

Resonance Raman Evidence for Tyrosine Involvement in the Radical Site of Galactose Oxidase*

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Resonance Raman data are reported for the redox-activated form of galactose oxidase from *Dactylium dendroides*. Excitation within the red (659 nm) and blue (457.9 nm) absorption bands leads to strong resonance enhancement of ligated tyrosine vibrational modes at 550, 1170, 1247, 1484, and 1595 cm^{-1} . The ring mode frequencies are unusually low, indicating a decreased bond order in the ring. The spectra clearly differ in both frequencies and relative intensities from those characteristic of known aromatic π -radicals. Enhancement of tyrosine ring modes on excitation within absorption bands previously associated with the presence of the radical in the active site suggests that the ligated tyrosine residue is present in the radical site and may stabilize this radical species through formation of a charge transfer complex. A dramatically different Raman spectrum is observed for the N_3^- adduct of galactose oxidase, exhibiting a single strong 1483 cm^{-1} feature. The intense visible-near IR absorption bands for galactose oxidase may derive from transitions within a charge transfer complex between an aromatic free radical and a tyrosine-copper complex.

Galactose oxidase catalyzes the oxidation of primary alcohols to the corresponding aldehydes coupled to the two-electron reduction of O_2 to H_2O_2 (1, 2). This mononuclear copper enzyme is distinct from other copper oxidases in exhibiting intense absorption features extending over the entire UV-visible-near IR spectral range (3). In a recent report we have shown that these features relate to a free radical associated with a cupric site in the active form of the enzyme (4). This unusually stable free radical has been implicated in the mechanism of redox catalysis for galactose oxidase (4), thus further extending the range of enzymes known to perform free radical active site chemistry (5). The increasing awareness of the importance of free radical chemistry in

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biology is just beginning to lead to detailed structural insights into the unusual stability of radicals in these enzymes. We are currently using a variety of spectroscopic approaches to study the nature of the radical site in galactose oxidase and its detailed role in the catalytic mechanism. We report here for the first time the observation of resonance-enhanced Raman scattering from a ligated tyrosine residue on excitation within the intense absorption features for galactose oxidase, implicating the presence of tyrosine in the radical site.

MATERIALS AND METHODS

Galactose oxidase (EC 1.1.3.9) was purified to homogeneity from culture filtrates of *Dactylium dendroides* (ATCC 46032) grown on sorbose media in the dark according to published procedures (2, 6). The purified enzyme was activated by treatment with potassium hexacyanoferrate(III) as previously described (4). Catalytic activity was determined by the direct assay procedure (7) using 3-methoxybenzyl alcohol which had been distilled under reduced pressure and stored under argon.

Resonance Raman spectra were obtained on instrumentation which has previously been described (8). Galactose oxidase (35 mg/ml) was purged with nitrogen before irradiation, and Na_2SO_4 (0.1 M) was added as an internal intensity standard. Data were collected with an intensified Reticon optical multichannel analyzer (EG&G Princeton Applied Research model 1420). Catalytic activity of the sample was measured before and after data collection.

RESULTS AND DISCUSSION

Previous studies using a combination of optical absorption, CD, and EPR spectroscopies on galactose oxidase have provided evidence for a unique coupled free radical-cupric center in this enzyme responsible for the two-electron redox chemistry in the catalytic mechanism (4). However, these studies have not been able to provide structural information on the nature of the radical site. Resonance Raman spectroscopy has proven an extremely powerful probe in structural analysis and assignment of spectral features both in small molecule and protein studies, complementing other methods by providing high resolution vibrational information on specific chromophores (9, 10). The selective resonance enhancements resulting from excitation within an absorption band permit local structural characterization. The application of resonance Raman spectroscopy to galactose oxidase is described below.

Resonance excitation at 659 nm into the red absorption band that dominates the near IR spectrum of galactose oxidase leads to strong enhancements of Raman scattering for bands at 1170, 1247, 1484, and 1595 cm^{-1} (Fig. 1, bottom spectrum) as well as 550 cm^{-1} (data not shown). The Raman intensity is calibrated by nonresonant scattering from the sulfate internal standard at 981 cm^{-1} . Similar features with comparable relative intensities are observed for excitation into the blue band (457.9 nm), but weak fluorescence from the sample reduces the signal-to-noise ratios. These features have much lower relative intensities at intermediate wavelength excitation (488.0 and 514.5 nm). For both 659 and 488.0 excitations, the signals disappear on sample reduction with ascorbate, which results in selective reduction of the radical site and elimination of the intense absorption features (4).

The pattern of five strongly enhanced features at 550, 1170, 1247, 1484, and 1595 cm^{-1} is strikingly similar to that observed for tyrosine-to-metal charge transfer excitation in a variety

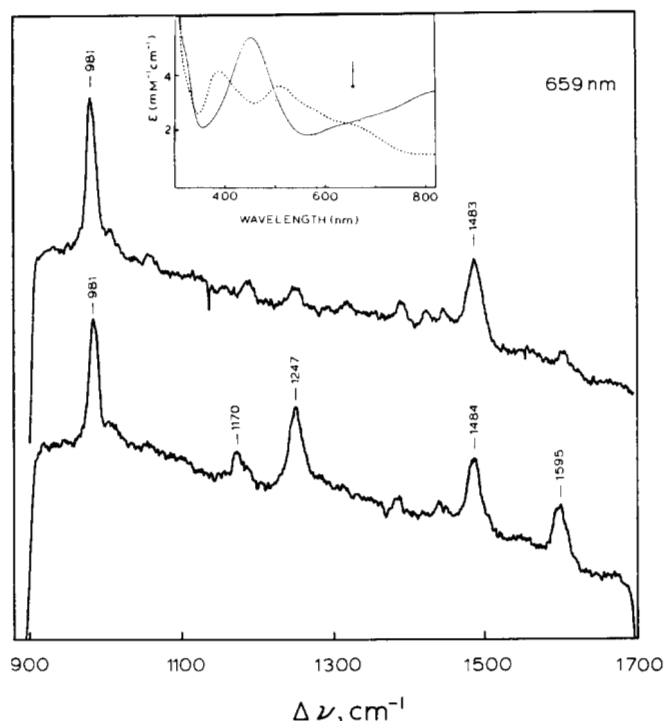


FIG. 1. Resonance Raman spectra for galactose oxidase. Bottom, native redox-activated galactose oxidase (35 mg/ml) in 50 mM NaPO₄ buffer, pH 7. Top, galactose oxidase after addition of NaN₃ to 1.0 mM. Excitation wavelength: 659 nm (approximately 160 milliwatts at sample). Inset, absorption spectra for redox-activated galactose oxidase (—) and azide complex (....) with arrow indicating Raman excitation wavelength.

of metalloenzymes in which tyrosine is coordinated to the metal ion (11, 12), which strongly suggests that tyrosine is bound to the cupric ion in galactose oxidase. All of the Raman ring mode frequencies observed for the tyrosine in galactose oxidase are, however, systematically and significantly lower than found previously for other metalloenzymes. Tyrosinate-metal complexes in these other proteins exhibit a narrow range of frequencies for these modes, from 1600 to 1607 cm⁻¹ and from 1494 to 1504 cm⁻¹, respectively, for the ν_{8a} and ν_{19a} in-plane ring stretching modes and from 1248 to 1293 cm⁻¹ for the ν_{7a} mixed C–O stretch/ring deformation mode. The systematically decreased frequencies we observe for the ring modes of the active site tyrosine in galactose oxidase, and in particular for the nearly frequency-invariant ν_{8a} and ν_{19a} modes, indicate a decreased ground state bond order in the tyrosinate residue. This is likely to result from either an increased electron density in a π^* orbital, or as is more likely (*vide infra*), a decreased electron density in the highest occupied bonding orbital.

Previous studies have provided evidence that the activation step which generates the unique intense absorption features of the active galactose oxidase involves one-electron oxidation of a group in the protein other than the metal center (4). The presence of a cupric metal ion in active galactose oxidase is supported by spectral features characteristic of this oxidation state (4), and this assignment is strengthened by recent copper K-edge x-ray absorption experiments on well characterized samples which define a Cu²⁺ center in both inactive and active enzyme species.¹ These combined spectral studies have led to the proposal of a stable free radical in the active site of galactose oxidase and a tentative assignment of the intense

absorption features to electronic transitions associated with this radical species. The enhancement of ligated tyrosine ring modes on excitation within these absorption features indicates that it is likely that the tyrosine-copper chromophore is present in the radical site and may be intimately involved in its stabilization.

The Raman features we have observed can be definitely assigned to ligated tyrosine, rather than a tyrosine radical species, based on the studies on metal-bound tyrosine cited above and upon additional vibrational studies on related non-metal-bound aromatic free radicals. Resonance Raman spectra for excitation within the low lying π - π^* transition of the model 4-methyl phenoxyl radical exhibit a single dominant Raman feature near 1500 cm⁻¹, assigned to a ν_{7a} mode with significant C–O double bond character, with lower enhancements for other Raman features (13). The vibrational frequencies observed for this model radical are quite distinct from those found for the singlet species (14). Thus, the spectra observed here for the native active galactose oxidase dramatically differ in both frequencies and relative intensities from the spectra observed for excitation of tyrosyl free radicals.

Addition of azide to the redox-activated galactose oxidase leads to perturbation of the absorption spectrum (Fig. 1, inset) with large changes in the molar absorptivities and λ_{max} values in both the visible and near IR spectral regions (4). Azide binding also results in a markedly different resonance Raman spectrum (Fig. 1, top spectrum). The single very strong feature near 1483 cm⁻¹ does not arise from an azide mode (15). The Raman spectral differences observed between active enzyme and the N₃⁻ complex may either result from resonance with a different chromophore and/or a different electronic transition in the active and azide-bound enzymes. In comparing the two Raman spectra, it is clear that while the enhancement patterns differ dramatically, many of the Raman features occur in both spectra (Table I). It is unlikely that the weak tyrosine-copper Raman features observed in the spectrum of azide-galactose oxidase simply arise from uncomplexed enzyme, because a saturating azide concentration is present. Further, an additional 5-fold increase in N₃⁻ concentration had no effect on the Raman spectrum. Thus, we conclude that all of the Raman spectral features derive from the azide complex of galactose oxidase. The superficial similarity of the azide com-

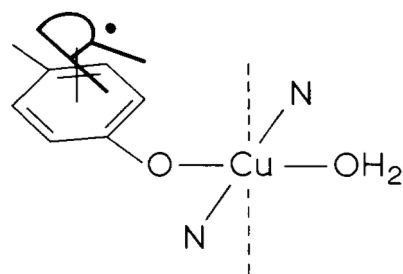
TABLE I
Vibrational frequencies for galactose oxidase complexes

Complex	$\Delta\nu$ cm ⁻¹	Assignment
Native redox-activated	1170	ν_{9a}
	1247 ^a s ^b	ν_{7a} C–O stretching and symmetric ring deformation
	1381 ^a	
	1437 ^a	
	1484 ^a s	ν_{19a} in-plane ring stretching
	1595 ^a s	ν_{8a} in-plane ring stretching
Azide	1054	
	1183	
	1246	
	1313	
	1384	
	1417	
	1440	
	1483 s	
	1597	

^a These features are observed in both forms of the enzyme.

^b s, strong.

¹ J. W. Whittaker and J. E. Penner-Hahn, unpublished results.



SCHEME I

plex relative intensities to those observed for excitation of the 4-methyl phenoxy radical (13) is likely fortuitous since all the vibrational frequencies of the azide adduct (Fig. 1, *top spectrum*) are close to the ligated tyrosine Raman frequencies, including features near 1417 and 1440 cm^{-1} , which have been previously reported in Raman spectra of tyrosinate-metal biological complexes. The differences between the azide-bound and native active galactose oxidase spectra thus most likely reflect a change in the relative enhancement pattern rather than a new feature from another chromophore. The enhancement pattern is, however, distinctly different from that previously shown for excitation within a tyrosine \rightarrow metal charge transfer transition. The possibility therefore also exists that the enhancements for tyrosine Raman modes in the N_3^- complex could result from excitation of a chromophore, other than tyrosine-ligated copper charge transfer, in which tyrosine participates.

Although a major tyrosine \rightarrow Cu^{2+} charge transfer contribution in both red and blue absorption features is indicated by the similar resonance Raman data obtained with excitation in both bands, these absorption bands markedly differ from any previously reported for tyrosine \rightarrow Cu^{2+} charge transfer in proteins and model complexes. Tyrosine \rightarrow Cu^{2+} charge transfer transitions typically occur at approximately 450 nm with molar absorptivities 5-fold less than that observed here (16, 17). Thus both the λ_{max} and the molar absorptivities are inconsistent with a simple tyrosine \rightarrow Cu^{2+} assignment. While distinct from any previously reported spectrum for a tyrosinate-copper complex, the galactose oxidase absorption spectrum (Fig. 1, *inset*) is, however, reminiscent of spectra of radical charge transfer complexes such as the mixed valent benzene dimer cation (C_6H_6) $_2^+$ which has been observed in hydrocarbon matrices at low temperature (18). The optical absorption spectrum observed for the benzene dimer cation exhibits both a blue absorption band assigned as a $\pi\rightarrow\pi^*$ electronic transition of the radical perturbed by stacking interactions in the charge transfer complex and a low energy intervalence transfer transition in the near IR region. These would then correspond to the two principal absorptions in the optical spectrum of galactose oxidase near 445 and 900 nm. This model suggests an analogous assignment of the near IR feature in galactose oxidase as an overlapping or mixed transition involving both tyrosine \rightarrow Cu^{2+} charge transfer and charge transfer with the putative radical chromophore, while the blue band would arise from an overlapping or mixed transition involving both the tyrosine \rightarrow Cu^{2+} charge transfer

and $\pi\rightarrow\pi^*$ ring transition of the putative aromatic radical chromophore. The resonance enhancement of tyrosine vibrational modes observed in both blue and red spectral regions indicates significant tyrosine-to-metal charge transfer character in both of these transitions. This potential mixing could result from admixture of the ligand-to-metal charge transfer excited state with nearby electronic excited states of the free radical complex.

Raman data combined with previous absorption and EPR data lead to a structural proposal for the stable radical site in galactose oxidase (Scheme I). The Raman data suggest the presence of a copper-coordinated tyrosine complexed with an as yet unidentified but possibly aromatic free radical ($\text{R}\cdot$). Charge transfer interactions in the complex could account for the stability of the radical site and its unusual absorption spectrum. Involvement of the tyrosine in terms of ground state charge transfer could also lead to the electron-deficient character of the tyrosine as reflected in the extraordinarily low vibrational frequencies observed for all the ring modes. Azide binding appears to strongly perturb the interactions in the active site complex, resulting in changes in both the near IR absorption and in the Raman spectrum. This indicates that the nature of the active site radical may be modulated by small molecule binding at the copper site. Further studies are currently in progress probing the structure and chemistry of the radical site in greater detail.

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