Ligand interactions with galactose oxidase: mechanistic insights

Mei M. Whittaker and James W. Whittaker
Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213 USA

ABSTRACT Interactions between galactose oxidase and small molecules have been explored using a combination of optical absorption, circular dichroism, and electron paramagnetic resonance (EPR) spectroscopies to detect complex formation and characterize the products. Anions bind directly to the cupric center in both active and inactive galactose oxidase, converting to complexes with optical and EPR spectra that are distinctly different from those of the starting aquo enzyme. Azide binding is coupled to stoichiometric proton uptake by the enzyme, reflecting the generation of a strong base ($pK_a > 9$) in the active site anion adduct. At low temperature, the aquo enzyme converts to a form that exhibits the characteristic optical and EPR spectra of an anion complex, apparently reflecting deprotonation of the coordinated water. Anion binding results in a loss of the optical transition arising from coordinated tyrosine, implying displacement of the axial tyrosine ligand on forming the adduct. Nitric oxide binds to galactose oxidase, forming a specific complex exhibiting an unusual EPR spectrum with all g values below 2. The absence of Cu hyperfine splitting in this spectrum and the observation that the cupric EPR signal from the active site metal ion is not significantly decreased in the complex suggest a nonmetal interaction site for NO in galactose oxidase. These results have been interpreted in terms of a mechanistic scheme where substrate binding displaces a tyrosinate ligand from the active site cupric ion, generating a base that may serve to deprotonate the coordinated hydroxyl group of the substrate, activating it for oxidation. The protein–NO interactions may probe a nonmetal O_2 binding site in this enzyme.

INTRODUCTION

Galactose oxidase is a fungal copper metalloenzyme that catalyzes the oxidation of primary alcohols to aldehydes, coupling that reaction to the reduction of dioxygen to hydrogen peroxide (1). Galactose oxidase exhibits a surprisingly low specificity for the primary alcohol but is completely regioselective, and no secondary alcohols are known to be substrates for this enzyme (1, 2). This regioselectivity of the enzymatic reaction suggests potential synthetic applications (3) and has raised interest in the design of small molecule catalysts that mimic this reactivity. Detailed studies of the active site of galactose oxidase and its catalytic mechanism can provide the basis for preparation of new reagents for alcohol oxidation.

The mechanism of galactose oxidase must account for a surprising feature of the enzyme structure: the enzyme contains a single copper ion in its active site (1), for which one-electron reactivity would be expected, yet both alcohol oxidation and O_2 reduction involve two-electron redox chemistry. This apparent redox mismatch between the catalytic metal center and the reactions it catalyzes has led to a systematic and quantitative study of this active site, ultimately resulting in the identification of a new type of catalytic group, a free radical-coupled copper complex in the active form of the galactose oxidase (4–7). Both the radical and the copper ion are redox active in this complex, composing a two-electron redox unit. The participation of a free radical in the galactose oxidase reaction is of interest with regard to the emerging new field of free radical enzymology (8–10). Stabilized free radicals formed from protein side chains are being identified in a growing number of enzymes, and free radical chemistry is being recognized as an essential component of the catalytic armamentarium in biochemistry. Radicals have been identified on tyrosine, tryptophan, and glycine residues, respectively, in ribonucleotide reductase (11), cytochrome *c* peroxidase (12), and pyruvate-formate lyase (13). We have used resonance Raman spectroscopy to identify a perturbed tyrosine ligand in the radical site of activated galactose oxidase (5), and it has proven possible to generate a protein-localized radical in the metal-free apoenzyme, unmasking the characteristic electron paramagnetic resonance (EPR) signal of a free radical that isotopic labeling experiments have shown is derived from a tyrosine residue (7). These spectroscopic studies recently have been complemented by crystallographic determination of the protein structure (14). One striking feature of the active site emerging from the crystallographic studies is a novel covalent modification of a tyrosine ligand, shown at near atomic resolution to be covalently cross-linked to a cysteinyl sulfur at a ring position ortho to the phenolic hydroxyl. This unusual structure is entirely consistent with the structural requirements of the active site radical defined spectroscopically.

The crystal structure provides a valuable starting point for developing a detailed understanding of the catalytic mechanism of this free radical enzyme but leaves important questions relating to the chemistry of the site unanswered. Electronic structural features of the active site complex, including assignment of oxidation state, description of bonding, and other aspects of structure closely relating to chemistry, lie beyond the level of resolution accessible in x-ray diffraction studies on proteins. Spectroscopic methods are, on the other hand, particularly sensitive to these electronic structural details. We have applied a combination of EPR, optical absorption, and circular dichroism (CD) techniques as ground and

Address correspondence to James W. Whittaker

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excited state spectral probes, using ligand binding reactions of the active site complex to develop a deeper understanding of metal and protein interactions with small molecules in the catalytic mechanism.

MATERIALS AND METHODS

Galactose oxidase from *Dactylium dendroides* was purified as previously described (4). Reductively inactivated and redox activated galactose oxidase were prepared as previously described (4). All reagents for the preparation of buffers and ligands were obtained from commercial sources. Ligand solutions for titration studies were prepared immediately before addition to the enzyme. Both ligand and enzyme solutions used in this study were in 50 mM sodium phosphate buffer, pH 7.0. Nitric oxide from Matheson Gas Products (Twinsburg, OH) was purified by successive passage through 12 N NaOH and 50% (vol/vol) H_2SO_4 scrubbing solutions and then dried with anhydrous $CaSO_4$ and P_2O_5 before injection into anaerobic protein samples. Proton uptake experiments were performed as described by Dunn (15). All solutions were prepared with CO_2-free deionized water, obtained by boiling deionized water for 10 min and cooling under an argon atmosphere. Buffer-free galactose oxidase in 50 mM NaCl was prepared by size exclusion chromatography under argon purge. An anaerobic cuvette containing 80 μM enzyme and 80 μM thymol blue indicator in 25 mM NaCl was adjusted to pH $\sim$ 8. The absorption change (ΔA_{600}) was calibrated by addition of aliquots of standard NaOH solution, and the uptake of protons on titration of the enzyme by 2 mM NaN_3 was monitored. In control experiments, the enzyme was omitted from the reaction mixture. Absorption spectra were recorded on a Varian (Sugarland, TX) Cary 5E UV-Vis-NIR spectrometer. For low temperature absorption measurements, samples were cooled in an Air Products (Allentown, PA) Heli-Tran optical cryostat. Circular dichroism spectra were recorded on a AVIV Associates (Lakewood, NJ) model 41DS UV-Vis-NIR CD/MCD spectrometer. EPR spectra were recorded on a Brüker (Billerica, MA) ER300 EPR spectrometer equipped with X-band microwave bridge and an Oxford Instruments (Concord, MA) ESR900 helium flow cryostat. A quartz flat cell was used to hold samples for room temperature EPR experiments. Quantitative EPR measurements were obtained as previously described (4). Copper analyses were performed on a Varian SpectrAA 20B atomic absorption spectrometer equipped with a GTA-96 graphite furnace.

RESULTS

Metal–ligand interactions: chemistry

The active site Cu^{2+} of oxidized and reduced galactose oxidase forms stable complexes with a variety of anions. In the reductively inactivated blue enzyme, where the isolated cupric ion is accessible to EPR spectroscopy, CN^- and F^- previously have been shown to coordinate directly to the metal center through observation of ligand hyperfine splittings in the EPR spectra for the complexes (16, 17). Azide also has been shown to form a complex with the active site metal ion by the observation of an intense ligand-to-metal charge transfer (LMCT) transition in the near ultraviolet (UV) absorption spectrum of the azide complex (4). Recently, acetate has been shown to bind to Cu in crystals of galactose oxidase grown at low pH, being replaced by a water to form an aquo complex at neutral pH (14). Although these basic features of coordination chemistry for the cupric site in galactose oxidase have been established, accurate data

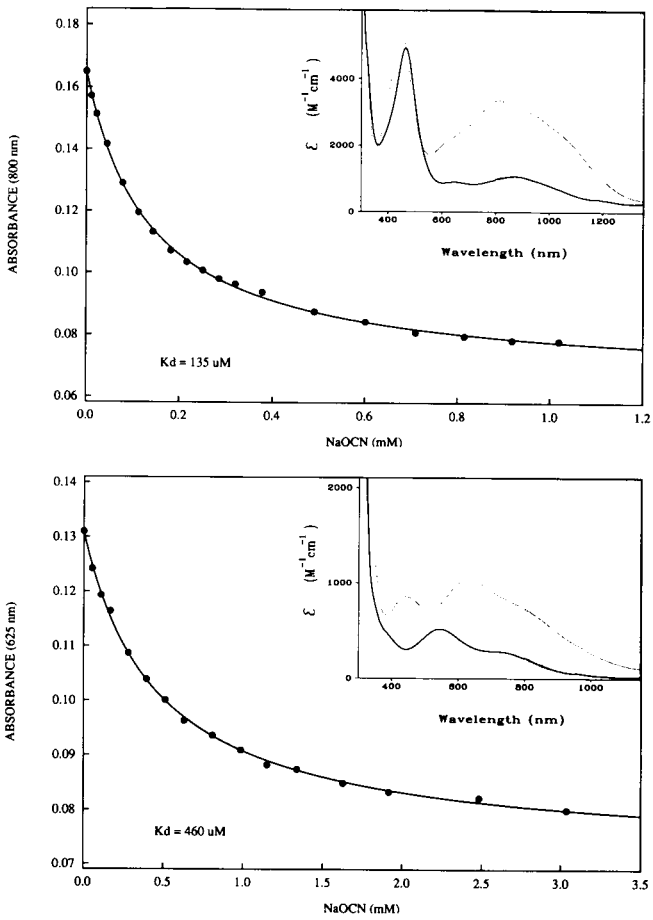

FIGURE 1 Cyanate interactions with galactose oxidase. (*Top*) Active enzyme titration data. (*Bottom*) Inactive enzyme titration data. Solid lines are theoretical single step binding isotherms. (*Insets*) Absorption spectra of starting enzyme (*dotted line*) and final complex (*solid line*).

for ligand affinities in these complexes are still lacking and the mechanistic significance of these interactions has yet to be explored.

Anion binding experiments are shown in Figs. 1 and 2 and Table 1. For each complex, the formation of the ligand adduct was monitored by the perturbation of the absorption spectrum of the enzyme, and the resulting titration data were analyzed in terms of a single step association reaction after correction of the raw data for fraction of ligand bound and dilution effects. In no case was any significant deviation from single annation observed, up to 0.1 M concentration of ligand in the case of thiocyanate. The dissociation constants for these complexes, evaluated from the data, are listed in Table 1. For each ligand, the inactive enzyme exhibits a 3- to 10-fold lower affinity than the active enzyme under identical conditions. Clean isosbestics were maintained between the spectra throughout the titration for all anions.

The stoichiometry of proton uptake by galactose oxidase when titrated by azide was measured in CO_2-free solutions buffered by thymol blue indicator dye. Addition of azide to a concentration 10 times the dissociation

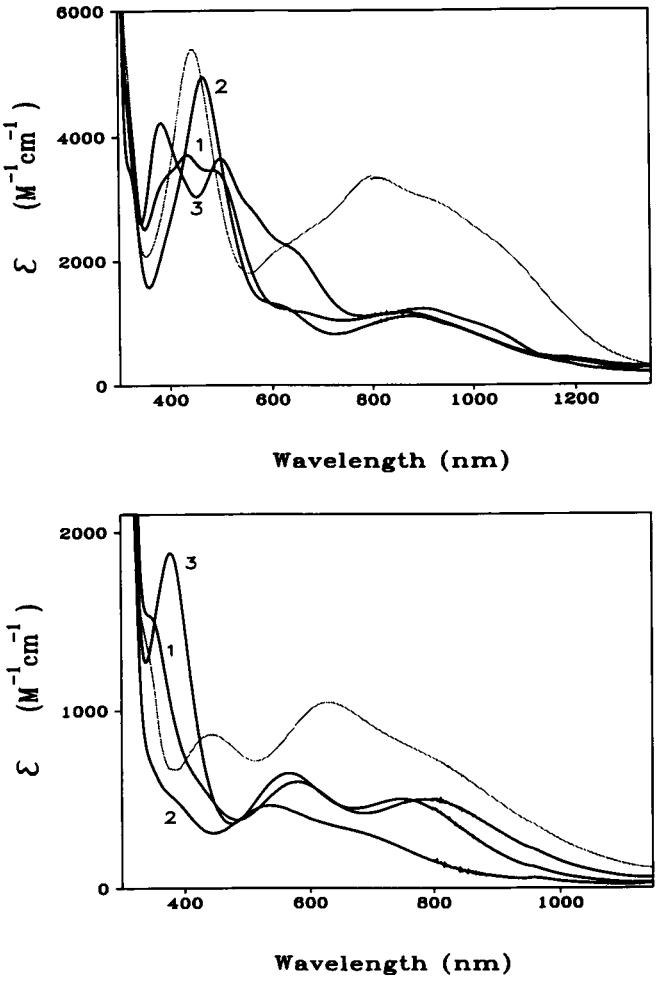

FIGURE 2 Anion interactions with active (*top*) and inactive (*bottom*) galactose oxidase. Absorption spectra of starting enzyme (*dotted line*) and final complexes (*solid line*) with (1) thiocyanate, (2) cyanide, and (3) azide.

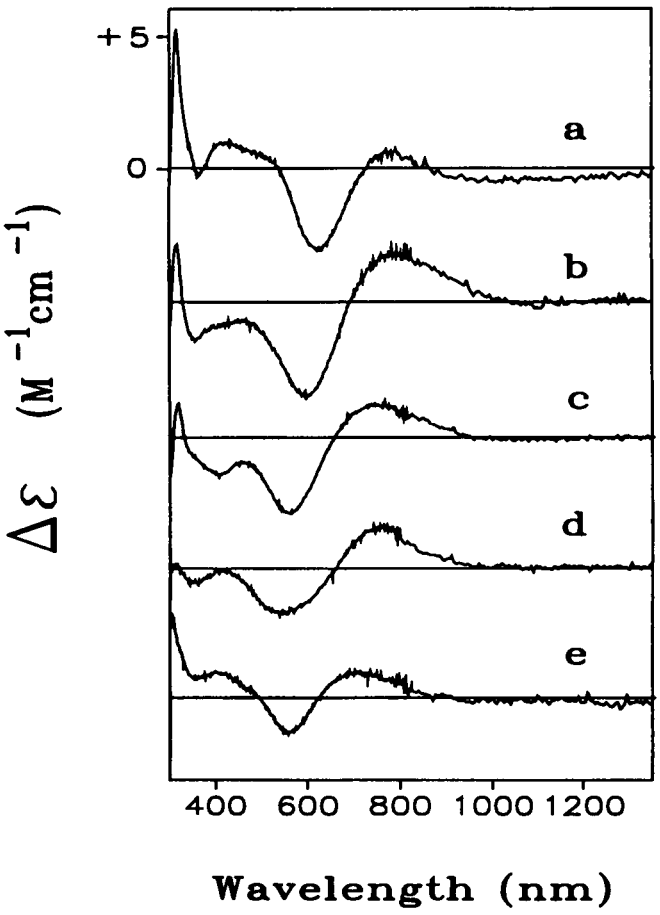

FIGURE 3 Circular dichroism spectra for complexes of inactive galactose oxidase. Protein in 50 mM $NaHPO_4$, pH 7. (*a*) Aquo; (*b*) KSCN; (*c*) NaN_3; (*d*) NaOCN; and (*e*) KCN complexes.

constant for the enzyme-azide complex resulted in the uptake of 0.8 protons per active site Cu ion. Further addition of azide had no effect. No proton uptake was observed in control experiments in which enzyme was omitted. Hydrolysis of azide was found to be insignificant under these conditions and enzyme was required for the proton uptake to be observed.

TABLE 1 Titration analysis of ligand interactions with galactose oxidase

$$EL \overset{K_d}{\rightleftarrows} E + L$$

Anion	Ligand affinity (K_d, mM)	
	Inactive enzyme	Active enzyme
Azide (N_3^-)	0.19	0.020
Thiocyanate (SCN^-)	4.3	1.8
Cyanate (OCN^-)	0.46	0.14
Cyanide (CN^-)	0.062	0.017

Metal-ligand interactions: spectroscopy

The optical spectra of both active and inactive enzyme change dramatically on formation of complexes (Figs. 1 and 2). The inactive blue aquo enzyme in the absence of anions exhibits unusually intense transitions in the ligand field spectral region (550–1000 nm) and a spectral feature at 440 nm that lies in the charge transfer region of the spectrum. On binding anions, the absorption intensity is decreased by >50% (Figs. 1 and 2, *bottom*), the ligand field spectra exhibit a shift to higher average energy, and the 440 nm absorption band disappears, resulting in a change in color from blue to red. Over the series of complexes containing N_3^-, SCN^-, OCN^-, and CN^-, subtler distinctions are evident in the details of the spectra. For the azide and thiocyanate complexes, two bands are resolved at low energy, whereas for the cyanate and cyanide complexes, the lowest energy band appears as a weak shoulder. Circular dichroism spectra of these complexes (Fig. 3) are dominated by an unusually strong CD band, which occurs near 620 nm in the aquo enzyme and shifts to higher energy in the anion complexes to

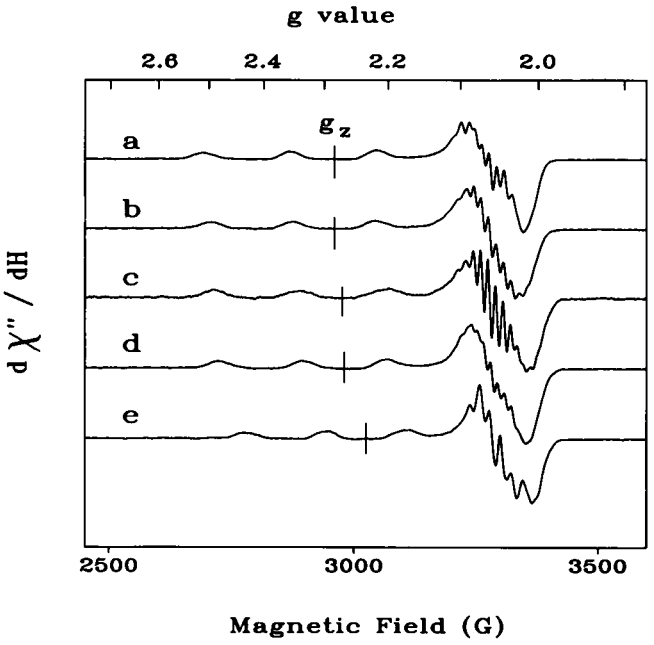

FIGURE 4 Low-temperature EPR spectra for complexes of inactive galactose oxidase. (Microwave frequency, 9.46 GHz; microwave power, 0.5 mW; temperature, 20 K; modulation frequency, 100 kHz; modulation amplitude, 10 G.) (*a*) Aquo; (*b*) cyanate; (*c*) thiocyanate; (*d*) azide; and (*e*) cyanide complexes.

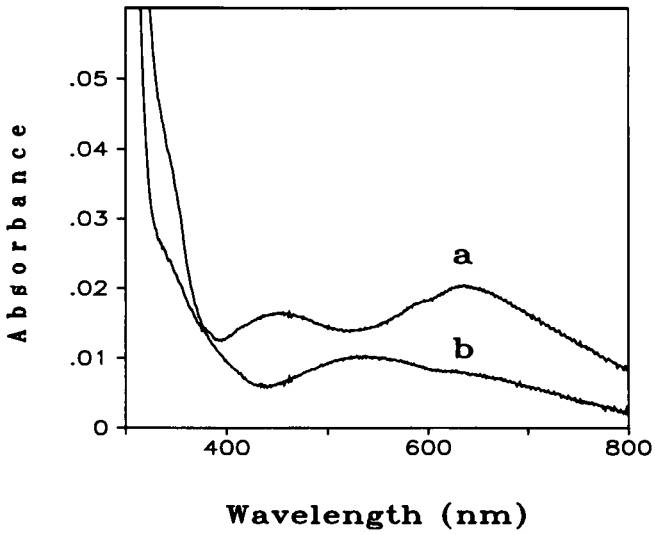

FIGURE 5 Absorption spectra for inactive galactose oxidase in 50 mM $NaHPO_4$, pH 7, containing 60% glycerol. (*a*) Room temperature (298 K); (*b*) low temperature (200 K).

near 550 nm. In the azide complex, an intense feature appears near 390 nm in the near UV region of the absorption spectrum. No corresponding features occur in the optical spectra for the other anions. No near infrared transitions are detected for the complexes beyond 1,200 nm in either absorption or CD. Low temperature EPR spectra for the complexes are shown in Fig. 4, with the shift in the g_z component indicated by the vertical markers in each spectrum.

The aquo complex of galactose oxidase exhibits a curious thermochromism, and reversible color changes are observed for protein samples stored at 77 K in a liquid nitrogen refrigerator. The inactive enzyme color changes from blue to red, and the active enzyme color changes from green to yellow on freezing at low temperatures. These changes are reversible on thawing the samples. The room temperature (298 K) and low temperature (200 K) absorption spectra for the inactive enzyme in 60% glycerol glassing solvent is shown in Fig. 5. As the temperature is decreased, the optical spectrum loses intensity and shifts to higher energy and the 440 nm absorption band is lost, paralleling the changes that occur on anion binding. EPR spectra for the inactive enzyme and its azide complex are also different at low temperature and room temperature (Fig. 6). EPR g values and hyperfine splittings both vary between solution and low temperature spectra of the two complexes. The solution spectra at room temperature exhibit $g_z = 2.27$, $a_{Cu} = 127$ G (H_2O) and $g_z = 2.24$, $a_{Cu} = 171$ G (N_3^-).

Absorption spectra of the active enzyme complexes (Figs. 1 and 2, *top*) are dominated by spectral features relating to the presence of the free radical in the active site and appear very different from the spectra of the inactive enzyme species. Distinct spectra are observed for the green active enzyme and its yellow anion complexes. In each case, the intense absorption band near 450 nm in the unliganded enzyme shifts to lower energy, whereas the broad absorption band centered near 800 nm decreases in intensity and resolves additional structure. In CD (Fig. 7), additional components of these

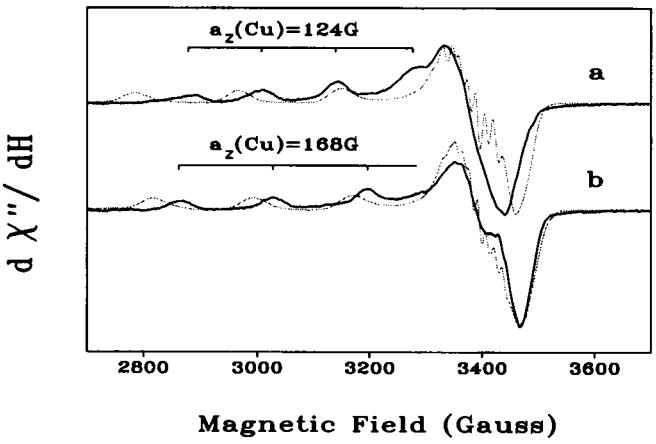

FIGURE 6 Solution EPR spectra for inactive galactose oxidase complexes. (*a*) Aquo complex in solution (*solid line*) with superimposed low temperature spectrum (*dotted line*). (*b*) Azide complex in solution (*solid line*) with superimposed low temperature spectrum (*dotted line*). Low temperature spectra are adjusted for the difference in acquisition frequency from solution spectra. (Microwave frequency, 9.76 GHz; microwave power, 10 mW; temperature, 298 K; modulation frequency, 100 kHz; modulation amplitude, 10 G.)

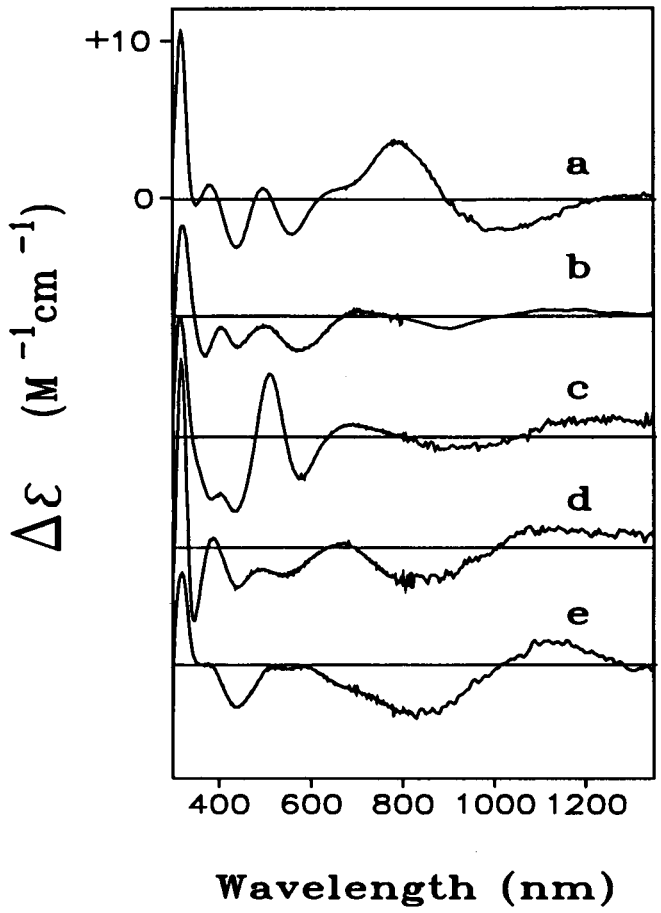

FIGURE 7 Circular dichroism spectra for complexes of active galactose oxidase. Protein in 50 mM $NaHPO_4$, pH 7. (*a*) Aquo; (*b*) KSCN; (*c*) NaN_3; (*d*) NaOCN; and (*e*) KCN complexes.

complex spectra are resolved, with at least five spectral features contributing beyond 500 nm. In the azide, thiocyanate, and cyanate complexes, relatively strong CD bands occur near 550 nm, comparable in transition energy and intensity with similar features in the corresponding complexes of the inactive enzyme. The azide complex of active galactose oxidase exhibits an intense absorption band near 390 nm. There is no evidence for spectral features beyond 1,200 nm in any of the ligand adducts.

Protein-ligand interactions: nitric oxide

Exposure of galactose oxidase in each of the three stable redox modifications (activated, reductively inactivated, and dithionite reduced) to NO converts the enzyme to a new form exhibiting an unusual EPR spectrum (Fig. 8 *e*) with all *g* values below 2. This spectrum is distinct from the spectrum of NO frozen in ice or in protein containing buffer (Fig. 8 *c*) and appears to arise from a specifically bound paramagnetic NO complex. The spectrum of this specifically bound NO in the galactose oxidase sample can be isolated at low temperature and high power through selective saturation of signals arising from the other species in the sample (the cupric ion and the solution NO spectrum), which evidently have longer relaxation times and are therefore more readily saturated. The spectrum of the galactose oxidase-NO complex exhibits axial *g* values ($g_{||}$ = 1.97, $g_{\perp}$ = 1.84) and lacks resolved Cu or N nuclear hyperfine splittings.

The low temperature EPR spectrum of inactive galactose oxidase in the presence of NO is shown in Fig. 8 *a*. This spectrum was recorded at low power to avoid saturation of the signals from the NO complexes. The observed spectrum can be simulated (Fig. 8 *b*) by combining three spectral components: the signal arising from the cupric site of the uncomplexed protein, the spectrum of nonspe-

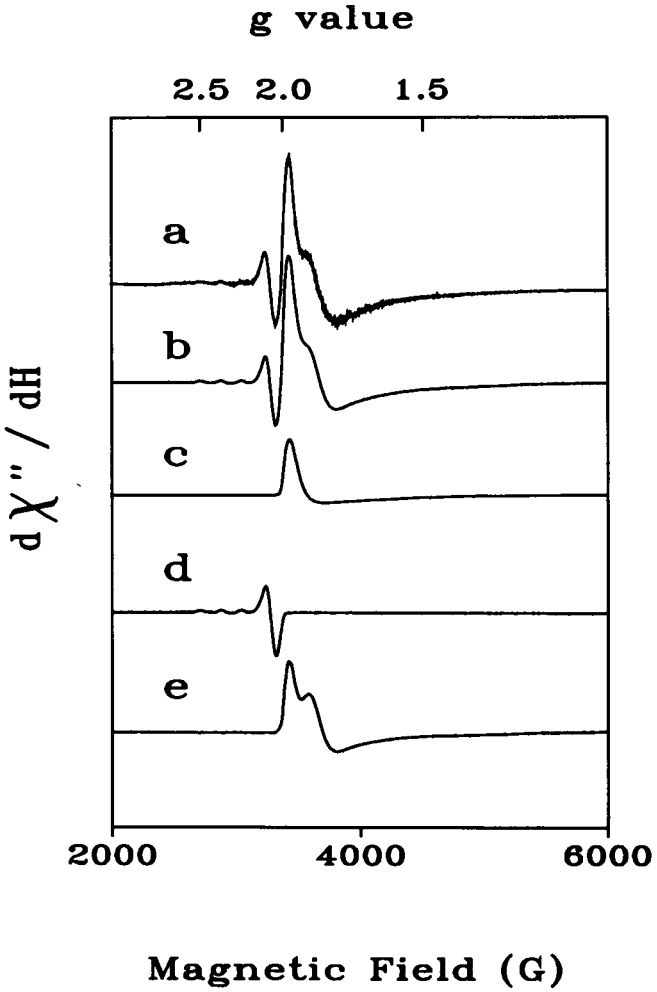

FIGURE 8 Resolution of spectral components in EPR spectrum for inactive galactose oxidase-NO complex. (*a*) Low temperature EPR spectrum of galactose oxidase-NO complex (microwave frequency, 9.46 GHz; microwave power, 1 mW; temperature, 4 K; modulation frequency, 100 kHz; modulation amplitude, 10 G). (*b*) Spectral reconstruction from components *c*–*e*. (*c*) Spectrum of NO in buffer containing bovine serum albumin. (*d*) Spectrum of inactive galactose oxidase sample before exposure to NO. (*e*) Spectrum of galactose oxidase-NO complex spectroscopically isolated at high power.

cifically bound NO in solution, and the specifically bound NO spectrum spectroscopically isolated at high power. Quantitation of these signals indicates that ~90% of the copper EPR spectrum is retained in the presence of NO, the nonspecific NO signal contributes a variable amount between sample preparations, generally quantitating in excess of one spin-one half per active site, and the specific NO signal integrates to approximately one spin-one half per active site. Similar quantitation is observed for specific and nonspecific NO signals in active and dithionite reduced galactose oxidase samples exposed to NO. There is no increase of the EPR-detectable cupric signal after treatment of active galactose oxidase with NO. The specifically bound NO signal exhibits a strong deviation from Curie law temperature dependence and virtually disappears above ~50 K, accounting for the lack of detection of this signal in an earlier study of Cu protein-NO at liquid nitrogen temperatures (18).

DISCUSSION

Investigations of ligand interactions with enzymes are important for understanding the chemistry of substrate binding and activation, providing essential insight into mechanisms of catalysis. For galactose oxidase, interactions with both organic substrate and dioxygen occur in the catalytic cycle. The pure redox modifications of galactose oxidase that are required for ligand binding studies only recently have been prepared and characterized (4). A blue form of the enzyme, generated by reduction of the radical under mild conditions, contains an isolated cupric center that is catalytically inactive. A green form of the enzyme, generated by mild oxidation of the enzyme, contains a free radical in addition to the copper, coupled antiferromagnetically to an EPR silent singlet (S=O) ground state (4). On reduction of the enzyme by substrate or sodium dithionite, a colorless cuprous form is produced. Ligand interactions in active and inactive (green and blue) forms of galactose oxidase are detected using optical (absorption and CD) or EPR spectroscopy to monitor the progress of a titration. The structural interpretation and assignment of spectra of these ligand complexes requires a correlation of spectroscopic methods and benefits from the available crystallographic data for galactose oxidase (14).

Titration data provide information on ligand affinities that can be used to explore another aspect of complex formation, the energetic factors that play an essential role in the activation steps in catalysis. The coordination chemistry of the active site metal center appears to be simple, titrating cleanly to monoanion complexes in each case we have examined based on the quality of fit to a single step association reaction. In spite of the relatively complicated spectra of some of these complexes and the possibility of linkage isomerism in coordination of pseudohalides, no evidence was found in the titration data for multiple equilibria, and clean isosbestics were maintained between the spectra. EPR spectra for the anion complexes also appear to reflect clean conversion to single species. Comparison of dissociation constants for the azide, thiocyanate, cyanate, and cyanide complexes of inactive and active galactose oxidase indicate a systematically higher affinity of the active enzyme complex for binding the exogenous ligand, nearly an order of magnitude higher for the azide complex. This clear distinction in the coordination chemistry of the active and inactive forms of the enzyme suggests differences in active site structure that are not detected in crystallographic studies (14). A charge difference in the active site for the two redox forms of the enzyme may contribute to the difference in anion affinities. The cupric ion in galactose oxidase is bound in the protein by two neutral ligands (histidine imidazoles) and two potentially anionic groups (tyrosine phenolates), canceling the Cu^{2+} charge and resulting in a neutral complex in the absence of exogenous ligands. Redox activation of galactose oxidase removes one electron from a directly coordinated metal ligand to generate the free radical–copper complex (4). As a consequence, the free radical complex should carry a net positive charge, contributing to the Coulomb stabilization of anion adducts in the active site and resulting in tighter binding to the active enzyme, as observed. This difference in charge would not be expected to be detectable at the resolution of protein crystal data. In addition, the crystallization conditions that have been used are incompatible with the existence of a free radical in the protein (14, 19), implying that the catalytically inactive blue form of the enzyme is the only structure available.

Proton uptake experiments reveal an additional aspect of active site chemistry relating to metal-exogenous ligand interactions. Titration of the active site cupric complex in the inactive form of galactose oxidase with azide leads to uptake of a single proton at pH 8, implying that formation of the azide complex is associated with generation of a base, most likely as a component of the active site. The observation that this base binds a proton at high pH (pH > 8) restricts the identity of the group responsible for this protonic equilibrium, since it requires a pK_a for this group greater than that of the indicator dye (pK_a = 8.8). The amino acid side chains tyrosine, cysteine, lysine, and arginine titrate in this pH range (pK_a = 10.1, 10, 10.5, and 12.5, respectively [20]), whereas the proton dissociation constant of histidine is near neutral pH (pK_a = 6.0) (20). Of these potential bases, only histidine and tyrosine are reported as components of the galactose oxidase active site. Displacement of the modified tyrosine ligand, the radical forming site, on anion binding to the cupric center appears unlikely based on studies of the active enzyme complexes. Anion binding perturbs the active enzyme complex without eliminating free radical–copper coupling. This electronic coupling is the origin of the EPR silent character of the active enzyme, and the fact that the active enzyme anion adducts remain

EPR silent indicates that the radical remains bound as a metal ligand in these complexes.

Spectroscopy of the anion complexes of active and inactive galactose oxidase reveals the effect of complex formation on the electronic structure of the active site metal center. The optical spectra of the inactive blue aquo enzyme and its anion complexes arise from the isolated cupric center in the protein. The combination of UV-visible-NIR absorption (Fig. 1, *inset*) and CD data (Fig. 3) permit the broad spectrum of the aquo complex to be resolved into four distinct ligand field or $d \rightarrow d$ transitions in the lower energy region. Overlapping components are resolved in the CD spectrum that also contributes to the assignment of the observed transitions in the absorption spectrum. The transition energies observed for the aquo complex are consistent (21) with the roughly square pyramidal geometry defined by the crystal structure (14) for the Cu site, and the unusually strong CD within the absorption band at 620 nm suggests assignment (4) to the $^2B_{1g}(x^2 - y^2) \rightarrow {}^2B_{2g}(xy)$ ligand field transition. Selection rules for a C_{4v} site symmetry assign the largest magnetic dipole character to this transition, which should thus acquire the largest anisotropy ($\Delta\epsilon/\epsilon$) and appear relatively strong in CD (4). The low symmetry of the Cu site may account for the relatively large extinction of the ligand field spectra for the aquo complex compared with simple cupric coordination complexes as a consequence of the relaxation of orbital selection rules in the absence of a center of symmetry (22).

At higher energy, beyond the range of ligand field spectra, the inactive enzyme complex exhibits a well-resolved transition near 440 nm, for which a tyrosine $\rightarrow Cu^{2+}$ LMCT assignment previously has been suggested (5). The identification of an axially coordinated tyrosine in the Cu binding site in galactose oxidase crystal structure (14) supports this assignment, and the low intensity of the LMCT transition can now be understood as a consequence of the apical coordination of this ligand, because both σ and π orbital overlaps between the coordinating phenolate oxygen and the $d_{x^2-y^2}$ ground state orbital of the Cu^{2+} ground state must vanish by symmetry, reducing the LMCT intensity proportionately with covalency. The energy of the transition is consistent with tyrosine $\rightarrow Cu^{2+}$ LMCT as reported in small molecule inorganic complexes. The LMCT transition expected for the modified tyrosine residue is not resolved in this spectrum and is expected at lower energy than that of an unsubstituted tyrosine because it is more readily oxidized. Although no discrete transition is resolved in the spectrum, LMCT from the modified tyrosine may contribute to the unusually intense absorption in the ligand field region of the spectrum. Higher energy transitions are expected to arise as LMCT excitations involving the coordinated histidine imidazoles (4, 23). The energy range of the $d \rightarrow d$ spectra of the cupric center rules out an effective tetrahedral copper geometry in galactose oxidase that has been suggested on the basis of the long Cu-water distance observed in the crystal (14). The relatively weak perturbation of the metal d-orbitals in a tetrahedral copper environment is associated with ligand field transitions below 8,000 cm^{-1} (beyond 1,250 nm) (24).

Binding azide to the inactive enzyme leads to the appearance of an intense absorption band near 390 nm in the near UV that has been assigned previously as azide-to-Cu^{2+} LMCT for an equatorially coordinated anion (4). The energy of the LMCT transition is characteristic of a tetragonal rather than a tetrahedral copper complex, as are the $d \rightarrow d$ spectra for this complex, which are shifted to higher average energy relative to the spectra of the aquo enzyme. In addition, the spectral feature we have identified as the LMCT transition arising from the axial tyrosine is no longer present. The increased tetragonality of the copper complex reflected in the ligand field spectra and the absence of the spectral feature associated with axial tyrosine ligation suggest that coordination of an anion results in displacement of the weakly bound axial tyrosine ligand.

Similar trends are observed in the spectra of thiocyanate, cyanate, and cyanide adducts, except that no LMCT transitions occur in the near UV-visible spectral range for these complexes. The perturbation of the $d \rightarrow d$ spectra increases in this series, with an increase of the highest energy $d \rightarrow d$ spectra evident in both absorption and CD. These results are consistent with an increased tetragonal distortion of the metal complex with ligands progressively higher in the spectrochemical series contributing a larger ligand field perturbation. In each case, coordination of the exogenous anion is associated with a decrease in the absorption intensity over the visible spectrum and a loss of the 440 nm absorption band and no additional transitions detected at low energy in either absorption or CD. The low temperature EPR spectra of these complexes reflects the trend toward higher ligand field energy for the $d_{x^2-y^2} \rightarrow d_{xy}$ orbital transition (increasing spectral energy for the strongest CD band) in the decreasing magnitude of g_z. The dominant contribution to the g shift in a tetragonally distorted copper complex arises from spin orbit mixing of $^2B_{1g}(x^2 - y^2)$ and $^2B_{2g}(xy)$ orbital states of the cupric ion, given to second order in perturbation theory by the expression (22)

$$g_z = 2 - \frac{8\lambda}{\Delta E_{x^2-y^2-xy}}.$$

As the energy splitting in the denominator increases, the overall g anisotropy is expected to decrease. Although the optical and EPR data for galactose oxidase does not support a quantitative correlation of $(g - 2)$ with ligand field transition energy at this level of analysis, the expected trend of decreasing g_z with increasing $d \rightarrow d$ transition energies is observed.

The corresponding results for anion binding of the ac-

tive enzyme are more difficult to interpret, because the observed spectra include contributions from both the copper and free radical in the active site. The intense absorption band near 450 nm characteristic of the presence of the free radical in the active enzyme species (4) (the "blue band") is retained on forming ligand complexes, consistent with EPR evidence for retention of a free radical-coupled Cu complex in the anion adducts. The most dramatic perturbation appears in the unusual, intense lower energy absorption band (the "red band"), which decreases in intensity in the anion complexes, most likely reflecting a sensitivity of the free radical site to ligand binding at the metal center. Although the complexity of the spectra preclude a detailed assignment, the possibility that the red band relates to transitions within a tetrahedral Cu complex is unlikely in view of the observation that the azide-to-Cu^{2+} LMCT transition in this complex occurs at an energy characteristic of tetragonal rather than tetrahedral Cu site. We previously have suggested that the blue band arises as an intra-radical transition within a stacked-aromatic chromophore and that the red band arises as a mixture of LMCT and charge resonance excitation between the aromatic π systems involved in the charge transfer complex (5). Stacking of a tryptophanyl indole ring over the tyr-cys cross-linked group is revealed by the crystal structure (14), supporting this interpretation. The combination of the modified tyrosine and the stacked tryptophan most likely compose the radical site probed in the electronic spectra.

The temperature-dependent color change characteristic of the aquo complexes of inactive and active galactose oxidase but not of their anion adducts provides further insight into the catalytic mechanism. Low-temperature absorption spectra can be recorded for the inactive enzyme, which is stable in glassing solvents, but not for the active enzyme, because the radical site is reduced under these conditions. However, the colors of both active and inactive enzyme in frozen samples at low temperature are characteristic of the anion complexes rather than the aquo complexes in solution at room temperature. The most likely explanation for this effect is a temperature-dependent proton-transfer equilibrium within the active site, which at low temperature favors coordinated hydroxide. The proton acceptor in this acid-base reaction would most likely be the tyrosinate ligand that is liberated in forming anion complexes. Note that a coordinated water is a structural analog for bound alcohol in the substrate complex, and our results suggest that deprotonation of the coordinated hydroxyl group occurs as a substrate activation step in catalysis. Solvent kinetic isotope effects in the turnover reaction previously have been interpreted in terms of a rate-limiting ionization of coordinated water in the active site (24), but this neglects the rapid exchange of substrate hydroxyl protons in deuterated water. The results are equally consistent with deprotonation of coordinated substrate, and we propose that substrate rather than water ionization is the step sensitive to solvent deuteration.

The temperature-dependent change in the aquo enzyme active site also can be followed by EPR spectroscopy, comparing the spectrum of the enzyme in solution at room temperature with the frozen solution spectrum recorded at low temperature (Fig. 6). The solution EPR spectrum is essentially that of an immobilized cupric center, because the rotational diffusion of a macromolecule the size of galactose oxidase, M_r = 68,000, is slow relative to the EPR transition frequency. From the molecular mass, the rotational diffusion coefficient for galactose oxidase may be estimated (25) ($\Theta \approx 10^6\ s^{-1}$) for comparison with the transition frequency ($\sim 10^{10}\ s^{-1}$ at X-band). For the aquo enzyme in solution, all four components of the Cu nuclear hyperfine splittings are resolved in the parallel region of the spectrum, with a distinctly smaller splitting than observed in the low temperature spectrum. The 30% decrease in hyperfine coupling constant at room temperature must reflect significant changes in the active site between the low and high temperature limits. For comparison, we also have recorded the room temperature solution EPR spectrum of the inactive enzyme azide complex (Fig. 6 *b*), showing values for the magnitude of the parallel Cu hyperfine virtually identical to those observed at low temperature. The EPR spectrum of the aquo enzyme at low temperature resembles both low and high temperature spectra of the azide complex, as expected from the optical spectra discussed above, supporting our description of the aquo enzyme at low temperature as a hydroxide complex.

The interactions with the second substrate, dioxygen, are also of key interest in understanding the catalytic chemistry of galactose oxidase. We have attempted to explore the interactions of galactose oxidase using nitric oxide (NO) as a probe for O_2 interaction site. In a number of O_2-reactive proteins, NO forms relatively stable, paramagnetic complexes accessible to characterization by EPR spectroscopy. For example, NO complexes of hemoglobin have provided information on the O_2 binding site (26). For galactose oxidase, we observe that similar EPR spectra are produced by exposing the active, inactive, or dithionite reduced enzyme to NO. In each case, we observe an unusual EPR signal with *g* values below 2, which is distinct from the signal of NO in ice generated in control experiments. Because NO is a paramagnetic gas, this signal is commonly observed in NO-binding experiments when excess ligand is present. The signal from this nonspecific NO occurs entirely below the free electron *g* value and characteristically displays the form shown in Fig. 8 *c*.

The latter spectrum does not seem to have received a detailed interpretation in spite of this species being a ubiquitous impurity in NO coordination studies. Based on the unusual *g* values of the EPR spectrum, it most likely arises within the low symmetry split $^2\Pi$ electronic ground state of the matrix bound NO molecule. Re-

moval of the axial symmetry of the free molecule would lift the degeneracy of the 2π antibonding orbital set occupied by the unpaired valence electron (27), quenching the orbital angular momentum and collapsing the EPR spectrum from $g = 0.8$ in the free molecule (28, 29) to the free electron g value ($g = 2.00$). Unquenching of orbital momentum through spin orbit coupling will lead to g shifts below 2 for the less-than-half filled antibonding shell of NO, as observed. The unusual NO spectrum detected in the presence of galactose oxidase (Fig. 8 *e*) also reflects a perturbed NO molecule, but the well-resolved axial g values indicate a more homogeneous environment for this complex. We have computed the Zeeman and spin orbit perturbations within the low lying electronic states of an orbitally perturbed NO molecule and find that a π-orbital splitting of a few hundreds of cm^{-1} will yield g values in the range observed in the experiment.

The NO interactions with inactive galactose oxidase can be interpreted most straight forwardly. Because the active site cupric ion is EPR detectable in this form, it has been possible to investigate the involvement of Cu in formation of the specific NO complex. The EPR spectrum of the fully formed NO adduct of galactose oxidase, shown in Fig. 8 *a*, is a superposition of three components, resolved in Fig. 8 *c–e*. Their combination, in Fig. 8 *b*, accurately reconstructs the experimental spectrum. Spectrum 8 *c* arises from nonspecific NO interactions. Spectrum 8 *d* is essentially identical to that initially obtained for the inactive enzyme sample before exposure to NO. This component represents ~90% of the original signal, indicating that most of the copper remains EPR detectable in the NO complex. Finally, the spectrum of the specifically bound NO, Fig. 8 *e*, quantitates to approximately one spin-one half per protein, consistent with a unique site for specific NO interactions in galactose oxidase. The observation of cupric EPR signal in this complex implies that, in contrast to many other metalloenzymes, the metal ion is *not* the NO binding site in galactose oxidase. The lack of resolved Cu nuclear hyperfine splittings in the NO spectrum reinforces this conclusion, because a crystallographically defined Cu nitrosyl that recently has been reported (30) exhibits well-resolved Cu hyperfine. The similarity of EPR spectra for NO complexes formed from three distinct redox modifications of galactose oxidase also points to a nonmetal interaction site for NO in that enzyme. There is, in fact, no direct evidence in our data that NO interactions actually occur in the active site, other than the observation that the specifically bound NO EPR spectrum saturates less readily than that of nonspecific NO, as would be expected if dipolar interactions between bound NO and the active site cupric ion provided a relaxation mechanism. Indeed, the absence of any significant change in saturation behavior of the Cu^{2+} EPR signal in the NO complex seems to argue for a more remote interaction site.

There are interesting similarities between this galactose oxidase NO complex and an analogous complex reported for another metalloenzyme, laccase (31), where unusual EPR spectra have been observed in the presence of NO. For laccase (31), the NO complex exhibits a spectrum with all g values below 2, as we observe for galactose oxidase, and also lacks resolved Cu hyperfine splittings, although nitrogen hyperfine splittings consistent with NO localization have been reported. This points to a nonmetal interaction site for NO in another O_2-reactive Cu metalloenzyme. The significance of the NO interactions in terms of the O_2 chemistry of these enzymes still needs to be established. For galactose oxidase, recent kinetic investigations of the catalytic mechanism indicate that the formation of a ternary complex of enzyme, alcohol, and O_2 is required for the most rapid reaction (32). Because the coordination chemistry we have outlined for the active site metal complex suggests that alcohols bind directly to Cu for activation, a nonmetal interaction site for O_2 would appear to be required for the formation of a ternary complex. The binding site might involve an aromatic residue in the vicinity of the active site that would form a relatively weak complex with dioxygen. This type of O_2 interaction has been reported for aromatic compounds (benzene, napthalene) (33). Formation of this complex could serve to stabilize molecular oxygen in the vicinity of the active site and would be expected to appear kinetically as a saturable step involving O_2. This scheme does not preclude direct O_2–metal interactions in a later stage of the reaction in which the product peroxide is formed. The possibility that enzymes may have evolved intrinsic O_2-interaction sites has not been extensively explored but appears to merit attention based on the preliminary results described above.

Our previous research on galactose oxidase (4–7, 19) has provided the basis for a catalytic mechanism involving a free radical coupled copper complex as a two-electron redox unit for alcohol oxidation and O_2 reduction. The present study permits an elaboration of this scheme, shown in Fig. 9, providing functional roles for the structural features identified spectroscopically and crystallographically. The free radical coupled Cu complex exhibits coordination chemistry, and we propose that alcohols directly coordinate to the metal as an initial step, forming an analogue of the coordinated water identified in the protein crystal structure. Displacement of the axially coordinated tyrosine appears to be a consequence of exogenous ligand binding, as reflected in spectroscopic and chemical studies, generating a strong base with a pK_a above 9. Just as a coordinating ligand perturbs the cupric ion, the hydroxylic ligand is itself perturbed in the complex and is expected to increase in acidity. This perturbation of the ligand is predicted to be greater in the radical containing Cu site, reflecting the higher affinity of the cationic complex for exogenous ligands. In the presence of the tyrosinate base displaced from Cu on binding sub-

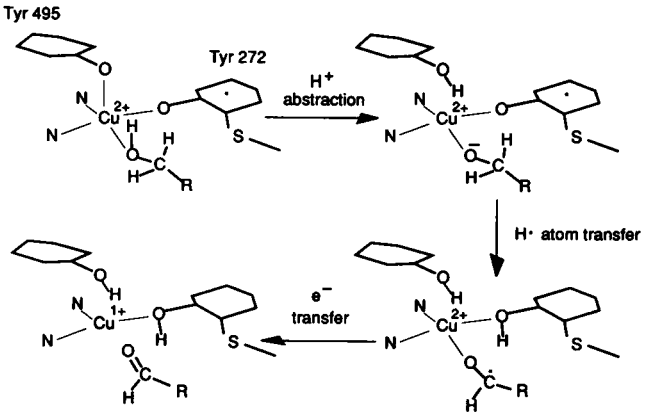

FIGURE 9 Mechanistic scheme for substrate oxidation by galactose oxidase.

strate, deprotonation of the hydroxyl group is expected to occur, activating the substrate for oxidation. The bound alkoxide could then undergo hydrogen atom transfer in free radical chemistry with the coordinated phenoxyl oxygen of the protein radical site as the acceptor. Based on electron-nuclear double resonance (ENDOR) studies on the apogalactose oxidase radical and on thioether-substituted phenol model studies (19), unpaired electron density on the phenoxyl oxygen is expected to be significant. Note that this scheme accounts for the stereospecific pro-S stereochemistry of hydrogen abstraction in the formation of aldehyde product that is experimentally observed (3). The resulting ketyl radical intermediate shown in Fig. 9 would then reduce Cu to form the experimentally characterized cuprous intermediate (4, 7) that reacts with O_2. This working model for the catalytic mechanism, based on the coordination chemistry of the active site and spectroscopic investigation of ligand interactions, provides a starting point for exploring the origins of alcohol oxidation catalysis in the active site and in the meeting the synthetic challenge of designing inorganic complexes that can serve as functional mimics of the enzyme.

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