Identification of Catalytic Residues in Glyoxal Oxidase by Targeted Mutagenesis

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Glyoxal oxidase is a copper metalloenzyme produced by the wood-rot fungus *Phanerochaete chrysosporium* as an essential component of its extracellular lignin degradation pathways. Previous spectroscopic studies on glyoxal oxidase have demonstrated that it contains a free radical-coupled copper active site remarkably similar to that found in another fungal metalloenzyme, galactose oxidase. Alignment of primary structures has allowed four catalytic residues of glyoxal oxidase to be targeted for site-directed mutagenesis in the recombinant protein. Three glyoxal oxidase mutants have been heterologously expressed in both a filamentous fungus (*Aspergillus nidulans*) and in a methylotrophic yeast (*Pichia pastoris*), the latter expression system producing as much as 2 g of protein per liter of culture medium under conditions of high density methanol-induced fermentation. Biochemical and spectroscopic characterization of the mutant enzymes supports structural correlations between galactose oxidase and glyoxal oxidase, clearly identifying the catalytically important residues in glyoxal oxidase and demonstrating the functions of each of these residues.

Glyoxal oxidase (GLOX) from the wood-rot fungus *Phanerochaete chrysosporium* is a secreted enzyme that functions as an extracellular factory for production of hydrogen peroxide, fueling peroxidases (lignin peroxidase and manganese peroxidase) that are responsible for microbial lignin degradation (1–3). Glyoxal oxidase catalyzes the oxidation of aldehydes to carboxylic acids, coupled to reduction of dioxygen to hydrogen peroxide,

\[ \text{RCOCHO} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCOOH} + \text{H}_2\text{O} \]  \hspace{1cm} \text{(Eq. 1)}

The enzyme has fairly broad specificity for the reducing substrate, and a variety of simple dicarbonyl and hydroxycarbonyl compounds have been shown to support turnover. However, there is biological evidence that *P. chrysosporium* specifically secretes simple dicarboxyls (glyoxal and methylglyoxal) to drive this reaction. Further metabolism of the glyoxylic acid product leads to formation of oxalic acid, which has been identified as a cofactor for manganese peroxidase turnover (4).

Previous studies of glyoxal oxidase (5) have demonstrated that it is a copper metalloenzyme containing an unusual free radical-coupled copper active site similar to that found in galactose oxidase (GAOX) (6, 7). In these enzymes, an amino acid side chain radical ligates the active site metal ion, forming a catalytic motif characteristic of a class of enzymes known as *radical copper oxidases*. This radical-copper complex acts as a two-electron redox active site, a distinction from other free radical enzymes (ribonucleotide reductase, etc.) (8–12) which typically exhibit single-electron reactivity. Glyoxal oxidase is isolated as an inactive, reduced form lacking the free radical, and requires treatment with a strong oxidant (e.g. Ir(IV) or Mo(V)) for activation (5).

The crystal structure of galactose oxidase (13) (Fig. 1) shows the active site metal ion coordinated by four protein ligands, including two histidines (His496 and His581) and two tyrosines (Tyr272 and Tyr495), forming a roughly square pyramidal metal complex. Two of the metal ligands (Tyr495 and His496) occur as consecutive residues in the protein sequence. The tyrosine ligands are distinct both in terms of coordination mode and, more especially, covalent modification of the side chain. In particular, one of the tyrosines (Tyr272) is cross-linked to a cysteinyl residue, forming a Tyr-Cys dimer site (13) that has been identified in spectroscopic (14, 15) and modeling (16–18) studies as the radical redox site in the enzyme. The second tyrosine (Tyr495) is bound axially to the active site metal ion, giving rise to distinctive features in the electronic spectra of the enzyme in the resting (TyrON) state and this residue has been shown to be displaced (producing a TyrOFF complex) when anions replace water in the inner sphere of the metal complex (19). This displacement is coupled to a protonation event that implies that Tyr495 can serve as a general base, capable of activating bound substrate by proton abstraction. A tryptophan residue (Trp290) stacked over the thioether side chain (13) is thought to contribute to the unusual stability of the free radical-containing form of this enzyme, which can persist for weeks in the absence of reductants (20, 21). Spectroscopic studies on the glyoxal oxidase (5) provide evidence for an active site structure nearly identical to that found in galactose oxidase, despite the distinct catalytic function. EPR spectroscopy has identified two nitrogen donor ligands in the Cu(II) complex, and a combination of optical absorption and resonance Raman spectroscopy have identified tyrosine and tyrosine-cysteine dimer residues in the active site.

Site-directed mutagenesis has been used to investigate the structural and catalytic roles of the active site residues in galactose oxidase (22–25). Conservative mutagenesis of the “axial” tyrosine to phenylalanine (Y495F) (22, 24) permanently converts the enzyme to a TyrOFF form (19) lacking the spectroscopic signatures of axial tyrosinate coordination and virtually eliminates catalytic activity. Elimination of the cysteinyl residue that is involved in the Tyr-Cys redox cofactor of the mature protein by C228G mutagenesis (23) likewise profoundly alters
both spectroscopic and catalytic properties of the copper center. Replacement of the active site tyrosine 272 by phenylalanine appeared to interfere with copper binding and protein stability, and the Y272F mutant of galactose oxidase could not be purified (23).

Crystallographic studies on glyoxal oxidase, a glycoprotein for which carbohydrate comprises approximately 15% of the total molecular mass (26), have been complicated by a twinning disorder in the protein crystals that may be related to the high degree of glycosylation.2 In the absence of high resolution structure information, theoretical sequence comparison makes it possible to identify putative active site residues (27). Using predictions from a comparative sequence analysis, we have undertaken a mutagenic program targeting the key active site residues in glyoxal oxidase. Characterization of these mutant proteins clearly identifies the residues important for metal binding and catalysis.

MATERIALS AND METHODS

Biological Materials—Pichia pastoris GS115 (his4) was a generous gift of Dr. James Cregg (Oregon Graduate Institute, Portland, Oregon) and was maintained on YPD agar. Aspergillus nidulans (argB; methHI; biA1) (28) was maintained on minimal medium agar.

Comparative Protein Sequence Analysis—Sequences for galactose oxidase and glyoxal oxidase were aligned by the BLAST local sequence similarity search program using the BLOSUM50 substitution scoring matrix for protein-protein comparison and default values for other constraints (29, 30).

Plasmids—The A. nidulans expression vector (pGLAGLOX) utilizes the promoter, secretion signal, and terminator of the A. niger glucoamylase gene (glaA) which is highly expressed and induced by maltose (28). The vector also features A. nidulans sequences (argB; ANS1) which permit efficient transformant selection on minimal medium (32). The pGLAGLOX plasmid was used as a template for the production of mutant plasmids using the Strategene QuickChange2 in vitro site-directed mutagenesis procedure for 20 cycles of amplification as described previously (31) with the appropriate primer set (C70A, 5′-CG-GTCCGTAGCCACTCTTTGGCAGGCCTGGCTGTCG-3′; Y135F, 5′-CCTAGGGACGCGTGTTCCTCATCGTCCGATTTTCC-3′; H135W, 5′-CATGATCATTGTTTCGTGGGTCCTCACACCGTTC-3′; E-109 X-Band EPR spectrometer equipped with an Air Products helium flow cryostat. g Value calibration was performed using a powdered sample of a,a′-diphenyl-β-picryl hydrazyl (g = 2.0037 (35)) as standard. Spin quantitation of paramagnetic samples was performed using a CuClO4 spin standard (10 m, in 2 m NaClO4). EPR spectral simulations were computed using the program sim15 (Quantum Chemistry Program Exchange QCPE265). Metal ion analyses were performed using a Varian Instruments SpectraAA atomic absorption spectrometer equipped with a GTA 96 graphite furnace for high sensitivity metal determinations.

RESULTS

Sequence Comparison—Glyoxal oxidase and galactose oxidase share a modest protein sequence similarity including 28% identity over their primary structures (Fig. 2). The sequence of glyoxal oxidase, the smaller of the two proteins, matches the C-terminal three-quarters of galactose oxidase, implying that glyoxal oxidase lacks a 150-residue N-terminal domain that is present in the larger enzyme. However, alignment of the protein sequences indicates that the critical active site residues characteristic of the radical copper oxidases (highlighted in Fig. 2) are conserved between these structures, consistent with spectroscopic comparisons (5) that demonstrate a close structural similarity of the active sites in these two enzymes.

Both sequences contain a unique pair of consecutive tyrosine and histidine residues (TyR495His496 in GAOX, Tyr477His478 in GLX). In galactose oxidase (13) both of these residues serve as copper ligands arising within a single turn in the protein fold. The metal binding loops in secreted and membrane-bound RXXH3 pentapeptide motif in the alignment with glyoxal oxidase, supporting the identification of Tyr477 and His478 in that enzyme as metal ligands corresponding to Tyr495 and His496 in GAOX. Similarly, a histidine residue that occurs approximately 90 residues later in both sequences may be assigned as the second conserved histidine ligand. The redo active tyro-

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2 R. D. Kidd, H. M. Baker, F. J. Kersten, M. M. Whittaker, J. W. Whittaker, and E. N. Baker, unpublished results.
Fig. 1. Organization of the active site of galactose oxidase. Four metal ligands lying nearly in-plane with the copper are shown bounded by a dashed square. Based on atomic coordinates obtained from the Brookhaven Protein Data Base (PDB ID, 1gog). Residues in the glyoxal oxidase sequence are indicated in parentheses.

sine (GAOX Tyr372) occurs in a hexapeptide context RXYXSS that is common to both proteins and leads to the prediction that, in glyoxal oxidase, Tyr135 is the radical-forming tyrosine metal ligand. The cysteinyl residue that forms the Tyr-Cys linkage in galactose oxidase (Cys228) also has a counterpart in the glyoxal oxidase sequence (Cys70). On the other hand, the tryptophan that overlies the Tyr-Cys thioether in galactose oxidase (Trp290) (Fig. 1) does not appear to be conserved, being replaced by histidine (His153) in the glyoxal oxidase sequence. However, this replacement is conservative in that it would preserve the hydrogen bonding characteristic of the indole ring in the pyrrole-type bonding of the imidazole ring, and the Trp and His residues both follow a conserved IGGS pattern in their respective sequence. These sequence correlations permit a strategy of targeted mutagenesis to be used to identify catalytic residues in GLOX by conservative substitution.

Heterologous Expression—Expression levels for GLOX C70A, Y135F, and Y377F mutant proteins in P. pastoris high-density fermentation cultures averaged 80-fold higher than the levels for the same protein variant in A. nidulans shake cultures (1–2 g/liter from Pichia cultures compared with 25–40 mg/liter for Aspergillus expression). GLOX(C70A), which had relatively low expression levels in both systems, was produced at 100 mg/liter in Pichia fermentation but was detectable in only trace amounts in Aspergillus culture. One of the mutants (H153W) could not be detected in either case.

Y377F—GLOX(Y377F) was isolated from expression cultures as a copper complex containing slightly substoichiometric amounts of metal ion (0.7 (Pichia) or 0.75 (Aspergillus) copper/active site) but with 4 orders of magnitude lower catalytic activity than the wild type enzyme (Table I). Optical spectra for the red-colored Y377F complex (Fig. 3, right, solid line) exhibit moderately strong absorption in the visible region, associated with the metal ion, that are resolved by oppositely-signed circular dichroism bands (ABS λ (nm) (ε (m⁻¹ cm⁻¹))): 340 (1180), 535 (1300), 700 (520); CD λ (nm) (ΔεCD (m⁻¹ cm⁻¹)): 360 (−1.0), 460 (−1.3), 560 (−3.1), 720 (+2.3)). Azide binding to the mutant (K_a = 7 nm) gave rise to characteristic absorption in the near UV close to 390 nm and slightly red-shifting the lower energy absorption bands (ABS λ (nm) (ε (m⁻¹ cm⁻¹)): 380 (2500), 558 (1050), 760 (710); CD λ (nm) (ΔεCD (m⁻¹ cm⁻¹)): 400 (−3.0), 565 (−3.0), 740 (+2.8)). The spectra of the complex reflect almost no sensitivity to pH over the range 5.4 to 8.2 (Fig. 4, top), with only a slight red shift of the absorption at the higher pH range. Treatment of the Y377F mutant with a powerful oxidant (Ir(IV)) leads to dramatic increase in the intensity of the visible absorption, with appearance of a strong band near 450 nm and a weaker, broader feature near 900 nm in the near IR (Fig. 3, left, solid line). Absorption λ (nm) (ε (m⁻¹ cm⁻¹)): 388 (4500), 485 (3200), 550 (2700), 650 (2700), 900 (1500); CD λ (nm) (ΔεCD (m⁻¹ cm⁻¹)): 555 (−1.6), 740 (−2.4), 540 (−2.6), 705 (+1.5), 895 (−1.9)). Azide was bound to this complex with high affinity (K_a < 15 μM), leading to the appearance of a near UV feature at 388 nm but almost no change in intensity in the near IR absorption band. Near UV absorption gave rise to unusual strong CD signals, whereas there was almost no change in CD in the near IR region in the presence or absence of anion (Fig. 3, left, dashed line) (ABS λ (nm) (ε (m⁻¹ cm⁻¹)): 388 (4500), 485 (3200), 550 (2700), 650 (2700), 900 (1500); CD λ (nm) (ΔεCD (m⁻¹ cm⁻¹)): 385 (−10.0), 500 (+4.5), 572 (−2.8), 875 (−1.4)).

The EPR spectrum of the Y377F mutant in the absence of oxidant (Fig. 5A) was associated with ground state parameters characteristic of a tetragonal Cu(II) complex: g_⊥ = 2.01, g_∥ = 2.27; a_g^2 = 174 G. Quantitation of the EPR signal (by double integration calibrated by a spin standard) indicates that it represents 0.6 spins/active site. The envelope of the m_1 = +½ copper hyperfine feature (ΓFWHM = 38 G) is structured, five components being resolved in the derivative of the experimental EPR spectrum in a symmetric pattern with 12 G splitting (data not shown). The g_⊥ region of the spectrum is richly structured. The EPR spectrum is perturbed in the presence of NaN_3 (Fig. 5B) (g_⊥ = 2.01, g_∥ = 2.25; a_g^2 = 176 G). Quantitation of the EPR signal (by double integration calibrated by a spin standard) indicates that it represents 0.6 spins/active site and the envelope of the m_1 = +½ copper hyperfine feature is broadened (ΓFWHM = 45 G). Addition of an excess of Ir(IV) to the sample virtually eliminated the Cu(II) EPR absorption and a new signal appeared near the free-electron g value (g = 2.0), the entire spectrum quantitating to 0.02 spins/active site (Fig. 5C). Expanding the g = 2 signal (Fig. 6) reveals a structured spectrum that can be simulated (Fig. 6, bottom) in terms of a nearly axial electronic g-tensor and hyperfine coupling to two dissimilar protons.

C70A—GLOX (C70A) was isolated as a metal-free apoenzyme (<0.1 copper/active site) that could be reconstituted by anaerobic incubation with CuCl to restore the full complement of copper to the enzyme (Table I). Both apo and metallated forms exhibited dramatically reduced catalytic activity, and a 100-fold increase in copper content on reconstitution resulted in less than 2-fold increase in the measured specific activity. The optical spectrum of the reconstituted C70A mutant (Fig. 4, lower, A) exhibited absorption across the visible region with a resolved band near 465 nm and a shoulder near 700 nm (ABS λ (nm) (ε (m⁻¹ cm⁻¹)): 465 (850), 700 (320)); the EPR spectrum of the reconstituted protein (Fig. 5D) lacks structure in the g⊥ region, with g_∥ = 2.05, g_⊥ = 2.26; a_g^2 = 178 G. This spectrum quantitates to 0.95 spins/active site.

### Table I

| Specific activity | Copper content |
|------------------|----------------|
| Units/mg | % | mol/mol active site |
| wt (Pichia) | 24.3 | 100 | 0.7 |
| wt (Aspergillus) | 24.6 | 101 | 0.75 |
| Y377F (Pichia) | 4.4 x 10⁻³ | 0.02 | 0.7 |
| Y377F (Aspergillus) | 2.8 x 10⁻² | 0.1 | 0.75 |
| C70A (apo) | 2.9 x 10⁻³ | 0.01 | 0.01 |
| C70A (+Cu) | 4.3 x 10⁻³ | 0.02 | 1.1 |
| Y135F (apo) | 2.4 x 10⁻³ | 0.01 | 0.03 |
| Y135F (+Cu) | 9.0 x 10⁻³ | 0.04 | 1.1 |

* Reconstituted with copper.
Correctly identifies active site residues is that these groups occur in a conserved context and that the spacings are well preserved between the two sequences, with the modularity of the super-barrel structure (27) determining the sequence distance between non-adjacent metal ligands. Thus, the gap between the two tyrosines that serve as metal ligands in galactose oxidase (Tyr<sup>272</sup> and Tyr<sup>495</sup>) is 223 residues, representing four wedges containing an average of 55 residues. A similar gap (242 residues) occurs between the (putative) active site tyrosines (Tyr<sup>135</sup> and Tyr<sup>377</sup>) in the GLOX sequence. The similarity of sequence context for the critical residues further supports the significance of the sequence correlations and their identification as structural motifs.

Mutagenesis of the putative active site residues provides a crucial experimental test of the predictions provided by these sequence comparison studies. While in vitro site-directed mutagenesis of structural genes has become straightforward, systematic expression of the protein products is more problematic. We have found it difficult to consistently prepare stable transformants for high level production of GLOX protein in the <i>A. nidulans</i> expression system that has been useful for production of wild type protein, and have explored the methylotrophic yeast <i>P. pastoris</i> (36, 37) as an alternative heterologous expression host. In <i>P. pastoris</i>, it is possible to directly select (by antibiotic resistance) for rare multicopy integrants that tend to produce the highest levels of protein through gene dosage effects. In addition, the strong induction provided by the alcohol oxidase (AOX1) promoter in <i>P. pastoris</i> and the efficient secretory pathways in this organism make it an extremely useful system for high level extracellular expression of recombinant proteins (36, 37), allowing us to produce up to 2 g/liter of protein in high density fermentation for three of the four mutants chosen for this study. For the three highly expressed mutants (C70A, Y135F, and Y377F) the mutation involves conservative substitution of the wild type Glyoxal oxidase (GLOX, accession number A48296) aligned as described under “Materials and Methods.”

Y135F—Like the C70A mutant, GLOX(Y135F) was isolated as metal-free apoenzyme but could be reconstituted to full metal content (Table I). Similarly, the enzyme exhibited less than 0.05% of the wild type glyoxal oxidase catalytic activity in either metallated or apoforms. The copper complex exhibited weak and poorly resolved absorption in the visible spectrum (α<sub>max</sub> = 160 μ<sup>-1</sup> cm<sup>-1</sup>) (Fig. 4, lower, B). The EPR spectrum (Fig. 5E) reflects the full complement of enzyme-bound copper (1.0 spins/active site), and exhibits a rhombic splitting of g values (g<sub>||</sub> = 2.05, g<sub>⊥</sub> = 2.26; α<sub>Cu</sub><sup>2+</sup> = 178 G) lacking resolved structure in the g<sub>⊥</sub> region.

**DISCUSSION**

The crystal structure for galactose oxidase (13) shows that its active site, comprising four metal binding residues (Tyr<sup>272</sup>, Tyr<sup>495</sup>, His<sup>581</sup>, and His<sup>582</sup>) together with a covalently attached cysteine (Cys<sup>228</sup>), is located in a distinct catalytic domain (Fig. 2). The sequence context for the critical residues further supports the significance of the sequence correlations and their identification as structural motifs.
perfine broadening in the EPR spectrum, due to the additional $^{14}$N interaction in the complex. Together, these results confirm the assignment of tyrosine Tyr$^{377}$ to the "axial tyrosine" in the GLOX active site corresponding to Y495 in GAOX. The GLOX(Y377F) mutant retains the ability to form a radical-Cu(II) complex (shown by the disappearance of the Cu(II) EPR signal) (5) but this complex is inactive, as expected based on our previous identification of the axial tyrosine requirement for substrate activation in the radical-copper oxidase turnover mechanism. The absence of any significant Cu(II) signal in the
oxidized complex contrasts with results reported for the axial tyrosine mutant of GAOX (Y495F) where only ~10% of the expected radical-coupled copper complex was formed (24).

The optical spectrum of the oxidized Y377F mutant (Fig. 3, left) exhibits strong absorption features that correspond to the Tyr\textsuperscript{OFF} form of the radical-containing wt GLOX (Fig. 3, left, inset). The absence of an intense near IR absorption feature of the oxidized wt GLOX, previously assigned to a ligand-to-ligand charge transfer absorption between axial and radical tyrosine ligands in galactose oxidase, provides additional evidence that Tyr\textsuperscript{377} is the axial tyrosine ligand in GLOX. The observation that azide binding does not affect the near IR absorption feature strengthens the correlation with the Tyr\textsuperscript{OFF} form of the active wt enzyme. The oxidized Y377F protein exhibits a minority free radical EPR signal (Fig. 5C) (<0.02 spins/active site) that probably arises within the ~30% apoprotein in the sample. A similar signal is observed in wt enzyme under the same conditions (5). The signal is characteristic of a phenoxy radical (g\textsubscript{av} = 2.006) and spectral simulation requires a relatively axial g tensor that is the signature of the Tyr-Cys radical species (Fig. 6). The g values are slightly larger than expected for the Tyr-Cys radical in galactose oxidase, indicating distinct radical environments in the two proteins. The observation of a redox active Tyr-Cys group in the protein demonstrates that although Tyr\textsuperscript{377} is required for turnover it is not essential for biogenesis of the radical cofactor.

Two mutants that are predicted to affect the equatorial ligation of copper (C70A and Y135F) are both isolated mainly as apoprotein, although reconstitution restores full metal content and permits spectroscopic characterization of the complex. Reconstituted C70A mutant exhibits an optical spectrum characteristic of Cu(II)-phenolate complexes (Fig. 4, bottom, A), with absorption near 465 nm typical of phenolate to Cu(II) ligand-to-metal charge transfer, but at longer wavelength and somewhat lower intensity than found for phenolate-Cu(II) spectra of wt GLOX (Fig. 3, right, inset). The corresponding galactose oxidase C228G mutant was also reported to contain substoichiometric copper as isolated and exhibited optical spectra similar to that found here although with slightly lower intensities. In GAOX this Cys residue forms a covalent bond to the redox-active Tyr, and its substitution by an unreactive amino acid prevents formation of the mature redox cofactor. The spectra in these complexes arise from a tetragonal Cu(II) having an unmodified Tyr phenolate coordinated in the equatorial plane. The EPR spectrum of the Cu(II) complex (Fig. 5D) is relatively broad and lacking resolved ligand hyperfine structure, implying a degree of heterogeneity in the metal-binding site that is consistent with the greater mobility afforded an unreacted tyrosine residue. Y135F mutagenesis has an even greater effect on the optical spectrum (Fig. 4, bottom, B), dramatically lowering the absorption intensity with loss of the 465 nm absorption assigned to equatorial tyrosinate coordination. These features, and the absence of radical forming cofactor in GLOX(Y135F) mutant allows identification of Tyr\textsuperscript{135} as a copper ligand and the radical redox site corresponding to Tyr\textsuperscript{272} in GAOX. EPR evidence for a tetragonal Cu(II) complex (Fig. 5E) may imply that a hydroxide ion substitutes for the tyrosinate oxygen in the mutant protein, forming a tetragonal complex together with a second water and the two nitrogenous ligands.

For all three mutants, replacement of residues predicted to be essential for catalysis dramatically reduces catalytic activity (Table I), leaving a small but detectable activity approximately 10-fold greater than the background observed for a nonspecific protein control (bovine serum albumin). This activity might reflect a residual activity of the mutant active sites, as concluded in previous studies of the corresponding mutants of galactose oxidase (23). However, that interpretation cannot account for the 5-fold difference in activity observed for Y377F mutants expressed in different hosts (Aspergillus and Pichia), for proteins that are nearly indistinguishable in terms of spectra and metal content. Furthermore, the residual activity of the mutant proteins does not appear to correlate with metal content. Thus, for the C70A mutant, a 100-fold increase in copper content resulted in only a doubling of the specific activity, and similarly a 30-fold increase in copper content was associated with only 4-fold higher specific activity for reconstituted GLOX(Y135F) (Table I). The residual activity is, in fact, so minute (0.01–0.04% of wild type) as to be consistent with a minority fraction of wild type enzyme that might result, for example, from unselected spontaneous reversion or, more likely, from missense translation errors (38, 39). Missense substitution and processivity errors have been reported to rise as high as 1% during overexpression of heterologous proteins (39).

In conclusion, mutagenesis of GLOX permits identification of three catalytic residues (Cys\textsuperscript{70}, Tyr\textsuperscript{135}, and Tyr\textsuperscript{377}) and demonstrates their respective functions in the active site. Based on these studies, the active site of glyoxal oxidase can be represented as shown in Fig. 1. As proposed for GAOX, an axial tyrosine (Tyr\textsuperscript{377} in GLOX) will function as a general base reactant directly to copper in the active site. Elimination of any of these groups removes an essential catalytic function and blocks turnover.

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**Fig. 6. EPR spectrum for the GLOX(Y377F) free radical.** Top, expansion of region near free-electron g-value in Fig. 5C. Instrumental parameters: microwave frequency, 9.162 GHz; microwave power, 0.1 nW; modulation amplitude, 0.5 G; temperature, 135 K. Bottom, simulated spectrum (g\textsubscript{a} = 2.0085, g\textsubscript{b} = 2.0075, g\textsubscript{c} = 2.0020; ax = 16 G, ay = 15 G, az = 15 G; g\textsuperscript{av} = 2.006, g\textsuperscript{xx} = 11 G, g\textsuperscript{yy} = 11 G; 9.162 GHz; gaussian line shape, Γ = 4 G).
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