a strong antiferromagnetic exchange splitting in the electronic ground state of the complex ($J > 200$ cm^{-1}) [43,52,53].

This analysis of the active site structure underlines the essential role of individual metal ligands in the reactivity of the complex, each serving a distinct function as redox cofactor (Tyr–Cys), ligand exchange site (H_2O) or a general base (Tyr495). However, interactions between ligands and with the extended outer sphere environment are clearly also important. Electronic coupling between ligands (Tyr–Cys–Cu; Tyr495 Cu) may play a role in tuning the reactivity of the site, and the outer sphere tryptophan residue (W290) (Fig. 1) appears to have a role in stabilizing the free radical complex, perhaps by shielding the Tyr–Cys from solvent.

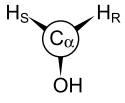
Reaction stereochemistry

The stereochemistry of substrate oxidation by galactose oxidase has been investigated using partially resolved mixtures of (*R*)- and (*S*)-[6^{2}H$_1$] monodeuterated galactose, in which either the pro-*R* or pro-*S* hydrogen of the α-methylene group in the substrate has been replaced by deuterium (Scheme 5). Analysis of the aldehyde product indicates a pro-*S* stereoselectivity for alcohol oxidation [54], although the degree of stereospecificity and the generality of the result for other substrates has not been established.

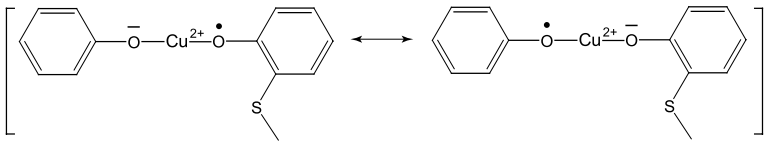
Inspection of the galactose oxidase active site provides some insight into the enzymatic stereoselectivity (Fig. 2). With the Tyr272 phenoxyl oxygen serving as a hydrogen acceptor (vide infra), stereoselective atom abstraction will depend on the relative proximity of the two α-hydrogens to the phenoxyl oxygen. The orientation of the C_α–H bond vectors is determined by the $\angle$Cu–O–C_α–C_β dihedral angle, (ω) (Scheme 6), which in turn depends on the position of C_β of the substrate. Access to the active site is constrained by several surface residues (Phe194, Phe464, P463, R330, and W290) that define a solvent channel opening asymmetrically above the metal complex. Computer modeling indicates that the steric bulk of these residues restricts the orientation of a metal-coordinated galactose as shown in Scheme 6 and Fig. 3, with the pro-*S* hydrogen projected towards the Tyr–Cys redox cofactor ($\omega = 100°$). The stereospecificity of galactose oxidase can thus be understood in terms of basic features of the active site structure.

Kinetic analysis

Kinetic studies of galactose oxidase are complicated by the existence of three distinct oxidation states of the enzyme (Schemes 2A–C) and the possibility of reversible activation/inactivation by interconversion between these states [26]. In practice, it is generally possible to avoid this complication by using freshly prepared, fully active enzyme, or by including oxidant in the reaction mixture to reactivate any enzyme inactivated during turnover [31]. For example, when the enzymatic reaction is monitored by oxygen uptake, ferricyanide is routinely included in the reaction mixture. At the concentration of oxidant used in the assay ($K_3Fe(CN)_6$, 1 mM) the rate of



Scheme 5.



Scheme 4.

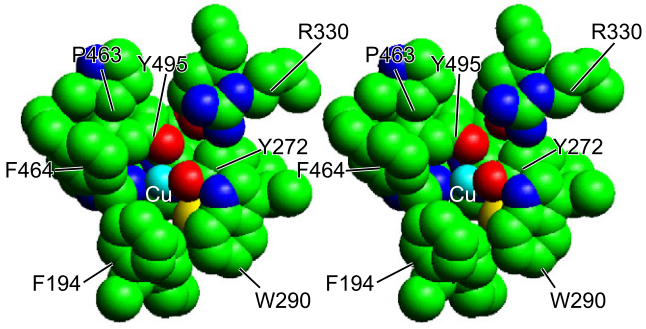
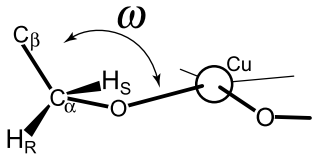


Fig. 2. Active site access at the surface of galactose oxidase. Space-filling stereoview of the solvent channel opening above the surface-exposed catalytic complex. Key residues in the extended outer sphere of the complex are identified. Heteroatoms are indicated by shading. Rendered using InsightII.



Scheme 6.

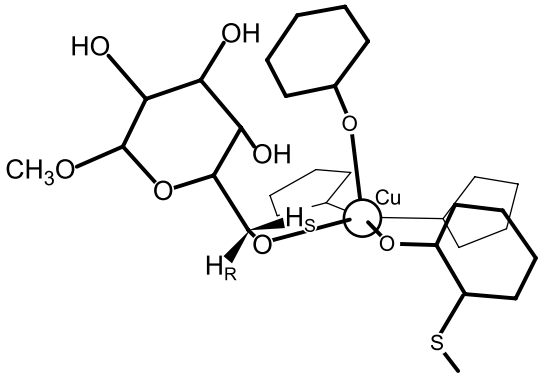
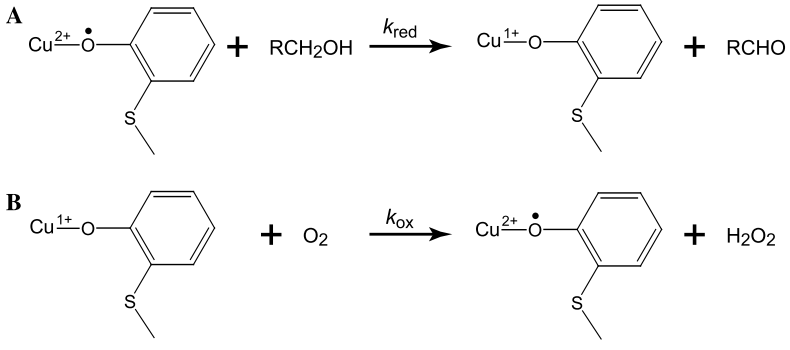


Fig. 3. Model for substrate complex with galactose oxidase. The orientation of the substrate is constrained by steric interference with Phe194, Trp290, Phe464, and Arg330. Prochiral hydrogens of the substrate α-methylene group are labeled.

reaction of fully reduced enzyme with dioxygen is $10^4\times$ faster than the rate of reaction with ferricyanide, ensuring that the its presence does not significantly affect the observed reaction rate [31]. Because ferricyanide activates the enzyme, these reaction conditions are not suitable for determining the activation state of the enzyme. For that purpose, the direct assay (oxidation of 3-methoxy benzyl alcohol in the absence of co-oxidant) is more appropriate [55]. Once formed, each of these states appears to be stable in the presence of the others [26,34,37], and auto-redox behavior reported for galactose oxidase [56] is likely to be an artifact of the enzyme reacting with stoichiometric impurities in the samples.

The overall catalytic reaction (Eq. (1)) may be written as separate reduction and reoxidation steps, consistent with a ping–pong mechanism [26]. In the first (reductive) half-reaction, the oxidized free radical–Cu^{2+} complex reacts with the primary alcohol (with rate constant k_{red}) to form two-electron reduced enzyme complex and the aldehyde product (Scheme 7A). In the second (oxidative) half-reaction, the reduced enzyme reacts with dioxygen (with rate constant k_{ox}), converting the active site to the metalloradical complex and forming hydrogen peroxide (Scheme 7B). The observation that the enzyme can be prepared in a stable, fully reduced form (Scheme 2C) that is competent for reaction with dioxygen supports this picture of independent reductive and oxidative turnover reactions, defined by distinct rate constants, k_{red} and k_{ox} (Scheme 7) [26]. The rate constants k_{red} and k_{ox} have been reported for reaction of the enzyme with both reducing substrates and dioxygen for a wide range of substrates [33]. For substrates exhibiting a relatively wide range of substrate oxidation rate constants ($k_{red} = 0.8\text{–}2.7\times10^4\,M^{-1}\,s^{-1}$), the reoxidation rate is remarkably constant ($k_{ox} = 0.98\text{–}1.02\times10^7\,M^{-1}\,s^{-1}$), which provides additional evidence for a ping–pong turnover mechanism [34].

Steady-state kinetics experiments have provided important information on catalytic constants for the reaction: K_m(galactose) = 175 mM at saturating O_2 concentrations; $K_m(O_2) > 3$ mM [32]. The high K_m for the



Scheme 7.

reducing substrate may reflect an enzymatic strategy for broadened substrate specificity at the expense of binding affinity, as a result of limited interactions between the protein and the substrate alcohol in the Michaelis complex; a coupling between substrate binding and energetics of active site reorganization that masks the true binding affinity; or high affinity binding ($K_D = k_2/k_1$) may be expressed in a large K_m value ($K_m = (k_2 + k_3)/k_1$) as a result of a relatively high commitment to catalysis (Eq. (2)).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} EP \tag{2}$$

In Eq. (2) k_1 and k_2 represent the on and off rate constants for substrate binding, respectively, and k_3 defines the commitment to the catalytic reaction.

Rapid reaction techniques have been used to investigate the reductive half-reaction (Scheme 7A) [34]. Under anaerobic conditions, the reaction between the active enzyme and galactose can be monitored as a decrease in the intense optical absorption of the enzyme on reduction of the metalloradical complex. Since the reduced enzyme reacts rapidly with any O_2 present, dioxygen must be rigorously excluded to ensure that the kinetics accurately reflect the substrate interactions and are not affected by turnover. This problem is especially challenging in the case of galactose oxidase, which is readily inactivated by the usual oxygen scrubbing agents, and requires that alternative approaches [57] be used. At low substrate concentrations (<10 mM galactose) the reaction is found to be essentially bimolecular, with an effective second order rate constant of $1.58 \times 10^4\ M^{-1}\ s^{-1}$ [34]. At higher substrate concentration, the rate begins to exhibit saturation effects consistent with a $K_m = 180$ mM, compared to K_m(galactose) = 175 mM found in the steady-state reaction. The absorption transient is a simple exponential decay with no evidence for intermediate states of the enzyme.

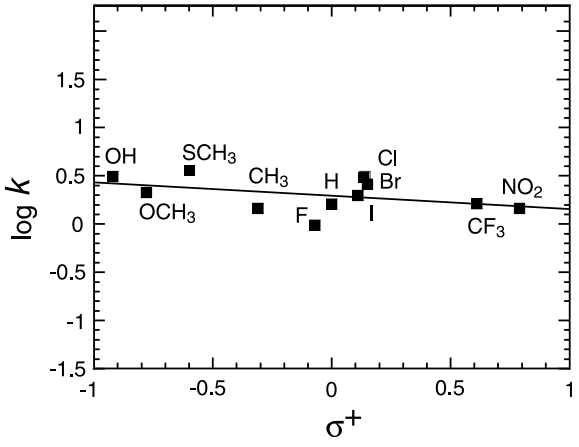


Fig. 4. QSAR analysis of the galactose oxidase turnover reaction. Rate data for enzymatic oxidation of a series of *para*-substituted benzyl alcohols is plotted versus the electronic substituent parameter Hammett σ^+. The correlation slope is $\rho = -0.09 \pm 0.32$.

Substrate reaction profiling

The nature of the catalytic reaction may also be probed by systematically varying the structure of the substrate. The relatively broad substrate specificity of galactose oxidase has made it possible to study turnover of a series of substituted benzyl alcohols with *para*-substituents including strongly electron-donating (HO–, CH_3O–, CH_3S–, CH_3–) and electron-withdrawing (F–, Cl–, Br–, I–, CF_3–, and NO_3–) groups [35]. This series spans a broad range of electronic substituent parameters, allowing the rate data to be analyzed by quantitative structure–activity relationship (QSAR) [58] methods (Fig. 4). A linear free energy relationship is clearly evident when the rate data (log k) is plotted versus the Hammett substituent parameter σ^+ (which is generally appropriate for benzylic oxidation processes), with a correlation slope near zero ($\rho = -0.09$). The absence of a strong substituent sensitivity is evidence for absence of a buildup of electronic charge on the C_α carbon during substrate oxidation and is considered a characteristic of a free radical transition state [59]. Similar behavior has been observed for a functional model complex designed to mimic the free radical–copper catalytic motif of galactose oxidase [60].

Isotope kinetics

The sensitivity of enzymatic reactions to isotopic perturbations of the substrates makes isotope kinetics one of the most powerful probes of catalytic reaction mechanism [61]. The magnitude, temperature dependence and site-dependence of the kinetic isotope effect (KIE) yields essential information on the nature of the catalytic transition state. Deuterium kinetic isotope effects tend to be relatively large, because of the large isotopic mass ratio ($^2H/^1H$) compared to carbon, for example ($^{13}C/^{12}C$). Kinetic isotope effects may be classified as primary or secondary, depending on whether or not the labeled atom is directly involved in bond making/bond breaking in the transition.

For an enzyme like galactose oxidase which follows ping–pong kinetics, the analysis of the reaction is complicated by occurrence of two distinct half reactions, each involving distinct bond-making and bond-breaking processes. Adjusting the concentrations of the substrates for the two half-reactions allows their rates to be independently varied. In practice, the extraordinarily fast reaction of galactose oxidase with O_2 ensures that the alcohol oxidation half-reaction is fully rate limiting for turnover in air-saturated buffer when the reducing substrate is kept below 10 mM. Conversely, the O_2 reduction half-reaction becomes fully rate-limiting for turnover at low concentrations of dissolved O_2 and high concentrations of the reducing substrate. Alternatively, the two

half-reactions may be resolved by monitoring single-turnover processes using rapid reaction techniques.

In the substrate oxidation half-reaction of galactose oxidase, two bonds (the hydroxyl O–H and methylene C–H) are broken in the conversion of alcohol substrate into aldehyde product. The two methylene hydrogens of galactose (and other primary alcohols) may be labeled by standard synthetic methods in order to determine the extent to which C–H bond cleavage contributes to the rate determining step of the catalytic reaction. For galactose oxidase, an unusually large KIE ($k_H/k_D = 23$) on V/K has been measured in the steady state under conditions where substrate oxidation is fully rate limiting to turnover, indicating that C–H bond cleavage dominates the chemistry of the catalytic oxidation process [34]. The KIE is anomalously large compared to the theoretical range for primary KIE predicted for C–H bond cleavage ($k_H/k_D = 2$–10) [62], an observation that may be interpreted as evidence for hydrogen tunneling. The strong temperature-dependence of the KIE is also consistent with a quantum mechanical contribution to the oxidation reaction [34].

In the experiments described above, both of the methylene hydrogens were labeled in the α,α′-[$^2H_2$] substrate. The observed KIE in this case is actually a product of primary KIE for the reaction (associated with C–H bond cleavage for the hydrogen abstraction) and a secondary KIE (associated with the hydrogen that is retained in the product). A significant secondary KIE might be expected for the oxidation reaction, since the methylene group undergoes a change in bonding from sp^3 to sp^2 when the alcohol is converted to an aldehyde. Secondary KIEs ranging from 1.1 to 1.4 are typically associated with this type of rehybridization [62]. In order to resolve the primary and α-secondary KIE contributions to the observed KIE it will be necessary to prepare stereospecifically mono-deuterated substrates in which the pro-*R* (or pro-*S*) is selectively labeled.

In contrast to the methylene hydrogens, which are stable elements of the substrate structure, the hydroxyl proton readily exchanges in solution. Thus, labeling the hydroxyl group involves performing the reaction in D_2O solvent and measuring an effective solvent deuterium kinetic isotope effect (SKIE) [63]. No detectable SKIE can be measured on the substrate oxidation reaction either in steady-state or transient kinetics when care is taken to avoid artifacts associated with reductive inactivation of galactose oxidase by impurities in the D_2O solution [34]. Earlier reports of a substantial SKIE for the galactose oxidase reaction [64] may have been the result of artifacts reflecting the sensitivity of this enzyme to trace amounts of reductant in the isotopic solutions.

The magnitude of the KIE depends on the substrate structure. In the homologous series of substituted benzyl alcohols described above, the substrate KIE was found to vary systematically from a low value of 3.63 (for the most readily oxidized, 4-OCH_3 derivative) to a high value of 12.3 (for the least easily oxidized, 4-NO_2 derivative) [35]. A strong log-linear correlation trend extends over the KIE values for all of these substrates when plotted versus the Hammett σ^+ parameter (Fig. 5). This empirical observation may be understood as evidence for a change in the nature of the transition state over this substrate series, effectively profiling the reaction by projecting over substrates with varying transition states. In this picture, C–H bond cleavage dominates the transition state for oxidation of 4-NO_2 benzyl alcohol, while other elements of the reaction begin to become kinetically important in the limit of 4-OCH_3 benzyl alcohol oxidation. This is supported by measurement of the solvent isotope sensitivity for these reactions. While no significant SKIE is detectable for the 4-NO_2 benzyl alcohol reaction ($k_{H_2O}/k_{D_2O} = 1.05$), a significant SKIE ($k_{H_2O}/k_{D_2O} = 1.22$) is found for oxidation of the 4-OCH_3 derivative, indicating a substantial contribution from proton transfer in the transition state for the latter reaction [35].

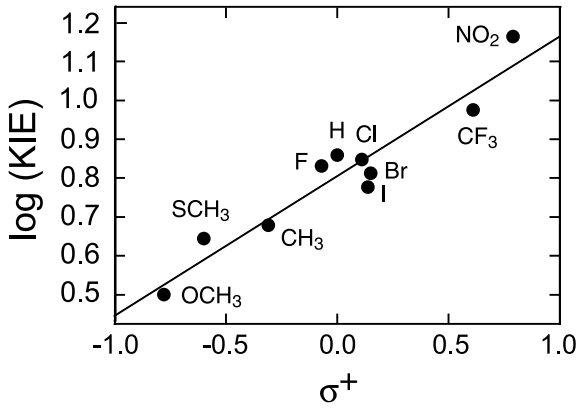


Fig. 5. Substituent dependence of the deuterium kinetic isotope effect for benzyl alcohol oxidation. Semi-log plot of observed k_H/k_D rate ratio versus the electronic substituent parameter Hammett σ^+ for a series of *para*-substituted benzyl alcohol derivatives.

Elements of the substrate oxidation reaction

Substrate oxidation may be conceptually decomposed into discrete elements based on the transformations that are known to occur in the substrate and the active site. The key transformations are:

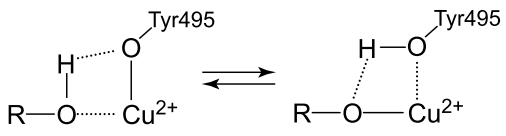
(a) removal of the hydroxylic proton of the substrate on conversion to aldehyde product (proton transfer, PT);
(b) removal of one of the hydrogens from C_α of the substrate (atom abstraction, HAT);
(c) reduction of the active site metal ion ($Cu^{2+} \rightarrow Cu^{1+}$) (single-electron transfer, SET);
(d) reduction of the Tyr–Cys free radical (Tyr–Cys$^\bullet$ → Tyr–Cys).

Proton transfer will be facilitated by the acidification of the substrate hydroxyl on coordination to Cu^{2+} ion in the active site. Coordination of a simple alcohol to a metal cation has a dramatic effect on the hydroxyl pK_a as a result of both Coulombic and covalency effects. For example, methanol coordinated to Zn^{2+} becomes a relatively strong acid, its effective pK_a being lowered by 10 pH units [65]. The coupling of proton uptake to anion binding in galactose oxidase suggests that the PT step may be facilitated in the substrate complex through this pertubation of the acidity of the coordinated alcohol as well as by the proximity of a proton acceptor, the general base that is unmasked by anion interactions in the active site. Previous assignment of this base to Tyr495 based on spectroscopic, biochemical and mutational analysis of the proton uptake process has led to a proposal for the existence of a direct proton transfer coordinate between the substrate hydroxyl and the Tyr495 phenolate (Scheme 8). The temperature dependence of a transition between Tyr495-bound and Tyr495-free forms of the enzyme has been used to estimate the thermodynamic parameters for PT along this reaction coordinate ($\Delta H = 4.9$ kcal/mol, $\Delta S = 19$ kcal/mol) [53].

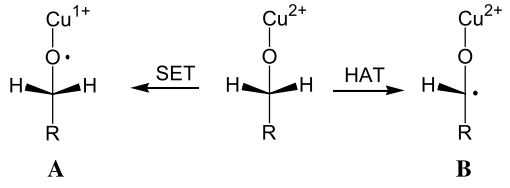
While abstraction of the hydrogen of the hydroxyl group is expected to occur by this proton transfer mechanism, abstraction of hydrogen from the methylene carbon must be associated with oxidative chemistry. The unusually large KIE observed for oxidation of α-deuterated substrates implies a hydrogen atom transfer (HAT) mechanism, representing one limit of a continuum of proton-coupled electron transfer (PCET) processes. A generalized PCET reaction involves transfer of one electron and one proton along a reaction coordinate that may span a spectrum of possibilities from concerted to step-wise e^-/H^+ transfer and even distinct acceptor sites for the electron and the proton. The fundamental principle underlying PCET is a change in the basicity of the acceptor group during oxidation–reduction, reflected in a change in bond enthalpies for the oxidized and reduced species [66,67]. Phenoxyl groups (including tyrosine and Tyr–Cys free radicals) undergo dramatic shifts in acidity for the phenolic oxygen on reduction (tyrosine phenoxyl p$K_a = -2$; phenol p$K_a = 10$; Δp$K_a = 12$) [68] and thus are well-suited to a role as a PCET site. This role is well-established in the tyrosine free-radicals of oxygenic photosynthesis and in ribonucleotide reductase free radical chemistry, and is likely an important aspect of the chemistry of the Tyr–Cys site in galactose oxidase. The fact that the Tyr–Cys oxygen appears to be coordinated to copper in both oxidized and reduced complexes raises a question regarding the protonation state of the Tyr–Cys in the reduced form. Although models for the Cu^{1+}–[Tyr–Cys] complex have been synthesized, the pK_a of the coordinated phenolic group is not known. The active site tryptophan (Trp290), which lies over the Tyr–Cys cofactor with the indole nitrogen adjacent to the coordinated oxygen of the Tyr272 side chain may play a role in the stabilization of the substrate-derived proton in this complex.

Reduction of the metal center most likely occurs via inner sphere single-electron transfer (SET). The rate of SET will depend on overlaps between the valence orbital on the coordinated substrate and the redox orbital (partly occupied d-orbital) on the Cu^{2+} ion. One-electron oxidation of a coordinated alcohol is predicted to yield an alkoxyl free radical. This redox equilibrium may be unfavorable for most substrates, although it is difficult to address the question quantitatively because of the lack of data on one-electron redox potentials for simple alcohols. However, the trend in one-electron oxidation potentials may be expected to parallel the trend in two-electron oxidation potentials that are relatively well-characterized. This would imply that in the benzyl alcohol series, an initial SET step would be less rate-limiting for the 4-OCH_3 derivative than for the 4-NO_2 derivative. However, it is found experimentally that the C–H bond cleavage, rather than SET, dominates in the latter case. Also, to the extent that an unfavorable initial SET is rate limiting in substrate oxidation, we might expect a significant equilibrium kinetic isotope effect on the redox equilibrium as a result of the decrease in C–H bonding in the alkoxyl form. This effect may be experimentally testable using monodeuterated substrates, which should exhibit half the equilibrium isotope effect of the doubly-labeled substrate.

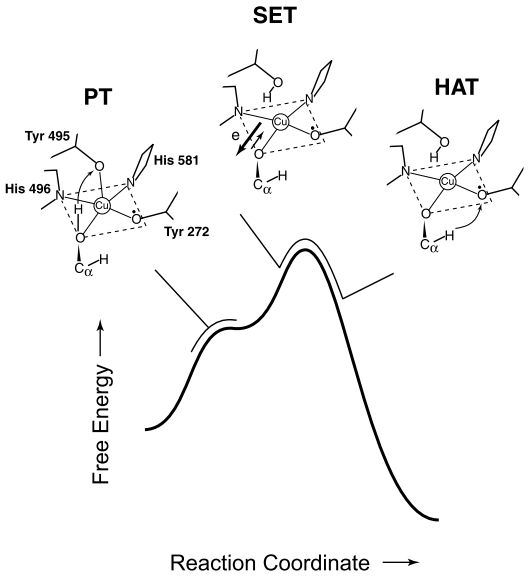
These two oxidation steps (SET and HAT) both generate free radicals, but with distinct structures. The substrate free radical formed by SET is an alkoxyl, with the unpaired electron localized on the oxygen atom (Scheme 9A), while the radical formed by HAT is a ketyl, in which the unpaired electron is localized on the C_α carbon of the substrate (Scheme 9B). There is no clear experimental evidence for the ordering of these steps in the reaction mechanism, and to some extent this



Scheme 8.



Scheme 9.



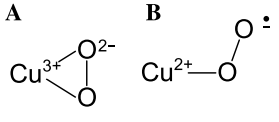
Scheme 10.

may not be a valid issue, since both processes are likely to contribute to the rate-determining step in catalysis as illustrated in Scheme 10.

Dioxygen reduction

The oxygen reduction half-reaction is the least-well characterized aspect of the galactose oxidase turnover mechanism. The reaction is known to be extremely fast, approaching the diffusion limit. The most important observation regarding the dioxygen reduction chemistry is that the enzyme is able to control the number of electrons delivered to O_2, avoiding the escape of one-electron reduction products (superoxide), although there is some evidence for an inactivation process resulting from loss of superoxide at a rate of one every 2000–5000 turnovers [31].

Synthetic chemistry has provided some valuable models for the nature of the oxygenated complex in galactose oxidase. Mononuclear copper coordination complexes have been prepared that contain dioxygen bound to the metal in two distinct coordination modes: side-on (Scheme 11A) [69] and end-on (Scheme 11B) [70]. Side-on coordination in the oxy complex is characteristic of strong metal–ligand covalent interactions and the bonding in the complex leads to a description as Cu^{3+}–peroxide, containing a highly oxidized trivalent copper ion. The end-on geometry, on the other hand, is described as a Cu^{2+}–superoxide adduct. Side-on oxygen-binding essentially requires the availability of two adjacent coordination sites on the metal ion, and therefore end-on ligation is favored in sterically congested complexes. As a result of the differences in valence electron distribution in these two classes of complexes, they are expected to exhibit distinct reactivity, with the side-on complex favoring inner sphere (metal-centered) reduction and the end-on complex favoring outer sphere (oxygen-centered) reduction.



Scheme 11.

Inspection of the active site of galactose oxidase shows that access to the metal center is quite restricted even for a diatomic substrate like O_2. His496, His581 and Tyr272 appear to occupy three of four equatorial positions in the complex, and the EXAFS evidence cited earlier suggests that all three ligands are retained in the reduced complex. The position of the Tyr496 phenolic oxygen is also quite constrained by the structure of the protein, and is unlikely to provide room for a second coordination position for oxygen binding at the front face of the metal center. Based on these structural considerations, the end-on oxygenated complex seems more likely in this case. The positions of crystallographically defined water molecules in the active site may provide a clue. In other oxygen-reactive enzymes, the pattern of interactions that stabilize the oxygenated intermediate also stabilize a special pair of water molecules in the absence of oxygen, forming a ''ghost'' of the peroxide molecule. Two water molecules in the active site of galactose oxidase, one being the metal-bound solvent, occupy positions that are separated by 3.2 Å, possibly defining the outline of the peroxide-stabilization site [71].

Reduction of dioxygen is expected to essentially reverse the sequence of steps involved in substrate oxidation (Fig. 6). Inner sphere reduction (SET) and outer sphere atom transfer (HAT) to an end-on bound dioxygen species will generate a Cu^{2+}–hydroperoxide adduct. Protonation of the proximal, coordinated oxygen atom (PT across the proton transfer coordinate from Tyr495) would serve to displace the coordinated peroxide and complete the turnover cycle.

Mechanism of cofactor biogenesis in galactose oxidase

The Tyr–Cys cofactor appears to be the key to the free radical chemistry of galactose oxidase and related enzymes. The origin of this feature in the mature protein has been the focus of considerable interest, particularly following a report that the Tyr–Cys cross-link can form spontaneously in vitro with formation of free radical coupled-Cu^{2+}-containing active enzyme product [72]. The reaction was reported to depend on the presence of Cu^{2+} and dioxygen and to be independent of any other proteins. Early work on self-processing of an

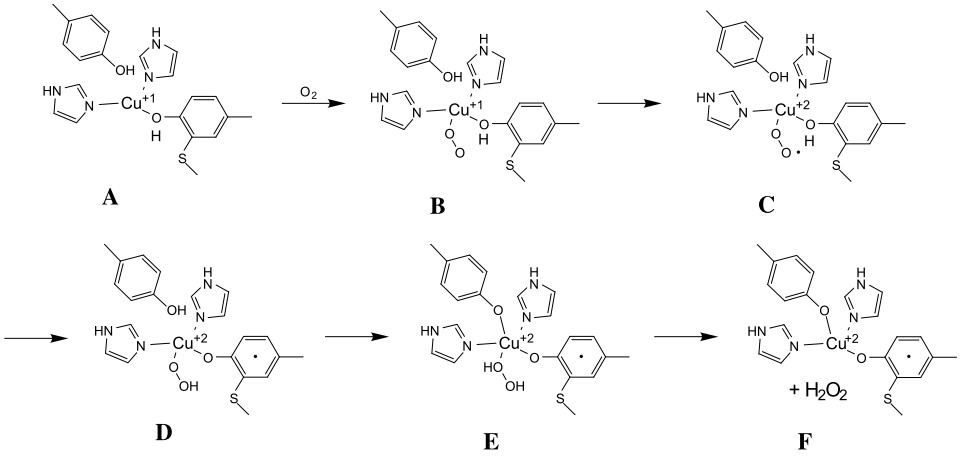
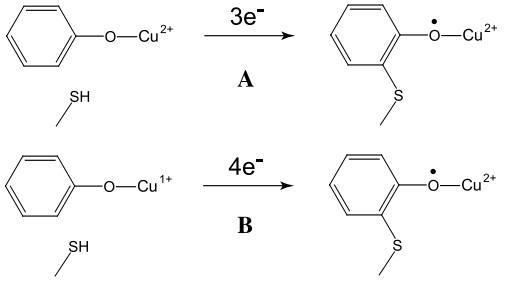


Fig. 6. Proposed mechanism for dioxygen reduction by galactose oxidase. Reaction of O_2 with the Cu^{1+} active site (A) is predicted to involve formation of an adduct (B) that will undergo single-electron transfer to form an oxy radical complex (C). Hydrogen abstraction reduces the complex to a hydroperoxide adduct (D) which may be protonated at the proximal oxygen (E) leading to displacement of the product (F).

immature form of galactose oxidase (retaining a 17-residue N-terminal pro-peptide extension and lacking the Tyr–Cys site) secreted by metal-starved *Aspergillus* cultures lead to a suggestion that the pro-peptide serves as a chaperone for delivery of copper to the active site and the maturation of the metal complex [73]. However, this proposal is at odds with the observation that galactose oxidase and related enzymes are efficiently expressed in their mature, functional form from chimeric fusion protein precursors containing widely divergent leader sequences [74].

Further progress on the mechanism of cofactor biogenesis in galactose oxidase has been made possible by the availability of large quantities of pregalactose oxidase (with a mature N-terminal but lacking the Tyr–Cys cross-link) resulting from high level expression in *Pichia pastoris* methylotrophic yeast fermentation cultures [37]. Using this well-defined precursor, the metal oxidation state dependence of the self-processing reaction has been investigated. Interestingly, the rate of Cu^{2+}-dependent cross-linking ($k_{xl} = 3.8 \times 10^{-5}\,s^{-1}$) is nearly $10^4\times$ slower than the Cu^{1+}-dependent rate ($k_{xl} = 0.4\,s^{-1}$), demonstrating that the reduced metal ion is required for the reaction to proceed efficiently. This sensitivity of the cross-linking reaction to metal oxidation state had actually been predicted on the basis of the overall electron count for conversion of Cu^{1+} or Cu^{2+} precursor complexes into the active enzyme, as illustrated in Scheme 12. The overall reaction involves oxidative coupling of tyrosine and cysteine side chains (a two-electron process) and further oxidation of the Tyr–Cys cofactor to the free radical. In the absence of metal-centered oxidation, this would be a three-electron process, which would require unfavorable odd-electron reduction of dioxygen. Metal-centered redox increases this to four-electron overall reaction which is more favorable for coupling to dioxygen, as shown in Eq. (2):



Scheme 12.

$$\left.\begin{array}{ll}\text{(a) oxidative coupling} & 2e^-\\ \text{(b) Tyr–Cys} \rightarrow \text{Tyr–Cys}^{\bullet} & 1e^-\\ \text{(c) } Cu^{1+} \rightarrow Cu^{2+} & 1e^-\end{array}\right\}$$
$$\text{Total} \quad 4e^- \qquad (3)$$

In fact, kinetic analysis of the cross-linking reaction is consistent with the slow Cu^{2+}-dependent process occurring via reduction to Cu^{1+} by adventitious reductants in the sample.

Analysis of the dioxygen stoichiometry for the cross-linking reaction gives further information on the cofactor biogenesis mechanism. Titration of an anaerobic solution of preformed pregalactose oxidase Cu^{1+} complex with O_2-containing buffer yielded a dioxygen stoichiometry of 1.8 O_2/active enzyme product, indicating that two molecules of dioxygen are consumed in the active site maturation process. This is a significant constraint on any detailed mechanistic proposal for this reaction, and on the basis of evidence for a four-electron overall reaction suggests that two molecules of hydrogen peroxide are generated in the process [37].

Transient kinetics studies of the Cu^{1+}-dependent biogenesis reaction have revealed a pH sensitivity, with the rate maximizing at higher pH above an effective pK_a of 7.5 characteristic of ionization of cysteine thiols in

solvent-exposed environments in proteins. A significant solvent kinetic isotope effect is also observed on the reaction at lower pH, which vanishes in the higher pH range, following a pH profile exactly mirroring the rate profile. This suggests that the proton giving rise to the isotope effect at low pH is the same proton that ionizes to give the rate enhancement at high pH.

The reaction of dioxygen with the Cu^{1+} precursor complex is expected to result in one-electron reduction of O_2, implying the involvement of free radical intermediates in the biogenesis reaction, although there is no spectroscopic evidence for build-up of radicals in the rapid reaction experiments and further investigation will be necessary to identify the radicals involved. A detailed mechanism consistent with the experimental results has been proposed (Fig. 7) [37]. The reaction is initiated by Cu^{1+} binding to a pre-organized active site (Figs. 7A and B). Dioxygen reacts with this complex to produce an oxygenated species (e.g., Schemes 11A or B). An end-on superoxo complex (Fig. 7C) would be expected to react in the outer sphere with the Cys228 thiol to form a thiyl free radical (Fig. 7D). The pH and isotope effect measurements described above may reflect this reactivity of the Cys228 side chain. Addition of the thiyl free radical to the Tyr272 ring system would break the aromatic conjugation of the ring (Fig. 7E) which would be restored on deprotonation and reduction of the metal ion (Fig. 7F). This mature, cross-linked complex corresponds to the fully reduced active site formed during turnover, and is expected to undergo very rapid reaction with a second molecule of O_2 to form the oxidized metalloradical complex. An alternative path, involving side-on bound oxy species and inner-sphere reaction of the initial complex, might proceed via a tyrosyl phenoxyl free radical intermediate (Scheme 13). This mechanism accounts for the metal oxidation-state dependence of the reaction, the O_2 stoichiometry, the pH and isotope sensitivity and the requirement for a metalloradical complex in the product.

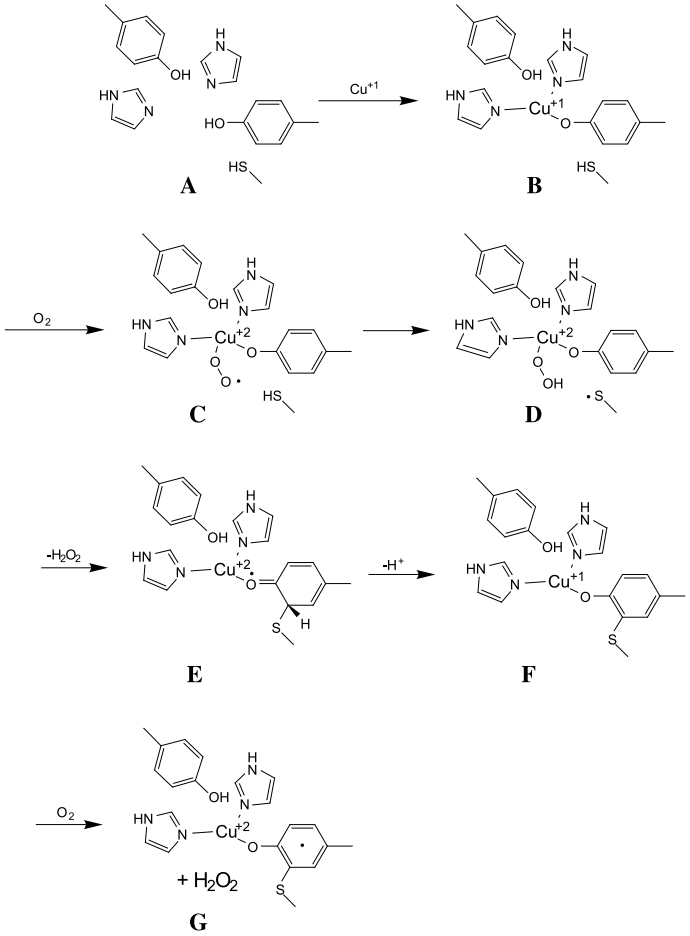
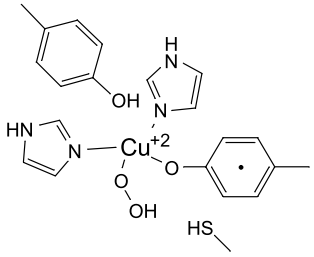
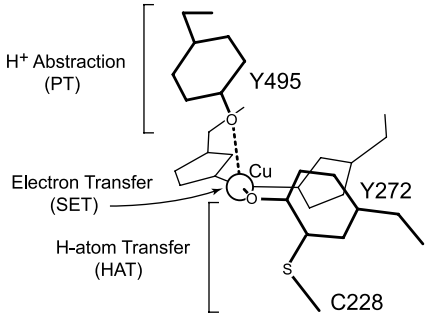


Fig. 7. Proposed mechanism for biogenesis of the Tyr–Cys cofactor in galactose oxidase. Cu^{1+} binds to a pre-organized active site (A) to form a reduced metal complex (B). Oxygen reacts directly with the metal center to form a reactive oxygenated adduct (C) capable of abstracting a hydrogen atom from the active site cysteine Cys228 to form a thiyl free radical (D). Addition of the free radical to the Tyr272 ring system (E) and loss of a proton restores aromaticity to the ring system and reduces the metal center (F) allowing a second molecule of O_2 to react to form the metalloradical complex (G).



Scheme 13.



Scheme 14.

Conclusions

The active site of galactose oxidase has evolved a structure capable of controlling the reactivity of free radicals for enzymatic oxidation chemistry. The two tyrosines in the active site (Scheme 14) each perform a distinct function in concert with the redox-active metal ion. Tyr495 serves as a general base that activates the reducing substrate by proton abstraction during the reductive half-reaction, and facilitates displacement of hydrogen peroxide product by protonation in the oxidative half-reaction. The metal ion functions as a one-electron storage site, complemented by the hydrogen-atom transfer reactivity of the Tyr–Cys cofactor. Together, these components form an efficient two-electron catalytic complex that can be turned on and off by reversible one-electron reduction, controlling the enzyme activity in the extracellular space.

Acknowledgment

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References

[1] J. Stubbe, W.A. van der Donk, Chem. Rev. 98 (1998) 705–762.
[2] P.A. Frey, Curr. Opin. Chem. Biol. 1 (1997) 347–356.
[3] P.A. Frey, Chem. Rev. 90 (1990) 1343–1357.
[4] P.A. Frey, Annu. Rev. Biochem. 70 (2001) 121–148.
[5] E.N. Marsh, Bioessays 17 (1995) 431–441.
[6] J.Z. Pedersen, A. Finazzi-Agro, FEBS Lett. 325 (1993) 53–58.
[7] R. Banerjee, Chem. Rev. 103 (2003) 2081–2083.
[8] A.F. Parsons, An Introduction to Free Radical Chemistry, Blackwell Science, Oxford, 2000.
[9] M.J. Perkins, Radical Chemistry – An Introduction, Oxford University Press, Oxford, 2000.
[10] B.-M. Sjöerg, Struct. Bond. (Berlin) 88 (1997) 139–173.
[11] S. Licht, G.J. Gerfen, J. Stubbe, Science 271 (1996) 477–481.
[12] X. Sun, S. Ollagnier, P.P. Schmidt, M. Atta, E. Mulliez, L. Lepape, R. Eliason, A. Graslund, M. Fontecave, P. Reichard, B.-M. Sjöberg, J. Biol. Chem. 271 (1996) 6827–6831.
[13] P.A. Frey, S.J. Booker, Adv. Protein. Chem. 58 (2001) 1–45.
[14] R. Banerjee, Chem. Rev. 103 (2003) 2083–2094.
[15] J. Knappe, A.F. Wagner, Adv. Prot. Chem. 58 (2001) 277–315.
[16] F. Berkovitch, Y. Nicolet, J.T. Wan, J.T. Jarrett, C.L. Drennan, Science 303 (2004) 76–79.
[17] P. Dorlet, S.A. Seibold, G.T. Babcock, G.J. Gerfen, W.L. Smith, A.L. Tsai, S. Un, Biochemistry 41 (2002) 6107–6114.
[18] J.E. Huyett, P.E. Dean, R. Gurbiel, A.L.P. Houseman, M. Sivaraja, D.B. Goodin, B.M. Hoffman, J. Am. Chem. Soc. 117 (1995) 9033–9041.
[19] J. Cheek, J.B. Broderick, J. Am. Chem. Soc. 124 (2002) 2860–2861.
[20] R.M. Cicchillo, D.F. Iwig, A.D. Jones, N.M. Nesbitt, C. Baleanu-Gogonea, M.G. Souder, L. Tu, S.J. Booker, Biochemistry 43 (2004) 6378–6386.
[21] O.T. Magnusson, P.A. Frey, Biochemistry 41 (2002) 1695–1702.
[22] J.W. Whittaker, in: A. Richard, E.B. Gralla (Eds.), Advances in Protein Chemistry, vol. 60, Academic Press, New York, 2002, pp. 1–49.
[23] J.W. Whittaker, Chem. Rev. 103 (2003) 2347–2363.
[24] M.J. McPherson, M.R. Parsons, R.K. Spooner, C.R. Wilmot, in: A. Messerschmidt, R. Huber, K. Wieghardt, T. Poulos (Eds.), Handbook of Metalloproteins, Wiley, Hoboken, 2001, pp. 1272–1283.
[25] G. Avigad, D. Amaral, C. Asensio, B.L. Horecker, J. Biol. Chem. 237 (1962) 2736–2743.
[26] M.M. Whittaker, J.W. Whittaker, J. Biol. Chem. 263 (1988) 6074–6080.
[27] N. Ito, S.E.V. Phillips, C. Stevens, Z.B. Ogel, M.J. McPherson, J.N. Keen, K.D.S. Yadav, P.F. Knowles, Nature 350 (1991) 87–90.
[28] M.M. Whittaker, J.W. Whittaker, J. Biol. Chem. 265 (1990) 9610–9613.
[29] G.T. Babcock, M.K. El-Deeb, P.O. Sandusky, M.M. Whittaker, J.W. Whittaker, J. Am. Chem. Soc. 114 (1992) 3727–3734.
[30] G.A. Gerfen, B. Bellew, R. Griffin, D. Singel, C.A. Ekberg, J.W. Whittaker, J. Phys. Chem. 100 (1996) 16739–16748.
[31] G.A. Hamilton, P.K. Adolf, J. de Jersey, G.C. DuBois, G.R. Dyrkacz, R.D. Libby, J. Am. Chem. Soc. 100 (1978) 1899–1912.
[32] L.D. Kwiatkowski, M. Adelman, R. Pennely, D.J. Kosman, J. Inorg. Biochem. 14 (1981) 209–222.
[33] M.P. Reynolds, A.J. Baron, C.M. Wilmot, E. Vinecombe, C. Stevens, S.E.V. Phillips, P.F. Knowles, M.J. McPherson, J. Biol. Inorg. Chem. 2 (1997) 327–335.
[34] M.M. Whittaker, D.P. Ballou, J.W. Whittaker, Biochemistry 37 (1998) 8426–8436.
[35] M.M. Whittaker, J.W. Whittaker, Biochemistry 40 (2001) 7140–7148.
[36] L.J. Farrugia, J. Appl. Crystallogr. 30 (1997) 565.
[37] M.M. Whittaker, J.W. Whittaker, J. Biol. Chem. 278 (2003) 22090–22101.
[38] M.M. Whittaker, Y.Y. Chuang, J.W. Whittaker, J. Am. Chem. Soc. 115 (1993) 10029–10035.
[39] S. Itoh, S. Fukuzumi, S. Takayama, Inorg. Chem. 36 (1997) 1407–1416.

[40] S. Itoh, K. Hirano, A. Furuta, M. Komatsu, Y. Ohshiro, A. Ishida, S. Takamuku, T. Kohzuma, N. Nakamura, S. Suzuki, Chem. Lett. (Jpn.) (1993) 2099–2102.
[41] K.E. Wise, J.B. Pate, R.A. Wheeler, J. Phys. Chem. B 103 (1999) 4764–4772.
[42] C. Anthony, Biochem. J. 320 (1996) 697–711.
[43] J.W. Whittaker, M.M. Whittaker, Pure Appl. Chem. 70 (1998) 903–910.
[44] B.A. Jazdzewski, W.B. Tolman, Coord. Chem. Rev. 200–202 (2000) 633–685.
[45] M.M. Whittaker, J.W. Whittaker, Biophys. J. 64 (1993) 762–772.
[46] M.P. Reynolds, A.J. Baron, C. Wilmot, S.E.V. Phillips, P.F. Knowles, M.J. McPherson, Biochem. Soc. Trans. 23 (1995) 510S.
[47] M.P. Reynolds, A.J. Baron, C.M. Wilmot, E. Vinecombe, C. Stevens, S.E.V. Phillips, P.F. Knowles, M.J. McPherson, J. Biol. Inorg. Chem. 2 (1997) 327–335.
[48] M.S. Rogers, P.F. Knowles, A.J. Baron, M.J. McPherson, D.M. Dooley, Inorg. Chim. Acta 275–276 (1998) 175–181.
[49] K. Clark, J.E. Penner-Hahn, M.M. Whittaker, J.W. Whittaker, Biochemistry 33 (1994) 12553–12557.
[50] K. Clark, J.E. Penner-Hahn, M.M. Whittaker, J.W. Whittaker, J. Am. Chem. Soc. 112 (1990) 6433–6434.
[51] M.L. McGlashin, D.D. Eads, T.G. Spiro, J.W. Whittaker, J. Phys. Chem. 99 (1995) 4918–4922.
[52] J. Müller, T. Weyhermüller, E. Bill, P. Hildebrandt, L. Ould-Moussa, T. Glaser, K. Wieghardt, Angew. Chem. Int. Ed. 37 (1998) 616–619.
[53] M.M. Whittaker, C.A. Ekberg, J. Peterson, M.S. Sendova, E.P. Day, J.W. Whittaker, J. Mol. Catal. B 8 (2000) 3–15.
[54] A. Maradufu, G.M. Cree, A.S. Perlin, Can. J. Chem. 49 (1971) 3429–3437.
[55] P.S. Tressel, D.J. Kosman, Methods Enzymol. 89 (1982) 163–171.
[56] C.D. Borman, C. Wright, M.B. Twitchett, G.A. Salmon, A.G. Sykes, Inorg. Chem. 41 (2002) 2158–2163.
[57] P.V. Patil, D.P. Ballou, Anal. Biochem. 286 (2000) 187–192.
[58] C. Hansch, A. Leo, Exploring QSAR. Fundamentals and Applications in Chemistry and BiologyAmerican Chemical Society, American Chemical Society, Washington DC, 1995.
[59] R.D. Gilliom, Introduction to Physical Organic Chemistry, Addison-Wesley, New York, 1970, p. 214.
[60] Y. Wang, J.L. DuBois, B. Hedman, K.O. Hodgson, T.D.P. Stack, Science 279 (1998) 537–540.
[61] W.W. Cleland, J. Biol. Chem. 278 (2003) 51975–51984.
[62] L. Melander, W.H. Saunders Jr., Reaction Rates of Isotopic Molecules, Krieger Publishing Co., Malabar, FL, 1987.
[63] K.B. Schowen, R.L. Schowen, Methods Enzymol. 87 (1982) 551–606.
[64] J.J. Driscoll, D.J. Kosman, Biochemistry 26 (1987) 3429–3436.
[65] E. Kimura, I. Nakamura, T. Koike, M. Shionoya, Y. Kodama, T. Ikeda, M. Shiro, J. Am. Chem. Soc. 116 (1994) 4764–4771.
[66] R.I.J. Cukier, Phys. Chem. 98 (1994) 2377–2381.
[67] J.M. Mayer, Annu. Rev. Phys. Chem. 55 (2004) 363–390.
[68] C. Aubert, P. Mathis, A. Eker, K. Brettel, Proc. Natl. Acad. Sci. USA 96 (1999) 5423–5427.
[69] N.W. Aboelella, E.A. Lewis, A.M. Reynolds, W.W. Brennessel, C.J. Cramer, W.B. Tolman, J. Am. Chem. Soc. 124 (2002) 19660–19661.
[70] K. Fujisawa, M. Tanaka, Y. Moro-oka, N. Katajima, J. Am. Chem. Soc. 116 (1994) 12079–12080.
[71] J.W. Whittaker, Essays Biochem. 34 (1999) 155–172.
[72] M.S. Rogers, A.J. Baron, M.J. McPherson, P.F. Knowles, D.M. Dooley, J. Am. Chem. Soc. 122 (2000) 990–991.
[73] S.J. Firbank, M.S. Rogers, C.M. Wilmot, D.M. Dooley, M.A. Halcrow, P.F. Knowles, M.J. McPherson, S.E.V. Phillips, Proc. Natl. Acad. Sci. USA 98 (2001) 12932–12937.
[74] M.M. Whittaker, J.W. Whittaker, Protein Expr. Purif. 20 (2000) 105–111.