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 N₅ 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 O₂ to H₂O₂ (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 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 48032) 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 Na₂S₀₄ (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
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 vsa 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 from 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 a decrease in the absorption spectrum (Fig. 1, inset) with large changes in the molar absorptivities and \( \lambda_{\text{max}} \) values in both the visible and near IR spectral regions (4). An 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 Nz 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 likely 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 \( \text{N}_2 \) 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-

| Complex                        | \( \Delta \nu, \text{ cm}^{-1} \) | Assignment                                      |
|--------------------------------|-----------------|---------------------------------------------|
| Native redox-activated         | 1170            | \( \nu_{\text{sa}} \)                  |
|                                | 1247*           | \( \nu_{\text{sa}} \) C-O stretching and symmetric ring deformation |
|                                | 1381*           |                                           |
|                                | 1437*           |                                           |
|                                | 1484*           | \( \nu_{\text{sa}} \) in-plane ring stretching |
|                                | 1595*           | \( \nu_{\text{sa}} \) in-plane ring stretching |
| Azide                          | 1054            |                                           |
|                                | 1185            |                                           |
|                                | 1246            |                                           |
|                                | 1313            |                                           |
|                                | 1384            |                                           |
|                                | 1417            |                                           |
|                                | 1440            |                                           |
|                                | 1483 s          |                                           |
|                                | 1597            |                                           |

*These features are observed in both forms of the enzyme.

\( \nu_{\text{sa}} \), strong.

1 J. W. Whittaker and J. E. Penner-Hahn, unpublished results.
The Radical Site of Galactose Oxidase

Although a major tyrosine-Cu" 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-Cu^2+ charge transfer in proteins and model complexes. Tyrosine-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 \( \lambda_{\text{max}} \) and the molar absorptivities are inconsistent with a simple tyrosine-Cu" 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\(_6\)H\(_4\))^+ 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 lower energy intercalation 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-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 tyrosine-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 (R'). 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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