A free radical-coupled copper complex has been identified as the catalytic structure in the active site of glyoxal oxidase from Phanerochaete chrysosporium based on a combination of spectroscopic and biochemical studies. The native (inactive) enzyme is activated by oxidants leading to the elimination of the cupric EPR signal and the appearance of a substoichiometric free radical-copper complex. Oxidation also leads to the appearance of a substoichiometric free radical EPR signal with an average g value ($g_{av} = 2.0055$) characteristic of phenoxyl or $\tau$-radicals arising from a minority apoenzyme fraction. Optical absorption, CD, and spectroelectrochemical measurements on the active enzyme reveal complex spectra extending into the near IR and define the redox potential for radical formation ($E_{1/2} = 0.64$ V versus NHE, pH 7.0). Resonance Raman spectra have identified the signature of a modified (cysteinyl-tyrosine) phenoxyl in the vibrational spectra of the active complex. This radical-copper motif has previously been found only in galactose oxidase, with which glyoxal oxidase shares many properties despite lacking obvious sequence identity, and catalyzing a distinct reaction. The enzymes thus represent members of a growing class of free radical metalloenzymes based on the radical-copper catalytic motif and appear to represent functional variants that have evolved to distinct catalytic roles.

The white-rot wood-metabolizing basidiomycete fungi are major degraders of lignin contributing essential chemistry to the global carbon cycle. Phanerochaete chrysosporium, the organism most extensively studied for its lignin-degrading ability, produces three classes of extracellular enzyme under ligninolytic conditions: lignin peroxidase, manganese peroxidase, and glyoxal oxidase (1, 2). In the presence of $\mathrm{H}_2\mathrm{O}_2$ (3), lignin peroxidases oxidize and partially depolymerize lignin or lignin model compounds (4–9). The oxidizing peroxide cosubstrate for this reaction must be generated in situ for efficient turnover of extracellular lignin peroxidase, a function performed by glyoxal oxidase, which catalyzes the oxidation of a number of aldehyde and alpha-carbonyl compounds, reducing $\mathrm{O}_2$ to $\mathrm{H}_2\mathrm{O}_2$ in the process. The enzyme exhibits a broad substrate specificity for oxidation of simple aldehydes to the corresponding carboxylic acids, as shown by Reaction 1 (10).

$$\text{RCHO} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{RCO}_2\text{H} + \text{H}_2\text{O}_2$$

**REACTION 1.**

Two of the substrates for glyoxal oxidase (glyoxal (CHOCHO) and methyglyoxal (CH$_3$CHOCHO)) are found in extracellular fluid of ligninolytic cultures (10) and are likely to represent physiological substrates for the enzyme in a complex extracellular metabolic scheme (9, 10). Purified glyoxal oxidase is catalytically inactive but can be activated by lignin peroxidase (11, 12), suggesting a possible extracellular regulatory circuit for the control of $\mathrm{H}_2\mathrm{O}_2$ production by glyoxal oxidase, and lignin peroxidase activity by $\mathrm{H}_2\mathrm{O}_2$.

P. chrysosporium glyoxal oxidase has been purified to homogeneity (11), doned for high level expression in Aspergillus nidulans (13), and its biochemical properties have been determined (11). Glyoxal oxidase is an acidic monomeric glycoprotein with a naked molecular mass of 57 kDa (calculated from the nucleotide sequence; Ref. 14) requiring one g-atom of copper for full activity. In its catalytic reaction and copper requirement, glyoxal oxidase resembles another extracellular fungal enzyme, galactose oxidase (15, 16) which efficiently catalyzes the conversion of alcohols to aldehydes with formation of hydrogen peroxide. Galactose oxidase (from Dactylium dendroides) contains copper and an essential protein free radical that is required for catalysis (16). The active site structure of galactose oxidase as revealed by crystallography (17) is illustrated in Scheme 1, showing the coordination of the copper ion by 2 histidine residues (His-496 and His-581), a simple tyrosinate (Tyr-495), and a covalently modified tyrosine (Tyr-272), cross-linked to a cysteinyl residue (Cys-228) to form a new, dimeric amino acid (cysteinyl-tyrosine). This feature has been identified by spectroscopic and modelling studies as the radical-forming site in galactose oxidase (18–20).

Comparison of the nucleotide sequences for glyoxal oxidase and galactose oxidase shows no obvious homology in primary structure (14, 21), with less than 20% amino acid sequence similarity based on standard sequence alignment algorithms. However, the present spectroscopic studies show a remarkable degree of similarity between the two enzymes at the level of active site structure and chemistry. Identifying a free radical-coupled copper catalytic motif in glyoxal oxidase. Spectroscopic and biochemical comparison of the two proteins suggests that glyoxal oxidase and galactose oxidase are functional variants catalyzing distinct reactions at nearly identical active sites.
MATERIALS AND METHODS

Recombinant P. chrysosporium glyoxal oxidase was isolated from growth medium of a stable transformant of A. nidulans (13) grown in Aspergillus transformant minimal medium containing 1% maltose as growth substrate, 70 mM NaNO₃, 50 mM KH₂PO₄, 7 mM KCl, 2 mM MgSO₄, 100 μg/ml methionine, 1 μg/ml biotin, and trace elements (22) at pH 4.6. Glyoxal oxidase was purified as described previously (11, 14) except that the protein was first concentrated from culture media by ammonium sulfate precipitation (23). Enzyme activity was measured polargraphically with an oxygen-sensitive electrode using methylglyoxal as substrate in the presence of the oxidant Na₂IrCl₆. For phosphate inhibition studies, glyoxal oxidase activity was measured using the oxygen electrode with dimethylsulfoxide buffer (pH 4.5) or MES¹ buffer (pH 6.0). Redox-activated glyoxal oxidase was prepared by addition of a slight excess of Na₂IrCl₆ to the enzyme solution. Protein concentration was determined by the method of Lowry (24).

Absorption spectra were recorded on a Varian Cary 5E UV-vis-NIR spectrometer interfaced to a microcomputer for data acquisition. EPR spectra were acquired on an ER300 EPR spectrometer equipped with X-band microwave bridge and a nitrogen flow system. Quantitative EPR measurements were performed as described previously (25). Circular dichroism (CD) spectra were recorded on an Aviv model 41DS UV-vis-NIR CD/MCD spectrometer (26). Copper analyses were performed on a Varian SPECTRAA 20B atomic absorption spectrometer equipped with a GTA-96 graphite furnace. Spectrophotometric titrations were performed on a Hitachi U2000 UV-vis absorption spectrometer. Spectroelectrochemical studies on glyoxal oxidase used a special thin layer optical cell based on modification of a commercial demountable IRE cell (Spectra-Tech demountable liquid sampling cell) similar in plan to a cell recently described for IR spectroelectrochemistry applications (27). The cell is constructed by supporting a 1000 lines/inch gold grid working electrode (Buckbee-Mears Co., St. Paul, MN) between quartz disks separated by a 0.2 mm polyvinylchloride gasket, using silver/saturated AgCl reference electrode (0.22 V versus NHE; Ref. 28) and a platinum wire counter electrode. Electroanalytical measurements were performed with an EG&G model 263 potentiostat interfaced with a microcomputer for data acquisition and analysis. Resonance Raman spectra were collected with a custom McPherson 2061/207 spectograph (0.67-m focal length, 600-groove grating, 7 cm¹ spectral resolution) using a Coherent Innova 302 krypton (647 nm) laser, a Kaiser optical super-notch filter, and a Princeton Instruments (LN-1100PB) liquid N₂-cooled CCD detector. Spectra were obtained on samples in glass capillaries at 278 K using a 90° scattering geometry and a 10-min accumulation time. Sample integrity was verified by the observation of the same absorption spectrum (typical of active enzyme) before and after laser irradiation.

RESULTS

Redox Characterization

Purified native glyoxal oxidase is catalytically inactive, but can be oxidatively activated by lignin peroxidase (11) or by high potential inorganic oxidants including molybdicyanide (K₃Mo(CN)₈) or hexachloroiridate (Na₂IrCl₆), or Mn₃EDTA (which reacts relatively slowly). The redox-activated enzyme is unstable and reversibly loses activity on dialysis or exposure to chromatographic media, reverting to the reduced, inactive form. Native glyoxal oxidase exhibits a cupric EPR signal at low temperatures, which is completely eliminated by addition of strong oxidants (Fig. 1). Spin quantitation of this EPR signal shows that it represents 0.7–0.8 equivalents of Cu²⁺/enzyme, identical to the total copper content determined by atomic absorption spectroscopy. The disappearance of the EPR signal on oxidation is similar to the behavior previously reported for galactose oxidase (25, 29) and is consistent with the formation of a free radical-copper center (25), resulting in an EPR-silent oxidized complex with an antiferromagnetically coupled electronic ground state (16). Like galactose oxidase (25, 30), glyoxal oxidase typically contains substochiometric copper (0.7–0.8 Cu²⁺/mol) and the oxidized protein exhibits a minority EPR signal arising from the presence of a small amount of apoenzyme radical (Fig. 2). The radical signals for the two enzymes are very similar, each having an average g value near 2.005 characteristic of phenoxyl radicals and revealing partly resolved hyperfine structure. In galactose oxidase the spectrum has been assigned by isotopic substitution (30) and ENDOR analysis (18) to the novel cysteinyl-tyrosinate phenoxyl (Cys-TyrO') with hyperfine interaction between the α-electron of the phenoxyl radical and one β-methylene proton and a single α-ring proton of the tyrosyl residue. The strong spectroscopic correlations between the two enzymes are developed in more detail below.

Spectroelectrochemical measurements on glyoxal oxidase (Fig. 3, left) provide an estimate of the redox potential of the radical-forming site. In these studies, the growth of intense absorption features associated with the radical-containing protein (see below) are used to monitor the progressive redox conversion of the sample, and the absorption/potential data are analyzed in terms of the Nernst equation (Equation 1).

\[ E = E_0^{ox} + \frac{RT}{nF} \ln \left( \frac{A - A_{red}}{A_{ox} - A} \right) \] (Eq. 1)

A is the sample absorbance, A_{red} and A_{ox} are the limiting absorbances for fully reduced and fully oxidized enzyme, respectively. R is the gas constant (8.314 J/K·mol), T is the absolute temperature, n is the number of electrons involved in the redox couple, and F is the Faraday constant (96.48 kC/mol). A Nernst plot of the absorption intensities through the oxidation step (Fig. 3, right) averaged between increasing and decreasing potential scans is linear (r² = 0.995) with a slope of 0.048 mV consistent with a single-electron step for the oxidation (n = 1.2) and a midpoint potential (E_{1/2} = 0.42 V versus Ag/AgCl; 0.64 V versus NHE) significantly higher than that found for galactose oxidase (0.4 V versus NHE at pH 7.3; ref. 31). This is consistent with the requirement for a relatively high potential oxidant for activation of glyoxal oxidase (Na₂IrCl₆ (0.892 V; Ref. 32) or K₃Mo(CN)₈ (0.798 V; Ref. 33)) compared to galactose oxidase, which is completely converted to the active, radical-containing form by milder oxidants like ferricyanide (0.424 V; Ref. 34). Activation of glyoxal oxidase by

¹ The abbreviations used are: MES, 3-(N-morpholino)ethanesulfonic acid; LMCT, ligand-to-metal charge transfer.
² E. S. Schweizer and J. W. Whittaker, unpublished results.
diffusible high potential manganese chelates (illustrated here by Mn$^{3+}$ EDTA oxidation) may have a physiological role in regulation of the extracellular activity of glyoxal oxidase through the metabolic networking of the ligninolytic enzymes (1, 2). The biological significance of this interaction is presently under further study. The stability of the redox-activated proteins (reflecting the stability of the free radical sites) differs between glyoxal oxidase and galactose oxidase. While the latter is stable for days at 4 °C (25), the stability of the active glyoxal oxidase is markedly lower, with a half-life of 4 h for the radical under similar conditions (50 mM sodium phosphate buffer, pH 6.5).

Spectroscopic Correlations between Glyoxal Oxidase and Galactose Oxidase

Optical Absorption Spectra—The optical spectra of redox-activated and native (inactive) glyoxal oxidase are shown in Fig. 4 (Table I), with the corresponding spectra previously obtained for galactose oxidase included for comparison. Blue, inactive glyoxal oxidase exhibits strong absorption in the visible spectrum at 451 and 678 nm (at 448 and 570 nm) that may be assigned as phenolate-to-Cu$^{2+}$ ligand-to-metal charge transfer (LMCT) and mixed LMCT and cupric ligand field (d → d) absorption, respectively, by analogy with the corresponding features in galactose oxidase (35). These assignments are further supported by resonance Raman spectroscopy (see below). Activation by oxidation of the protein results in conversion to a green form with the appearance of intense absorption bands in the blue ($\varepsilon_{448} = 5700 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and red ($\varepsilon_{433} = 4300 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The unusual red absorption band extending deep into the near IR region has been assigned in galactose oxidase as arising from ligand-to-ligand charge transfer associated with the presence of both a Cys-Tyr free radical ligand and an unmodified tyrosinate in the active site metal complex (36). The spectra for galactose oxidase and glyoxal oxidase are strikingly similar, suggesting nearly identical environments for the active site metal ions, in spite of the lack of obvious sequence homology between the two proteins (34).

The sensitivity of the active site of glyoxal oxidase to protons is reflected in spectrophotometric titrations. While the spectrum of the redox activated glyoxal oxidase is nearly unchanged over a wide pH range (4.5–8.5), more dramatic pH-dependent changes occur in spectra of the inactive enzyme (Fig. 5). Titration of inactive glyoxal oxidase in 100 mM potassium phosphate buffer converts the enzyme to a purple form at low pH ($\varepsilon_{522} = 1365 \text{ M}^{-1} \cdot \text{cm}^{-1}$), maintaining two isosbestic points (at 504 and 568 nm) that identify the reaction as a simple two-state transition. More detailed analysis by Henderson-Hasselbach fitting of the titration data gives an estimate for the pKₐ of the protein group involved in the protonation step (pKₐ = 7.7) (Fig. 5, inset). The spectral changes associated with the pH-dependent structure change (loss of 451 nm phenolate-to-Cu$^{2+}$ LMCT absorption at low pH) suggest that the titratable group is a coordinated tyrosinate, ionizing more readily in the metal complex than is typical of the free amino acid (pKₐ = 10.1; Ref. 37) and dissociating from the metal on protonation. The assignment to the unmodified tyrosinate (corresponding to Tyr-495 in the active site of galactose oxidase, Scheme 1) is supported by the observation that resonance Raman profiles at low pH reveal only vibrational modes of a Cys-Tyr group, whereas at high pH contributions from both tyrosinates are observed (see below).

In glyoxal oxidase, the acid titration of the coordinated tyrosinate reflects a perturbation of the phenolate basicity by approximately 2.5 pH units in the metal complex. The lack of sensitivity to protons for the corresponding low and high pH spectra of the redox activated enzyme implies a more dramatic perturbation of phenolate basicity in the radical-copper complex. This difference can be understood in terms of the decrease in overall charge of the ligand set associated with oxidation of the coordinated cysteinyl-tyrosinate anion to generate the neutral (Cys-TyrO$^-$) phenoxyl, making the copper a more strongly acidic metal center (38). The modulation of ligand acidity by metal interactions is an essential feature of ligand participation in catalysis in galactose oxidase (16) (see below). In that...
enzyme, the spectra of both active and inactive forms are relatively insensitive to pH, but there is evidence for a temperature-dependent proton transfer equilibrium in the active site and for proton-coupled anion binding (38).

Anion Interactions—Glyoxal oxidase is inhibited by phosphate in a pH-dependent fashion with $K_i$ values in the millimolar range ($K_i$ (pH 4.5) $\approx 5.1\text{ mM}$; $K_i$ (pH 6.0) $\approx 1.0\text{ mM}$) correlating with the ionization of the weak acid and preferential binding of the dibasic ion. Unlike azide, which blocks turnover by binding to copper (see below), phosphate has no significant effect on the optical spectrum of redox-activated enzyme, indicating that the inhibition is not a consequence of direct interaction with the active site metal ion and may result from steric effects, such as blocking substrate access to the active site. Inhibition of enzyme activity by phosphate may be physiologically important for regulation of glyoxal oxidase activity and can be expected to be a significant factor in environmental chemistry.

Azide acts as an analog of peroxide in oxygen-metabolizing enzymes, forming stable complexes that mimic reactive oxygen species. Addition of azide to both native and redox-activated forms of glyoxal oxidase leads to the appearance of new spectral features (Fig. 6, Table I), that are again similar to those observed for the azide complexes of galactose oxidase and other copper proteins (39–42). By analogy, the intense absorption near 380 nm is assigned to azide-to-Cu$^{2+}$ LMCT. The lower energy absorption bands have more metal-centered d character, as indicated by their relatively large CD anisotropy (see below). As previously observed for galactose oxidase (25, 38), there is a significant difference in affinity for azide between active and inactive forms; active glyoxal oxidase binds azide much tighter ($K_D = 0.5\text{ mM}$) than the native enzyme ($K_D = 25\text{ mM}$). Although the trend in affinity parallels the results for galactose oxidase, the dissociation constants for both forms are approximately 25 times (active) and 130 times (native) larger than the corresponding values found for that enzyme.

The similarity in the shifts in absorption bands between the two enzymes on binding exogenous ligands is particularly interesting in view of the evidence for pseudorotation of the copper complex and an intrinsic proton transfer coordinate in the active site of galactose oxidase, relating to substrate activation in catalysis (16, 38). Previous studies on galactose oxidase have demonstrated that coordination of an exogenous ligand results in a displacement of the unmodified tyrosinate (Tyr-495) through a fluxional interchange of strong and weak interactions in the metal complex that can be regarded as a pseudorotation distortion of the active site (16). Displacement of the tyrosinate ligand increases its basicity, allowing it to serve as a general base for a proton abstraction step in catalysis (38). In galactose oxidase, the pseudorotation mechanism leads to a thermodynamic linkage between anion binding and proton uptake (16). The close correspondence in the spec-
troscopic behavior of the two proteins under ligand perturbations indicates that not only are the two active sites structurally related but that these dynamical motifs are reproduced as well.

Circular Dichroism Spectra—Because of the signed character of CD intensity, circular dichroism spectroscopy increases the resolution of absorption spectra. The CD spectrum of active glyoxal oxidase (Fig. 7A) resolves six visible absorption bands, closely matching features observed in CD for the aquo complex of galactose oxidase (25, 38), but shifted to lower energy consistent with a weaker ligand field for the copper complex in the former enzyme. Even at this higher resolution, the similarity between the two enzymes is preserved in both intensity and sign of the polarization. For native glyoxal oxidase (Fig. 7B), the strongest polarization occurs in the ligand field absorption bands at 650 and 800 nm. In the redox-activated enzyme, the ligand field spectra, identified by their relatively large CD anisotropy ($\Delta \alpha / \alpha$) are shifted to higher energy (600 nm) compared to the inactive form. Azide binding shifts the ligand field spectra of both enzyme forms to higher energy as expected for conversion to a more nearly tetragonal complex, an important feature of active site ligand interactions previously studied in galactose oxidase (38, 43). The optical spectra of glyoxal oxidase and galactose oxidase are thus similar in both general features and essential details.

Resonance Raman Spectra—Resonance Raman spectroscopy is an important structural tool that provides detailed vibrational information for a specific chromophore through resonance enhancement of Raman intensities on excitation of electronic transitions. The resonance Raman spectra for glyoxal oxidase in its redox-activated form (Fig. 8A) and its azide adduct (Fig. 8C) are remarkably similar to those obtained for the corresponding forms of galactose oxidase (Fig. 8, B and D). In the case of active galactose oxidase (Fig. 8B), the features at 1170, 1246, 1499 (sh), and 1603 cm$^{-1}$ were assigned to the phenolate modes of the Tyr-495 ligand (Scheme 1) on the basis of their similarity to phenolate vibrations in other metal-tyrosinate proteins and model complexes (36). The dominant intensity of the 1246 cm$^{-1}$ C-O stretch is also typical of metal-tyrosinates (44). The absence of these four modes in the Raman spectrum of the azide adduct (Fig. 8A) suggests that this Tyr is displaced from copper due to the increased ligand field strength arising from replacement of the coordinated water by azide. Glyoxal oxidase exhibits remarkably similar resonance Raman spectra. Excitation of the active enzyme at 647 nm (Fig. 8A) reveals vibrational modes at 1170, 1249, and 1604 cm$^{-1}$, which, by analogy, can be assigned to a coordinated phenolate. These modes become the dominant RR spectral features with 458 nm excitation (data not shown), revealing that they are all due to the same chromophore and that the absorption in the 460 nm region is due primarily to phenolate $\rightarrow$ Cu$^{2+}$ LMCT. Azide

![Fig. 7. Circular dichroism spectra for glyoxal oxidase complexes.](image_url)

![Fig. 8. Resonance Raman spectra obtained with 647 nm excitation.](image_url)
binding to glyoxal oxidase again causes the disappearance of these modes (Fig. 8C), suggesting a similar release of the axial phenolate.

The copper-coordinated phenoxyl radical (Cys-Tyr 272) in active galactose oxidase is associated with another set of vibrational modes at 1382, 1487, and 1595 cm$^{-1}$ (Fig. 8B), which persist in the azide adduct (Fig. 8D) (36, 45). These three modes are characteristic of the Raman spectra of phenoxyl radicals, with the 1487 cm$^{-1}$ feature being assigned to the C-O stretch on the basis of its isotope dependence in model compounds (36, 46). The residual features at 1185, 1246, 1312, 1417, and 1439 cm$^{-1}$ in the azide complex (Fig. 8D) are also assigned to vibrations of the phenoxy radical. Active glyoxal oxidase exhibits a similar set of major features at 1375, 1486, and 1591 cm$^{-1}$ with 647 nm excitation (Fig. 8A). Both these and the minor features at 1250, 1309, 1421, and 1444 cm$^{-1}$ are again found in the azide adduct (Fig. 8C). All of these modes have diminished intensity with 458 nm excitation (data not shown), indicating that they are all due to the same chromophore. The similarity of the vibrational frequencies and intensities to those of galactose oxidase allows us to conclude that active glyoxal oxidase has a similar (Cys-Tyr) phenoxyl radical as a copper ligand.

The native (inactive) form of glyoxal oxidase exhibits distinctive resonance Raman features for both the unmodified tyrosinate and Cys-Tyr ligands (Table II). At pH 8.1 where both ligands are coordinated, 458 nm excitation selectively enhances the vibrations of the unmodified tyrosinate at 1171, 1260, and 1591 cm$^{-1}$, whereas 647 nm excitation selectively enhances the vibrations of the Cys-Tyr ligand at 1257, 1481, and 1596 cm$^{-1}$. At pH 6.1, only vibrational modes of the Cys-Tyr are observed at 1240, 1486, and 1596 cm$^{-1}$. The decrease in energy of the C-O stretch from 1267 to 1240 cm$^{-1}$ is explained by an increase in the Cu-O bond strength of the Cys-Tyr ligand on protonation and dissociation of the other Tyr ligand. Similarly, in the presence of azide, only Cys-Tyr modes are observed at 1272, 1483, and 1599 cm$^{-1}$. Thus, as in the case of the oxidized enzyme, the resonance Raman profiles again indicate that the unmodified tyrosinate ligand no longer binds to copper in the presence of azide and also does not bind at low pH. The enhancement of the Cys-Tyr modes with 647 nm excitation demonstrates that the unusually intense absorption bands in the 600–650 nm region of the reduced enzyme (Figs. 4 and 6) have substantial Cys-tyrosinate → Cu$^{2+}$ charge transfer character in addition to the Cu$^{2+}$ ligand field character indicated by CD spectroscopy (Fig. 7).

EPR Spectra—The EPR spectrum recorded for native glyoxal oxidase (Fig. 1) is characteristic of a cupric ion in a tetragonal ligand environment. The g values measured at the turning points of the EPR absorption spectrum ($g_1 = 2.28$; $g_2 = 2.08$; $g_3 = 2.03$) and the copper nuclear hyperfine splitting ($a_1$ (copper) = 172 G) closely reproduce the values for the inactive cupric galactose oxidase complex (25, 38). Additional structure present on the copper hyperfine features can be resolved into five components with a uniform splitting of 10.5 G consistent with a small transferred ligand hyperfine interaction arising from a pair of equivalent I = 1 nuclei (2nI_1 +1 = 5; n = 2). An interpretation of the nearly identical structure observed in EPR spectra for galactose oxidase in terms of ligation by 2–3 nitrogen nuclei (14N, I = 1) (47) has been confirmed by the results of protein crystallography showing coordination of the active site copper by 2 histidines (17). Based on the extraordinary spectroscopic correlations between the two enzymes, it appears likely that this structural pattern also appears in the metal binding site of glyoxal oxidase.

Contrasts between the Two Enzymes

In spite of the spectroscopic similarities of glyoxal oxidase and galactose oxidase, significant differences exist that clearly distinguish the two enzymes, including the redox potential and stability of the free radical, anion affinity, and catalytic chemistry. The reactions catalyzed by the two enzymes are distinct and there is little overlap between their substrates. Galactose oxidase selectively oxidizes primary alcohols (15) (although slow oxidation of aldehydes has recently been detected; Ref. 48), while the reverse is true for glyoxal oxidase, with the best aldehyde substrate (methylglyoxal) supporting nearly 100% turnover (11). The substrates recognized by glyoxal oxidase in terms of ligation by 2–3 nitrogen nuclei (14N, I = 1) (47) has been confirmed by the results of protein crystallography showing coordination of the active site copper by 2 histidines (17). Based on the extraordinary spectroscopic correlations between the two enzymes, it appears likely that this structural pattern also appears in the metal binding site of glyoxal oxidase.

Conclusions

Spectroscopic and biochemical characterization of the active sites of glyoxal oxidase and galactose oxidase reveals an amazing degree of similarity between two proteins essentially lack-
ing any obvious sequence homology and catalyzing distinct reactions. As a result of the spectroscopic similarities between the two enzymes, much is already known about the active site of glyoxal oxidase by analogy with the crystallographically characterized galactose oxidase structure. Aside from the redox tuning of the free radical side chain, the basic features of the catalytic complexes appear to be nearly identical in the two proteins. Resonance Raman spectra provide strong evidence for catalytic complexes and demonstrate that this unusual covalent modification occurs in contexts other than galactose oxidase. Crystallographic studies on glyoxal oxidase currently in progress\(^3\) can be expected to provide the basis for more extensive structural comparisons of the two proteins.

\(^3\) H. Baker, M. M. Whittaker, J. W. Whittaker, P. J. Kersten, and E. P. Baker, unpublished results.

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