Models for the Redox Active Site in Galactose Oxidase

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Received April 27, 1993®

Abstract: Modeling approaches have been used to develop insight into the nature of the redox active site of the free-radical-containing copper metalloenzyme galactose oxidase. The optical spectrum of the free radical generated by low-temperature UV irradiation of (methylthio)cresol is nearly identical to that observed for the free-radical site in metal-free apo galactose oxidase, supporting the assignment of the protein radical to a novel tyrosine–cysteine covalent cross-link structure recently reported from X-ray crystallographic studies. Basic characterization of the chemistry for this new type of biological redox group includes measurements of the substituent effects on phenolic proton acidic and observation of the oxygenation of (methylthio)cresol with peroxides. The latter reaction models a possible inactivation pathway for the enzyme. Low-temperature absorption and EPR spectra were obtained for free radicals formed from the methylthio and methylsulfinyl derivatives that are most important as models for the biological active site for comparison with the parent cresol radical. A series of simple copper complexes has been prepared with cresol, (methylthio)-cresol, and (methylsulfinyl)cresol ligands to explore the effect of substitution on coordination chemistry. Structural and spectroscopic data obtained for these inorganic models contribute to an understanding of the active site metal interactions, providing evidence that the thio ether sulfur of the protein redox group is noncoordinating in the crystallographically defined enzyme complex.

Galactose oxidase is a fungal copper metalloenzyme that catalyzes redox electron transfer between primary alcohols and O_2:[1]

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$$

Previous spectroscopic and biochemical studies[2] have shown that a free radical is stabilized as an essential component of the active enzyme, forming a free-radical-coupled copper active site. This novel structure extends the range of free-radical involvement in biochemical catalysis in the emerging new field of free-radical enzymology.[3] The presence of a free radical in galactose oxidase is not immediately apparent, since no EPR signals are observed from either Cu^{2+} ion or free-radical sites in the active complex, a consequence of strong antiferromagnetic exchange between these two paramagnetic centers. Within this interacting complex, both the metal ion and the protein free radical are redox active and participate directly in catalysis, forming a two-electron redox unit.[2] While the free radical in the active enzyme is spectroscopically masked by interactions with the cupric ion, a radical generated in the metal-free apoenzyme is directly accessible for detailed characterization.[4] EPR studies on isotopically labeled protein have demonstrated that this radical site is biosynthetically derived from a tyrosine residue but that the radical is distinct from the simple tyrosine phenoxyl radicals identified in ribonucleotide reductase[5] and in photosynthetic systems.[6] Crystallographic data recently reported for the inactive form of galactose oxidase reveal that one of the Cu ligands is a covalently modified tyrosine cross-linked to a cysteinyl sulfur from a residue remote in the polypeptide chain,[7] and spectroscopic information on the radical site is consistent with this tyrosine–cysteine dimer being the protein redox group.[2,8] Recently, ENDOR studies of the apoenzyme free radical have provided detailed information on spin distribution in the radical site and permitted a comparison with data on a model radical formed from (methylthio)cresol.[8] In parallel with the ongoing physical and chemical analysis of galactose oxidase, we are extending these model studies to explore specific features of this biological active site, providing insight into the chemistry of a new class of redox cofactors.

Materials and Methods

All reagents used in the preparation of complexes and glassing solvents were obtained from commercial sources. Solvents used for synthesis were redistilled and stored under argon. Freshly distilled methylene chloride was used for optical studies. For low-temperature optical experiments, a mixture of freshly distilled propionitrile:butyronitrile (1:1 molar ratio)[9] was degassed before using by freeze–thaw cycling under vacuum. This solvent forms a rigid glass below 100 K.[9] Absorption spectra were recorded on a Varian Cary 5E UV–vis–near-IR spectrometer. For low-temperature absorption measurements, samples were cooled in an Air Products Heli-Tran optical cryostat. EPR spectra were recorded on a Bruker ER300 EPR spectrometer equipped with a X-band microwave bridge and an Oxford Instruments ESR900 helium flow cryostat. Microwave frequency was measured with a Hewlett-Packard Model 5253B frequency counter. Magnetic field calibration was provided by the resonance from a DPPH crystal (g = 2.0037). Quantitative EPR measurements were performed as previously described,[2] taking precautions to avoid power saturation of the EPR signal. Free radicals were generated by irradiation of glassy samples prepared in a propionitrile:butyronitrile glassing solvent containing 1 equiv of tetrabutylammonium hydroxide with the full unfiltered output of a 150-W xenon arc lamp for 0.5 h. An

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® Abstract published in *Advance ACS Abstracts*, October 15, 1993.

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Table I. Summary of Crystal Data[a]

Cu complex	1	2	3	4
formula wt	475.5	489.5	505.5	641.7
crystal sys	monoclinic	monoclinic	monoclinic	monoclinic
space group	$P2_1 - C_2^2$	$P2_1/c - C_{2h}^5$	$P2_1/c$-C_{2h}^5	$P2_1/c$-C_{2h}^5
a, Å	8.635(3)	7.929(2)	8.012(2)	20.091(4)
b, Å	16.515(7)	23.199(7)	23.008(8)	9.671(2)
c, Å	8.650(3)	12.882(4)	12.796(4)	15.534(4)
β, deg	109.66(2)	103.84(2)	104.35(2)	90.92(2)
V, Å^3	1125(1)	2301(1)	2285(1)	3018(1)
Z	2	4	4	4
d_{calc}, g/cm^3	1.403	1.413	1.469	1.412
radiation, Å	0.71073	0.71073	0.71073	1.54184
μ, mm^{-1}	1.12	1.19	1.20	3.48
T, K	293 ± 1	293 ± 1	293 ± 1	293 ± 1
transm factors	0.877–1.000	0.447–0.840	0.900–1.000	0.358–1.000
total reflctns collectd	2849	3860	3830	4687
no. of indept absorptn corrected reflctns ($I > 3\sigma(I)$)	1545	1987	2124	2834
no. of refined params	307	298	284	398
R_1	0.044	0.044	0.042	0.047
R_2	0.051	0.051	0.052	0.059

[a] All calculations were performed on a Data General Eclipse S-200 or S-230 computer using versions of Nicolet E-XTL or SHELXTL interactive crystallographic software packages or on a DEC Microvax II computer or an IBM compatible 486 personal computer using Siemens SHELXTL-PLUS or SHELXTC-PC interactive software packages as modified by Crystalytics Co.

evacuated quartz optical Dewar was used to hold EPR sample tubes in liquid N_2 during irradiation. EPR spectra were simulated using a standard program (SIM15, obtained from the Quantum Chemistry Program Exchange as QCPE265) modified to permit all nuclear hyperfine terms to be treated nonaxially. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. Commercial crystal structure analyses were obtained from Crystalytics Co., Lincoln, NE.

Syntheses. (Methylthio)cresol was synthesized according to a published procedure,[10] and its structure was confirmed by NMR spectroscopy (δ 2.28 (s), 2.34 (s), 6.47 (s), 6.89 (d), 7.04 (d), 7.28 (s), in $CDCl_3$) and mass spectrometry (M^+ = 154).

(Methylsulfinyl)cresol was synthesized by the following two procedures. **(A)**. The addition of 1 equiv of 30% aqueous hydrogen peroxide to (methylthio)cresol in an equal volume of methanol was followed by reaction for 5–6 h at room temperature. The progress of the reaction was monitored by fluorescent silica gel TLC using chloroform as the solvent. Needle-shaped crystals formed during evaporation of the methanol were filtered and washed with distilled water, with a yield greater than 90%. **(B)**, After the addition of 1 equiv of performic acid and allowing the mixture to stand at 0 °C for 2 h, the reaction mixture was lyophilized twice at 0 °C and the lyophilized product dissolved in methanol. Needle-shaped crystals formed on the addition of cold water to the chilled methanol solution (yield ~60%). The product, (methylsulfinyl)cresol, was characterized by NMR spectroscopy (δ 2.28 (s), 2.94 (s), 6.85 (d), 7.18 (d), 7.26 (s), 10.0 (s), in $CDCl_3$) and mass spectroscopy (M^+ = 170). Anal. Calcd for (methylsulfinyl)cresol, $C_8H_{10}O_2S$: C, 56.47; H, 5.88. Found: C, 56.65; H, 5.93.

(Methylsulfonyl)cresol was synthesized according to procedure **B** for (methylsulfinyl)cresol (see above) except that 4 equiv of performic acid (0.86 M) was used. The lyophilized product from this reaction was purified by sublimation at 50–55 °C, and white crystals were obtained in good yield (>70%). No significant amount of (methylsulfonyl)cresol was formed when up to 8 equiv of hydrogen peroxide (approximately 0.6 M) was used instead of performic acid, although the time required to form (methylsulfinyl)cresol was proportionately decreased. The product, (methylsulfonyl)cresol was characterized by NMR spectroscopy (δ 2.35 (s), 3.15 (s), 6.95 (d), 7.35 (d), 7.50 (s), 8.65 (s), in $CDCl_3$) and mass spectrometry (M^+ = 186). Anal. Calcd for (methylsulfonyl)cresol, $C_8H_{10}O_3S$: C, 51.61; H, 5.38. Found in two analyses: C, 51.63, and 51.60; H, 5.47 and 5.23.

[Cu(1,1,4,7,7-pentamethyldiethylenetriamine)]$(ClO_4)_2$·2CH_3CN ([Cu(PMDT)]$(ClO_4)_2$·2CH_3CN) was prepared according to a published procedure.[11]

[Cu(PMDT)(cresol)](ClO_4)·CH_3OH (complex **1**) was synthesized by the addition of 1 equiv of cresol and potassium hydroxide to a boiling solution of [Cu(PMDT)]$(ClO_4)_2$·2CH_3CN in dry methanol, refluxing under argon for 20 min. After the solution was cooled to room temperature, a white precipitate was filtered off under argon and the filtrate was placed in a −20 °C freezer. Extra precautions required to exclude moisture in the synthesis of the cresol complex were not essential in the other syntheses described below. Long, rectangular, reddish-brown diffraction-quality crystals formed in the reaction mixture. Crystals were collected from the cold reaction mixture under a dry argon atmosphere. After filtration, the crystals were stored in a tightly sealed container to avoid loss of CH_3OH of crystallization (80% yield). Anal. Calcd for [Cu(PMDT)(cresol)](ClO_4)·CH_3OH, $C_{17}H_{34}N_3O_6ClCu$: C, 42.95; H, 7.16; N, 8.84. Found: C, 42.81; H, 7.43; N, 8.95.

[Cu(PMDT)((methylthio)cresol)](ClO_4) (complex **2**) was synthesized according to the same procedure as [Cu(PMDT)(cresol)](ClO_4)·CH_3OH. Dark blue crystals formed with 75% yield. Rectangular diffraction-quality crystals for X-ray structure determination were obtained from the reaction mixture and by ether diffusion into methylene chloride solution. Anal. Calcd for [Cu(PMDT)((methylthio)cresol)](ClO_4), $C_{17}H_{32}N_3O_5SClCu$: C, 41.72; H, 6.54; N, 8.59. Found: C, 41.60; H, 6.65; N, 8.50.

{Cu(PMDT)(methylsulfinyl)cresol)](ClO_4) (complex **3**) was synthesized according to the same procedure as [Cu(PMDT)(cresol)](ClO_4)·CH_3OH except that (methylsulfinyl)cresol was dissolved in methanol before being added to the reaction mixture. Green diffraction-quality crystals for X-ray structure determination formed directly in the reaction mixture, 80% yield. Anal. Calcd for [Cu(PMDT)((methylsufinyl)cresol)](ClO_4), $C_{17}H_3N_{32}O_6SClCu$: C, 40.40; H, 6.34; N, 8.32. Found: C, 40.09; H, 6.44; N, 8.26.

[Cu(PMDT)((methylthio)cresol)$_2$](ClO_4) (complex **4**) was prepared by the addition of (methylthio)cresol (1 equiv) to [Cu(PMDT)((methylthio)cresol)](ClO_4) in hot CH_3CN. The reaction mixture was allowed to stand for about 1 week at room temperature. Progress of the reaction was monitored by fluorescent silica gel TLC, using chloroform as the solvent, following the addition of a small amount of water to aliquot samples. Dark purple crystals were formed by ether diffusion into the reaction mixture. [Cu(PMDT)((methylthio)cresol)$_2$](ClO_4) also appeared as a side product during the synthesis of [Cu(PMDT)((methylthio)-cresol)](ClO_4), using CH_3CN in place of CH_3OH as the reaction solvent. No complex 4 formed when CH_3OH was used as the solvent or in the absence of copper. Diffraction-quality crystals for X-ray structure determination were formed by ether diffusion into methylene chloride solution (yield 70%). Anal. Calcd for [Cu(PMDT)((methylthio)cresol)$_2$]-(ClO_4), $C_{25}H_{40}N_3O_6S_2Cu$: C, 46.80; H, 6.24; N, 6.55. Found: C, 46.44; H, 6.42; N, 6.65.

X-ray Crystallography. X-ray data for Cu complexes **1–4** were measured at 293 ± 1 K on a computer-controlled Four-Circle Nicolet (Siemens) Autodiffractometer by using full (1.00°-wide) ω scans and graphite monochromated Mo Kα radiation, except complex **4** for which Ni-filtered Mo Kα radiation and θ–2θ scans were used. Crystal parameters and the details of the data and reduction are given in Table I. Structures were solved by direct methods using Siemens SHELXTL-PC software package as modified by Crystalytics, Inc. The resulting structure

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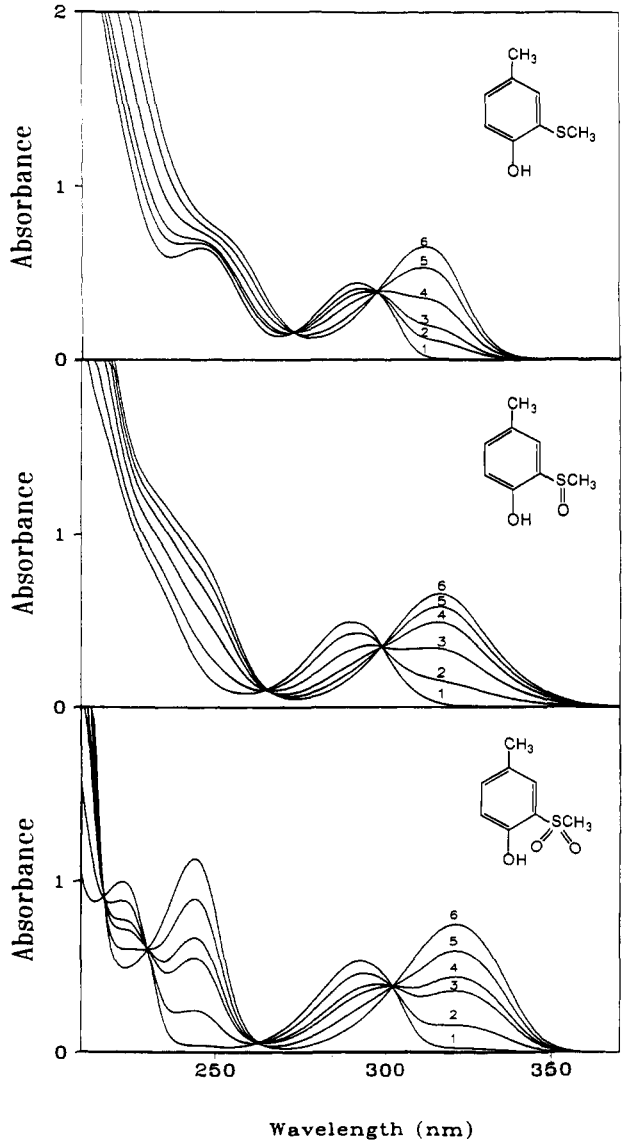

Figure 1. Top: (methylthio)cresol (145 μM) in 50 mM Na phosphate buffer; (1) pH 6.57, (2) pH 8.62, (3) pH 9.06, (4) pH 9.49, (5) pH 9.95, and (6) pH 11.84. Middle: (methylsulfinyl)cresol (145 μM) in Na phosphate buffer; (1) pH 4.71, (2) pH 7.06, (3) pH 7.58, (4) pH 8.18, (5) pH 8.63, and (6) pH 9.95. Bottom: (methylsulfonyl)cresol (145 μM) in Na phosphate buffer; (1) pH 6.17, (2) pH 7.09, (3) pH 7.63, (4) pH 7.83, (5) pH 8.24, and (6) pH 9.82.

parameters have been refined to convergence using counterweighted full-matrix least-squares techniques and a structural model which incorporated anisotropic thermal parameters for all non-hydrogen atoms and isotropic thermal parameters for all hydrogen atoms. Methyl groups were included in the structural model as rigid rotors with a conventional geometry and a C–H bond length of 0.96 Å. The remaining hydrogen atoms were fixed at idealized sp^2- or sp^3-hybridized geometries with a C–H bond length of 0.96 Å. Perchlorate counterions in lattices for complexes **1** and **2** are disordered with two preferred orientations. In complex **1**, hydrogen atoms on the methanol solvent molecule of crystallization were not included in the structural model. The correctness of the enantiomeric description was verified in cycles of least-squares refinement in which the multiplier of $\Delta f''$ refined to a final value of 0.4(2). In complex **4**, coordinates for the phenolic hydrogen of the noncoordinating phenol were determined from a difference Fourier map. Since this hydrogen atom could not be satisfactorily refined as an independent isotropic atom, its coordinates were fixed at this position in the refinement.

Results

Reactions and Spectra of Model Phenols. (Methylthio)cresol is converted smoothly and quantitatively to (methylsulfinyl)-cresol by treatment with a stoichiometric amount of H_2O_2 or performic acid at or below ambient temperature. Treatment with an excess of performic acid (but not H_2O_2) results in the formation of (methylsulfonyl)cresol. Figure 1 shows the results of optically detected pH titrations for these three substituted phenols. Analysis of these data yields pK_a values for the phenolic groups of (methylthio)cresol (pK_a = 9.5), (methylsulfinyl)cresol (pK_a = 7.7), and (methylsulfonyl)cresol (pK_a = 7.7). Ionization of the phenols results in a red shift in the lowest energy absorption features from 290, 288, and 293 nm to 310, 316, and 322 nm, respectively, for the methylthio, methylsulfinyl, and methylsulfonyl derivatives.

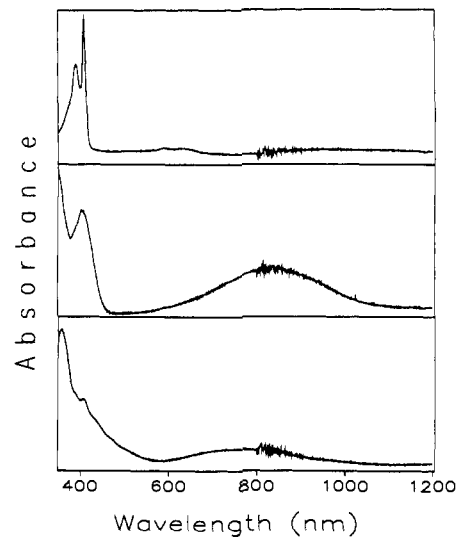

Figure 2. Low-temperature absorption spectra for the products of UV irradiation of (top) 5 mM cresol, (middle) 7 mM (methylthio)cresol, and (bottom) 5 mM (methylsulfinyl)cresol in propionitrile:butyronitrile at 77 K.

Low-temperature absorption spectra of the products of UV irradiation of cresol, (methylthio)cresol, and (methylsulfinyl)-cresol in nitrile glassing solvent at 77 K are shown in Figure 2. Carrying out the sample irradiation and spectral measurements at helium temperature (10 K) compared to liquid N_2 temperature does not lead to an increase in the optical absorption intensity. The product from irradiation of cresol is identified from the absorption spectrum (transitions at 408 and 388 nm) as the phenoxyl free radical.[12] The optical absorption spectrum of the product of UV irradiation of (methylthio)cresol strongly resembles the spectrum of radical-containing apo galactose oxidase, with a near-UV absorption band near 400 nm and a broad absorption in the red at 830 nm.[4] Irradiation of (methylsulfinyl)cresol under these conditions yielded a distinct product, characterized in optical absorption by transitions near 355 and 755 nm. Helium-temperature EPR spectra of radical species formed under these conditions are shown in Figure 3, together with spectral simulations from which ground-state Zeeman and hyperfine parameters have been evaluated. Spectra for all three radical products exhibited an average g-value near 2.005, characteristic of phenoxyl free radicals. The spectrum for the cresol radical product is the same as has previously been reported, and the spectrum of the (methylthio)cresol phenoxyl radical is similar to that observed for the radical prepared in 0.1 M KOH:LiCl glassing mixture.[8] The yield of free radical generated by 0.5 h of irradiation in nitrile glassing solvent at 77 K was consistently on the order of 5% for all three compounds on the basis of EPR spin quantitation. This yield is not optimized, and higher levels of conversion have been achieved by increasing the extent of UV irradiation and with sample dilution. More than 15% conversion to radical products occurs on prolonged exposure to UV light but with the appearance of additional signals in the spectrum near g = 2.04.

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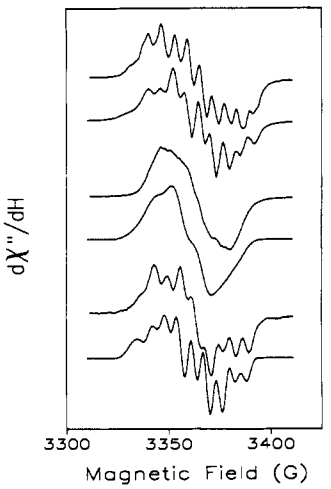

Figure 3. EPR spectra for the radical products of (top) 5 mM cresol, (middle) (methylthio)cresol, and (bottom) (methylsulfinyl)cresol in nitrile glassing solvent following 0.5 h of UV irradiation at 77 K. Instrumental parameters: temperature, 10 K; microwave frequency, 9.435 GHz; microwave power, 10 μW; and modulation amplitude, 1 G. The upper spectrum in each pair is experimental data, and the lower spectrum is simulated using the following parameters: (top) g_{xx} = 2.0060, g_{yy} = 2.00050, g_{zz} = 2.0023; three equivalent β protons (a_{xx} = 13.2 G, a_{yy} = a_{zz} = 12.0 G); two sets of two equivalent α protons each (a_{xx} = 9.75 G, a_{yy} = 3.25 G, a_{zz} = 6.5 G; a_{xx} = 2.55 G, a_{yy} = 0.8 G, a_{zz} = 1.7 G); line width 3G; (middle) g_{xx} = 2.0095, g_{yy} = 2.0070, g_{zz} = 2.0023; three equivalent β protons (a_{xx} = 11 G, a_{yy} = 10 G); one *ortho* α proton (a_{xx} = 8.3 G, a_{yy} = 2.75 G, a_{zz} = 5.55 G); two equivalent *meta* α protons (a_{xx} = 3 G, a_{yy} = 1 G, a_{zz} = 2 G); line width 6 G; (bottom) g_{xx} = 2.0085, g_{yy} = 2.0080, g_{zz} = 2.0023; three equivalent β protons (a_{xx} = 13 G, a_{yy} = 12 G); one *ortho* α proton (a_{xx} = 9.75 G, a_{yy} = 3.25 G, a_{zz} = 6.5 G); two equivalent *meta* α protons (a_{xx} = 2.8 G, a_{yy} = 0.8 G, a_{zz} = 1.5 G); line width 3 G.

Both the EPR signals and the yellow color for all of these radicals disappear on warming the samples above 100 K, consistent with the glassing temperature reported for this solvent.[9] The observation that the free radicals formed from these compounds exhibited negligible power saturation under the experimental conditions suggests a relatively short relaxation time compared to many organic free radicals. For the thio-substituted compounds, rapid spin relaxation may reflect sulfur contributions in the ground-state electronic wave function for the radical, with increased spin-orbit interaction providing a mechanism for spin-lattice coupling.

Coordination Chemistry of Cresol, (Methylthio)cresol, and (Methylsulfinyl)cresol. $[Cu(PMDT)](ClO_4)_2 \cdot 2CH_3CN$ forms 1:1 complexes with cresol, (methylthio)cresol, and (methylsulfinyl)cresol. Structures of these three complexes defined by X-ray crystallography are shown in Figures 4–6. Within complexes involving an identical ligand complement provided by the PMDT trischelate, the three phenolic ligands express distinct coordination chemistry, The complex $[Cu(PMDT)(cresol)](ClO_4) \cdot CH_3OH$ exhibits a four-coordinated structure, while five-coordination is observed for both [Cu(PMDT)((methylthio)cresol)](ClO_4) and [Cu(PMDT)((methylsulfinyl)cresol)](ClO_4). Lattice constants and unit cell parameters for the crystalline products are listed in Table I.

The possibilities of linkage isomerism in the complexes of the substituted phenols provide these structures with a special interest relating to interactions between the redox cofactor and copper in the galactose oxidase active site. In [Cu(PMDT)((methylthio)-cresol)](ClO_4), the thioether sulfur is found to directly coordinate to copper, with a relatively long 2.55-Å bond distance, and the methyl sulfide side chain is twisted out of the plane of the phenolic ring system. This (methylthio)cresol–copper complex reacts with excess (methylthio)cresol in boiling acetonitrile, yielding a complex containing an *ortho–ortho*-coupled (methylthio)cresol

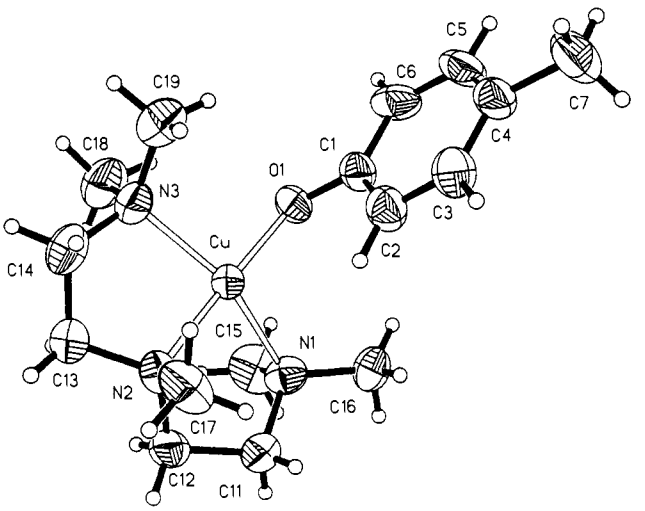

Figure 4. Structure of $[Cu(PMDT)(cresol)](ClO_4) \cdot CH_3OH$ (complex **1**).

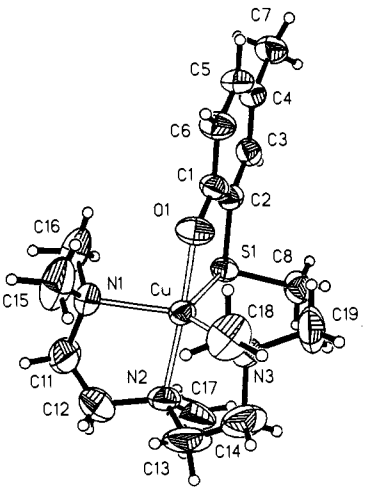

Figure 5. Structure of [Cu(PMDT)((methylthio)cresol)](ClO_4) (complex **2**).

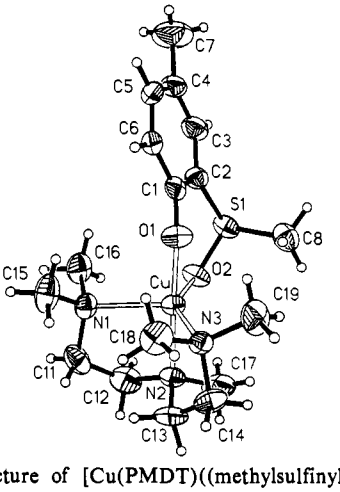

Figure 6. Structure of [Cu(PMDT)((methylsulfinyl)cresol)](ClO_4) (complex **3**).

dimer (complex **4**, Figure 7). Only one of the two available phenolic oxygens coordinates to a metal ion in this complex. In $[Cu(PMDT)((methylthio)cresol)_2](ClO_4)$ (Figure 7), the coordinating (methylthio)cresol is essentially identical to the corresponding structure in the monomer complex. In contrast, the dangling thioether side chain for the uncoordinated (methylthio)-cresol group lies in the plane of the phenolic ring. In [Cu(PMDT)-((methylsulfinyl)cresol)](ClO_4), the phenolate and sulfoxide

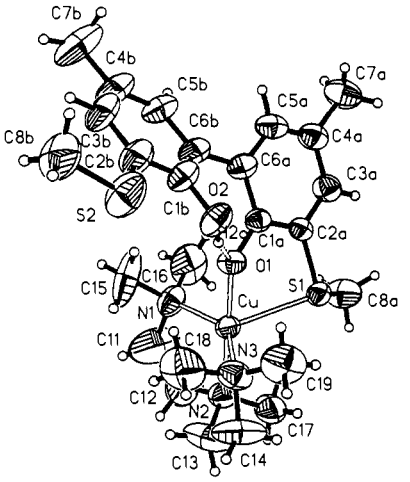

Figure 7. Structure of [Cu(PMDT)((methylthio)cresol)$_2$](ClO$_4$) (complex **4**).

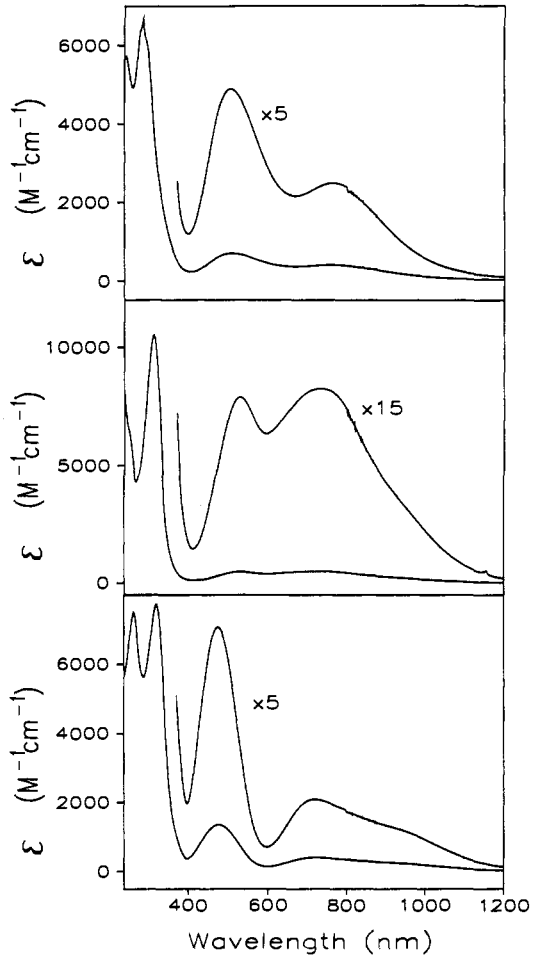

Figure 8. Room-temperature absorption spectra of (top) complex **1**, (middle) complex **2**, and (bottom) complex **3** in CH_2Cl_2.

oxygens coordinate to copper but not the sulfur. Both oxygens have short bond distances (2.057 and 1.903 Å, respectively) to Cu. The (methylsulfinyl)cresol ligand is chiral, and enantiomeric complexes are paired in the $Z = 4$ unit cell of the centrosymmetric space lattice for crystals of this complex prepared from an unresolved racemic mixture. Crystals of the (methylthio)cresol– and (methylsulfinyl)cresol–copper complexes are highly pleochroic in polarized light.

Room-temperature solution absorption spectra of these complexes in methylene chloride are shown in Figure 8. Intense near-UV features occurring for all three complexes correspond to

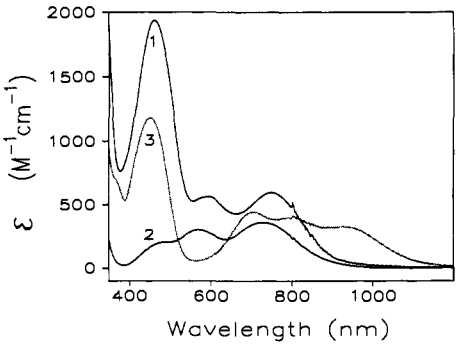

Figure 9. Low-temperature (100 K) absorption spectra of (1) complex **1**, (2) complex **2**, and (3) complex **3** in nitrile glassing solvent.

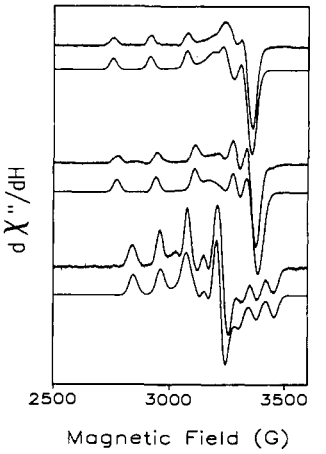

Figure 10. EPR spectra for 1 mM (top) complex **1**, (middle) complex **2**, and (bottom) complex **3** in nitrile glassing solvent. Instrumental parameters: temperature, 80 K; microwave frequency, 9.46 GHz; microwave power, 10 μW; and modulation amplitude, 10 G. The upper spectrum in each pair is experimental data, and the lower spectrum is simulated using the following parameters: (top) $g_{xx} = 2.050$, $g_{yy} = 2.065$, $g_{zz} = 2.250$; a_{xx}(Cu) = a_{yy}(Cu) = 34 G, a_{zz}(Cu) = 161 G; line width 40 G; (middle) $g_{xx} = 2.030$, $g_{yy} = 2.060$, $g_{zz} = 2.225$; a_{xx}(Cu) = 34 G, a_{yy}(Cu) = 50 G, a_{zz}(Cu) = 169 G; line width 40 G; (bottom) $g_{xx} = 2.228$, $g_{yy} = 2.138$, $g_{zz} = 2.012$; a_{xx}(Cu) = 45 G, a_{yy}(Cu) = 118 G, a_{zz}(Cu) = 74 G; line width 35 G.

intraligand π–π transitions for the phenolic ring systems and occur at energies observed for the unprotonated phenoxide forms in solution. Ligand-to-metal charge-transfer (LMCT) transitions occur at lower energy. In the cresol complex, a strong absorption band is observed near 500 nm that is consistent with the phenolate-to-copper charge-transfer assignment. No resolved charge-transfer spectra are observed for the (methylthio)cresol complex. For the methylsulfinyl complex, a transition of moderate intensity (ϵ = 1420 M^{-1} cm^{-1}) appears near 475 nm. Relatively weak ligand field or d → d absorption bands occur at lower energy, near 765 nm for the tetragonally coordinated copper–cresol complex. For the pyramidal (methylthio)cresol complex, ligand field spectra are observed near 525 and 734 nm (ϵ = 535 M^{-1} cm^{-1}). The roughly trigonal-bipyramidal copper complex of (methylsulfinyl)cresol exhibits ligand field transitions near 715 and 920 nm. Additional structure is resolved in the spectra of these complexes in nitrile glassing solvent at low temperature (100 K) (Figure 9) and is particularly evident in the absorption spectrum of the (methylsulfinyl)cresol complex, in which three distinct transitions (near 700, 810, and 935 nm) may be resolved and the charge-transfer absorption is shifted to 450 nm. For the (methylthio)cresol complex, a weak absorption band is resolved near 470 nm at low temperature.

EPR spectra for these complexes recorded at liquid nitrogen temperature in nitrile glassing solvent are shown in Figure 10. The best-fit simulations (Figure 10) for the experimental spectra of cresol and (methylthio)cresol complexes are obtained using

spin Hamiltonian parameters characteristic of cupric complexes having a $d_{x^2-y^2}$ orbital ground state, reflecting an approximately tetragonal (square-pyramidal) effective geometry for these complexes. In contrast, the parameters required to simulate the EPR spectrum of the methylsulfinyl–copper complex ($g_{x,y} > g_z \sim 2.0$) are characteristic of a trigonally distorted cupric complex having a predominantly d_{z^2} ground-state electronic wave function.[13] In the latter complex, the appearance of a minor feature in the experimental spectrum not reproduced in the simulation may reflect a small degree of heterogeneity in the frozen sample.

Discussion

The free-radical site in galactose oxidase is a unique feature of the protein structure that forms the basis for its biological activity as an alcohol oxidation catalyst. Biological free radicals are generally short-lived reactive species occurring as transient intermediates in redox reactions. In contrast, the radical site in galactose oxidase is unusually stable, surviving for days in solution in the presence of O_2. The stabilization of this biological free radical and the factors controlling its formation at a low redox potential and its reactivity in catalysis may be traced to the structure and the interactions of the protein redox group in the active site. The most likely suspect for the radical-forming group in the active site is a tyrosine–cysteine cross-link site that has been identified crystallographically.[7] Understanding the reactivity of this new type of protein side-chain redox group is expected to provide important insight into the chemistry of the galactose oxidase active site.

The reactions involved in the formation of this redox cofactor are presently unknown. It is possible that the covalent cross-link may form nonenzymatically, the result of free-radical coupling between tyrosine and cysteine within a proenzyme complex. Once formed, the linkage must be quite stable in the protein toward reaction with O_2 and toward nucleophilic displacement of the side chain, as is typical of the robust character of thioethers. Modification of the redox site in the protein is expected to result in the loss of enzymatic activity, and we have begun to explore reactions of the model compound that may shed light on possible pathways for inactivation of the enzyme. Inactivation of galactose oxidase is a serious problem for both spectroscopic and chemical characterization as well as in possible synthetic or analytical applications. A reversible inactivation of galactose oxidase following exposure to mild reductants such as ferrocyanide or ascorbate is known to be a consequence of one-electron reduction of the protein-derived free radical.[2] However, other, irreversible, inactivation steps may also occur. Quantitative studies have shown that a variable fraction of cupric enzyme that is otherwise indistinguishable from the rest is not susceptible to activation by oxidants,[2] possibly representing species in which the redox cofactor has been covalently modified. The reaction of (methylthio)-cresol with hydrogen peroxide and peracids to form sulfoxides may model inactivation of the enzyme active site by reaction with the product hydrogen peroxide. For the (methylthio)cresol model compound, a smooth transfer of oxygen occurs with either H_2O_2 or performic acid, forming the (methylsulfinyl)cresol product. Conversion to a sulfone derivative does not occur to a significant extent with H_2O_2, a product of galactose oxidase turnover, even at elevated concentrations and requires a more powerful oxidant such as performic acid for its preparation. In contrast to sulfoxide formation, generation of a sulfone is unlikely to be a significant pathway for inactivation of the redox group in galactose oxidase.

Effects of substitution of the phenolic ring system in the model compounds have been observed in the acid–base chemistry of the methylthio, methylsulfinyl, and methylsulfonyl derivatives. Perturbation of acidity of the phenolic proton in these model compounds will relate to biological functions of the corresponding protein derivatives as a consequence of the importance of proton equilibria in enzyme catalysis and the effect of ligand basicity on metal–ligand interactions. Basic characterization of these models thus includes measurement of the effects of substituents on the acidity of the phenolic group. Titration of the phenolic groups reveals a progressive increase in the acidity of the hydroxyl group by 1 pH unit for thioether substitution and 2 pH units for both the sulfinyl and sulfonyl derivatives, relative to the parent cresol ($pK_a = 10.2$).[14] Distinctive changes in the electronic spectra of these substituted phenols are also evident in the titration data, providing reference data for comparison with protein spectra. A blue shift of the lowest energy electronic transition results from oxygenation of the thioether, while ionization to form the phenolate results in a red shift of the low-energy transitions in both compounds.

Cresol, (methylthio)cresol, and (methylsulfinyl)cresol form phenoxyl free radicals under UV irradiation at 77 K in glassy solution, displaying distinctive EPR and optical absorption spectra. The generation of the known free-radical derivative of the cresol parent compound[8] establishes the experimental conditions for preparing phenoxyl radicals in this series. The EPR spectra of all three products of UV photolysis also support assignment to phenoxyl radicals, exhibiting average g-values near 2.005, characteristic of the phenoxyl group, and permit quantitation of the extent of conversion. Neither the methylthio- nor the methylsulfinyl-substituted radicals exhibit a significant g-shift that would reflect substantial sulfur orbital contributions in the electronic ground state. This is understandable in terms of earlier ENDOR studies that indicate an odd alternant spin distribution for the (methylthio)cresol phenoxyl radical,[8] resulting in unpaired electron density on the oxygen and on the *ortho* and *para* carbons but not on the adjacent sulfur atom. The EPR spectra are not significantly solvent-dependent, and EPR data obtained for the (methylthio)cresol radical in CH_2Cl_2:toluene glassing solution are very similar in terms of both line shape and yield to data for samples in the propionitrile:butyronitrile mixture. Both also closely resemble spectra previously reported for this radical in KOH:LiCl glass.[8] The nitrile solvent appears to have important advantages, however, forming a glass that is less susceptible to fracturing, resulting in more reliable optical spectra. In addition, an artifact EPR signal that is present in the toluene glass at a lower extent of conversion is apparent in the nitrile samples only at higher levels of UV exposure. Finally, the spectral window for both photolysis and spectral measurements extends further into the UV for nitriles than for the toluene-containing solvent. Base was required for forming radicals in both solvent systems.

The optical absorption spectrum of the free radical formed from (methylthio)cresol is nearly identical to the spectrum of radical-containing oxidized apo galactose oxidase.[4] The striking resemblance between model and protein free radicals provides strong support for the identification of the radical site in apo galactose oxidase with a thioether-substituted phenol such as the crystallographically determined tyrosine–cysteine dimer.[7] The similarity of the optical spectra exhibited by these radicals indicates that in the apoenzyme, at least the radical is localized almost exclusively on the modified tyrosine, as indicated previously in ENDOR studies,[8] rather than delocalized over a larger structural unit. The formation of the free-radical phenoxyl derivative of the methylthio-substituted phenol at relatively low potential ($E_{1/2} = 0.45$ V)[1,2], nearly half a volt lower than that required to oxidize an unsubstituted tyrosine, suggests a functional significance for this structure in biology may be the selective accessibility and stabilization of the one-electron oxidation product, since further oxidation does not appear to occur up to at least 0.8 V.[1] In contrast, other redox cofactors such as flavins[15]

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and topaquinones[16] have a relatively accessible second oxidation level.

The coordination chemistry of the thioether- and sulfinyl-substituted phenols has been explored in order to better understand the interactions of the redox cofactor with the cupric ion in the active site of galactose oxidase. The cresol complex **1** models the coordination of the unmodified tyrosine ligand (Tyr-495) in the copper binding site of galactose oxidase as well as the interactions of the unmodified precursor tyrosine (Tyr-272). In a broader context, complex **1** provides a general model for tyrosinate–copper interactions in proteins. The geometry adopted by the phenolate ligand is interesting in that it orients the aromatic ring system perpendicular to the equatorial plane of copper ligands, maximizing the overlaps between the p π orbitals on the coordinated oxygen with both a $d_{x^2-y^2}$ orbital on copper and the π system of the ring. For the complex we have prepared, in which the phenolate is best described as occupying an equatorial ligation site, a strong absorption band is observed near 460 nm with an intensity consistent with the assignment as a phenolate-to-Cu LMCT. The intensity of this LMCT band is more than twice that observed in the inactive form of galactose oxidase, for which crystallographic data imply an axial site for interactions with the unmodified tyrosine (residue 495).[7] Charge-transfer intensity is expected to be relatively weak for this axial ligand as a consequence of the symmetry-required cancellation of orbital overlaps for interactions between the O valence orbitals and a perpendicularly oriented $d_{x^2-y^2}$ orbital, accounting for the relatively low intensity in the enzyme complex as calibrated by the inorganic model. Beyond this spectroscopic correlation, there are also parallels in the water sensitivity of this copper–cresolate complex and the chemistry of the active site of galactose oxidase, where small-molecule binding is coupled to a proton-uptake step that appears to result from displacement and protonation of a coordinated tyrosine phenolate.[17]

(Methylthio)cresol displays a distinct bidentate coordination to copper in complex **2**, involving both the phenolate oxygen and the thioether sulfur in interactions with the metal ion, demonstrating that the *ortho* thioether substitution pattern permits chelation of a metal center. The Cu–S bond distance is relatively long, consistent with weak thioether interactions observed for a range of other copper complexes.[18] EPR data support a predominantly $d_{x^2-y^2}$ orbital description for the ground state consistent with a predominant tetragonal perturbation of the cupric ion in the complex. An electronic perturbation of the thioether group on forming the complex is reflected in the bond angles around S, which show a pronounced deviation toward pyramidal geometry, resulting in a torsion angle (C_3–C_2–S–C_8) of 66.2°. Similar geometric distortions have been reported for complexes of Cu with other thioether-donating ligands where the effects of S bonding on the valence electronic structure are reflected in the geometry around the sulfur atom.[18] A useful reference point for a noninteracting (methylthio)cresol group is provided by the dangling group in the dimer complex **4** where the coplanar side-chain geometry expected for the unperturbed thioether is retained with a torsion angle of less than 2°. These observations confirming that the pyramidal distortion for the ligated group reflects coordination are of interest in terms of defining the interactions between Cu and the redox cofactor in the active site of galactose oxidase. The 1.7-Å X-ray crystal structure has been solved to show a near-planar *cis* geometry about the thioether group, with a torsion angle (C_δ–C_ϵ–S–C_β) of 7° and a Cu–S bond distance apparently greater than 3 Å.[7] On the basis of the model studies, this implies that the thioether is not coordinated in the form of the enzyme in the crystals, which most likely represent the reductively inactivated form of the protein.[7,8,17] However, there are no intervening atoms between the thioether sulfur and Cu, and coordination of the thioether may be favored in another oxidation state.

Spectroscopic characterization of complex **2** has also contributed to understanding the electronic spectra of the biological active site. The optical absorption spectra for the model exhibit a relatively low-energy electronic transition near 500 nm of low intensity, which may be attributed to ligand-to-copper charge transfer from the thioether-substituted ligand. The absence of a strong phenolate-to-Cu^{2+} LMCT in the model parallels the lack of resolved spectra from the coordinated cofactor in galactose oxidase. For the enzyme complex, the lower energy transitions are relatively intense and may include contributions from the unresolved LMCT from the modified tyrosine. This curious lack of a strong LMCT is currently under investigation. The oxygenated (methylsulfinyl)cresol ligand also coordinates to the metal ion, and the structure obtained for complex **3** shows that relatively strong interactions occur with both the sulfoxide and phenolate oxygens in the ligand. The optical spectrum of this complex exhibits a strong absorption band near 450 nm that must arise from the phenolate-to-Cu^{2+} LMCT. This assignment is supported by the observation that previously characterized cupric sulfoxide complexes do not exhibit charge-transfer excitations beyond 350 nm.[19] Chelation of copper by the sulfoxide ligand imposes a trigonal-bipyramidal geometry at the metal center, and the low-temperature EPR spectrum observed for this complex is propionitrile:butyronitrile solution is characteristic of a predominantly d_{z^2} orbital ground state, with $g_\perp > g_\parallel \sim 2.0$, consistent with the crystallographically defined geometry.

In addition to providing calibration for structural and spectroscopic studies on the galactose oxidase active site, the chemistry of these inorganic complexes may lead to functional models as well. The possibility that the (methylthio)cresol Cu complex might serve as a functional mimic for the catalytic active site is suggested by its reaction with excess (methylthio)cresol in acetonitrile to form an *ortho* linked dimer compound (complex **4**) whose structure is characteristic of the products of oxidative phenol coupling reactions that are believed to occur through free-radical mechanisms.[20] Free (methylthio)cresol does not undergo this dimerization reaction under the same conditions, suggesting that the copper complex **2** may be capable of transiently stabilizing a free-radical complex, leading to the observed reactivity.

Acknowledgment. This work was supported by the National Institutes of Health (GM-46749).

Supplementary Material Available: Lists of bond distances and bond angles for complexes **1**, **2**, **3**, and **4** (16 pages); listings of observed and calculated structure factors (34 pages). Ordering information is given on any current masthead page.

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