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Stereoelectronic Properties of Metalloenzymes. 5. Identification and Assignment of Ligand Hyperfine Splittings in the Electron Spin Resonance Spectrum of Galactose Oxidase

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Abstract: Time-averaged electron spin resonance spectra for native galactose oxidase suggested two nitrogen-liganded atoms based on the partial resolution of superhyperfine structure in the parallel region of the spectrum. Electron spin resonance data obtained for ^{63}Cu-galactose oxidase as well as the $^{19}F^-$-enzyme complex and imidazole-enzyme complex indicate that not only are there two nitrogen atoms present in the native protein but that these arise from liganded imidazole (histidine) nitrogen atoms. Interpretation of these spectra is dependent on the observation of superhyperfine structure in the parallel, perpendicular, and "overshoot" regions of all the spectra. A detailed discussion and an explanation for the interpretations are presented.

Galactose oxidase, a single chain copper protein, is unique because it contains a single non- or low-blue copper(II) atom.[1-4] Thus, its spectral properties exemplify those characteristic of type II copper(II) atoms[5] without interference by other prosthetic groups. As such, it offers an opportunity to gain information concerning the structure of the large class of type II sites in multi-nuclear copper proteins, especially those where detailed spectroscopic studies are made difficult by the type I or "blue" copper(II) atom(s) present.[5]

The characterization of the copper(II) coordination site in a complex ion or protein, in principle, can be achieved in large measure by the determination of the spin Hamiltonian parameters of the system. For example, for a copper(II) containing protein, the magnitudes of the principal g value and metal hyperfine coupling together have been correlated with the charge and chemical nature of the ligand atoms.[5,6] Furthermore, the overall symmetry can be suggested by the degree of anisotropy in the three g values and the exact number and chemical nature of some ligand atoms can be deduced from ligand superhyperfine structure.[5]

Blumberg et al.[1] first reported the electron spin resonance (ESR) spectrum of galactose oxidase. More recently, our laboratories[4,7] and others[8] have examined in detail this spectrum and its response to exogenous ligands.[7,9] However, to date, no hyperfine splittings due to ligand atoms have been assigned unambiguously nor has the complex "perpendicular" region of the spectrum been adequately interpreted. In this report, such assignments are made and a reasonable model for the metal coordination site in the protein is suggested.

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Experimental Section

Galactose oxidase was purified from shake flask cultures of *Dactylium dendroides* as previously reported.[2] ^{63}Cu-enzyme was obtained by growing fungus in media depleted of all trace metals by passage over columns of Chelex-100 (Bio-Rad) followed by metal supplementation using reagent grade metal salts and with ^{63}CuO (Oak Ridge) as the copper source. The metal content of the medium was monitored by graphite furnace atomic absorption (Perkin-Elmer Model 360); endogenous copper was reduced to less than 10^{-9} M prior to addition of ^{63}Cu(II). The alternative method of preparing ^{63}Cu-galactose oxidase from the apoenzyme is by nature a destructive process. Preparation by growth of the isotopically pure enzyme routinely gave more well-resolved ESR spectra and was the method employed here.

Enzyme samples for spectral studies were prepared in 0.1 M sodium phosphate buffer (pH 7.0), with an enzyme concentration of ~0.5 mM. Ligand solutions were prepared in the same buffer. Imidazole was a Sigma product, twice recrystallized from benzene after initial treatment with Norite. KF was zone-refined and kindly supported by Bell Laboratories, Murray Hill, N.J. All other chemicals were reagent grade. Spectral titrations were carried out as previously described.[7] All enzyme samples regained full activity and spectral characteristics after F^- or imidazole was removed by dialysis. Thus, F^- and imidazole binding was completely reversible as previously seen with other anions and neutral molecules.

Electron spin resonance spectra were obtained with a Varian E-9 spectrometer operating near either 9 GHz (X-band) or 35 GHz (Q-band) microwave frequencies at 100 K. X-band spectra were calibrated employing diphenylpicrylhydrazyl (DPPH) as an internal

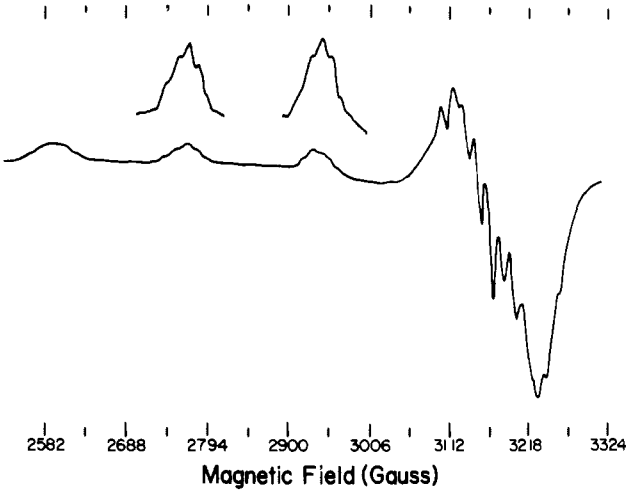

Figure 1. ESR spectrum of $^{63/65}$Cu-galactose oxidase at 9.090 GHz. This figure represents an average of six scans as described in the Experimental Section. The insert shows the $M_I \pm 1/2$ transitions at higher signal level.

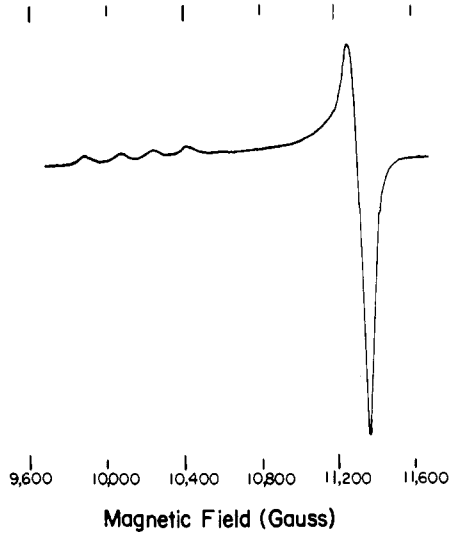

Figure 2. ESR spectrum of $^{63/65}$Cu-galactose oxidase at 34.758 GHz.

standard in conjunction with a Hewlett-Packard microwave frequency meter. Spectral averaging was carried out using a Nicolet Lab 80 CAT. Microwave power was routinely 20 mW and the modulation amplitude was 4.0 G. The Q-band field was precalibrated as was the microwave frequency. Such spectra were recorded at 4.0-G modulation amplitude with 16 dB microwave attenuation. The 35-GHz spectra were obtained at the University of New Hampshire; we are grateful to Professor Dennis Chasteen for allowing us the use of his spectrometer.

Results and Discussion

Figure 1 and the insert represent a time-averaged ESR spectrum of galactose oxidase containing ^{63}Cu/^{65}Cu in natural abundance. This spectrum is similar to those previously reported by us[4] and others[1,8,9] except that hyperfine structure due to ligand atoms (shfs) is clearly evident in the parallel region of the spectrum, particularly the $M_I = \pm 1/2$ transitions as would be expected. Resolution of this structure is not improved by isotropic substitution apparently because of the inherent line width and splitting values for the shfs in this region. The spectrum is indicative of a coupling pattern due to two nitrogen ligand atoms ($I = 1$). Since the shfs due to covalent ligands bound to copper is often nearly isotropic,[10] then splitting of a similar magnitude and number *should* be evident in the perpendicular region of the spectrum. Thus, five lines in the "complex" perpendicular region must be due to ligand shfs also.

Further evidence of this shfs appears in the intense spectral feature at ~3240 G. This "extraneous" or "overshoot" line is often observed[10] in X-band ESR spectra of copper(II) systems and can be a valuable diagnostic aid in obtaining the spin Hamiltonian parameters for a complex spectrum. This overshoot line represents a class of unique intermediate orientations of the copper(II) complex between the parallel and perpendicular orientations of the g and A tensors ($0 < \xi < \pi/2$) relative to the external magnetic field. (That this line is in fact an "overshoot" line can be determined by comparing the line widths of the entire perpendicular envelope at X- and Q-band frequencies. The differences must be due to an "extra" peak at high field in the X-band spectrum.) It often is the most intense feature of the spectrum. Qualitatively, this feature can be thought of as a weighted average of the fourth parallel and perpendicular lines ($M_I = -3/2$) and as such will contain any hyperfine splittings due to ligand atoms exhibited by either (or both) of these two lines. The magnitude of the splittings will be of some intermediate value when shfs is present in all orientations, as is the case here. Most valuable, however, is the fact that this line occurs at the maximum field position in copper spectra and the splitting pattern is not complicated by metal hyperfine splittings. In the case of galactose oxidase, a simple five-line pattern (see Figure 1) is observed on this overshoot line and is again indicative of a coupling to two nitrogen atoms. As expected, the magnitude of this splitting on the "overshoot" line (14.8 G) is intermediate between that observed on the parallel lines (14.5 G) and that observed in the perpendicular region (15.1 G). We have previously suggested that the complex perpendicular region of this spectrum might also be due to coupling to the copper nucleus in addition to shfs.[4] This possibility can now be eliminated by examining the 35-GHz spectrum of galactose oxidase (Figure 2). The perpendicular region of the spectrum shows neither g value anisotropy nor a metal hyperfine pattern. In addition, experience has shown that shfs which is clear at X-band frequencies[11] is often not detected at Q-band frequencies. This is not true of metal hyperfine splittings. Thus, all of the hyperfine splitting in the complex perpendicular region of the ESR spectrum of galactose must be due to shfs of the perpendicular spectral component and shfs of the high field ($M_I = -3/2$) parallel line which also lies in this region.

Confirmatory evidence for this interpretation can be obtained by inspection of the shfs in the spectra of galactose oxidase in the presence of added exogenous ligands. Ligand addition has resulted in the enhancement of shfs exhibited by other natural[12] and artificial copper(II) proteins.[13,15] We have previously shown that certain (CN^- and SCN^-) exogenous ligands form tight 1:1 complexes with galactose oxidase,[7] and, more importantly, it has not been possible to form a 2:1 complex with the Cu(II) center. This stoichiometry is indicated by either the appearance of a new and unique ESR spectrum upon the addition of a 1:1 molar ratio of ligand to copper if a strong complexing ligand is employed or, in certain favorable cases, by the appearance of shfs due to the added ligand which is also characteristic of a 1:1 complex. This latter case is dramatically illustrated by the X-band spectrum of ^{63}Cu-galactose oxidase in the presence of 0.22 M KF (Figure 3A). Hyperfine coupling due to one $^{19}F^-$ ($I = 1/2$) is clearly evident, especially on the $M_I = +3/2$ transition in the parallel region (see insert) indicating effective coordination to only one such ion ($A_F^{\parallel} = 41.0$ G). Of particular value is the observation that the shfs due to nitrogen is better resolved in this case ($A_N^{\parallel} = 11.2$ G) than in the native protein. This well-resolved pattern again can be confidently assigned to only two equivalent nitrogen atoms. In addition, a striking new feature consisting of two well-separated sets of

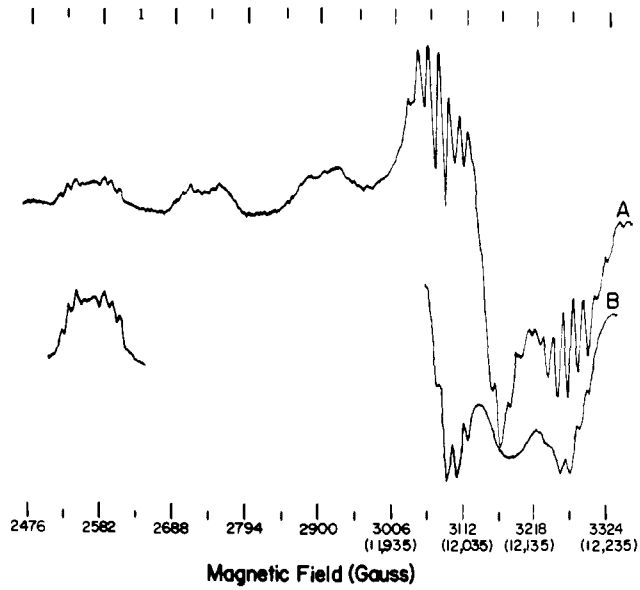

Figure 3. ESR spectrum of ^{63}Cu-galactose oxidase-$^{19}F^-$ complex at (A) X- and (B) Q-band frequencies (perpendicular region only); [KF] = 0.22 M. The insert shows the $M_I = +3/2$ transition at X-band at higher signal level.

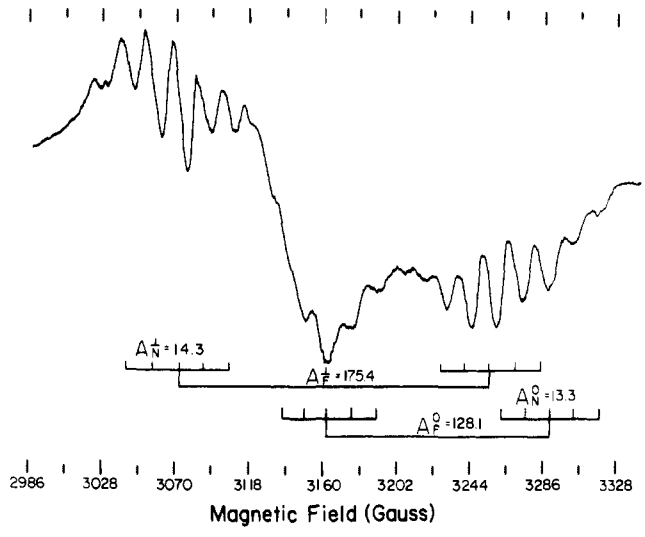

Figure 4. Perpendicular region of the ESR spectrum in Figure 3A.

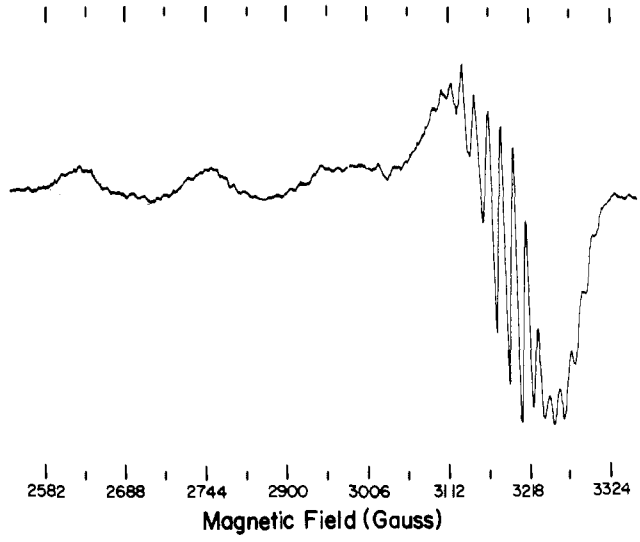

Figure 5. ESR spectrum of ^{63}Cu-galactose oxidase-imidazole complex; [Im] = 0.12 M.

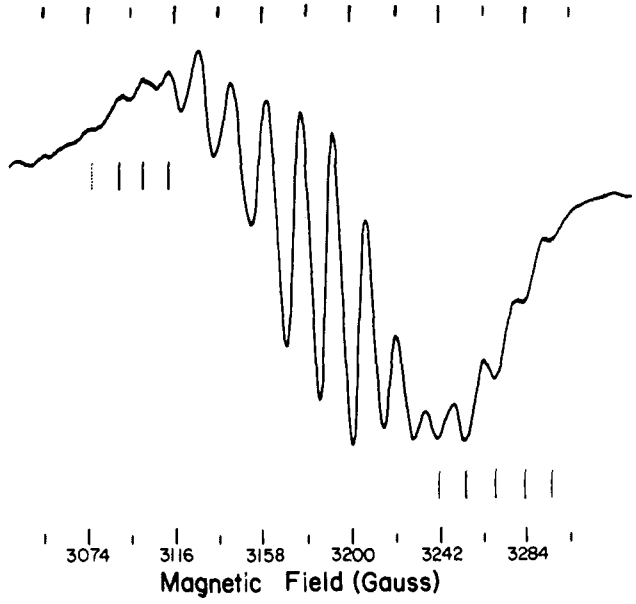

Figure 6. Perpendicular region of the ESR spectrum in Figure 5.

five lines appears in the perpendicular region of the spectrum (Figure 4). This could be due to a strong rhombic symmetry imposed by the F^- ion or to a strong dipolar coupling of the $^{19}F^-$ in perpendicular orientations ($A_F^{\perp}$ = 175.4 G). The 35-GHz spectrum is virtually superimposable on the X-band spectrum in this region (Figure 3B) which rules out the first alternative.[16] The overshoot line which is also split by the $^{19}F^-$ again clearly shows a five-line pattern (A_F^{O} (overshoot) = 128.1 G) with a hyperfine splitting value (A_N^{O} = 13.3 G) intermediate between that in the parallel region ($A_N^{\parallel}$ = 11.2 G) and the perpendicular region ($A_N^{\perp}$ = 14.3 G). Note that the ^{19}F coupling in the overshoot line is of an intermediate magnitude, as well.

The exact identification of the endogenous nitrogen ligands can be deduced in the following manner. Addition of imidazole as an exogenous ligand yields a more complex hyperfine splitting pattern in the perpendicular region than is exhibited by the native enzyme (Figure 5). However, the 35-GHz spectrum (not shown) shows no new rhombic distortion (g_{xx} remains nearly equal to g_{yy}) nor the appearance of an apparent copper hyperfine in the perpendicular envelope. Thus, the changes observed at 9 GHz must be due entirely to extra shfs and must be from only one additional ligand.[4,7] Again, the overshoot line can be a valuable guide interpreting the spectrum (Figure 6). In this case, one can find four lines of what clearly is a seven-line pattern (the maximum intensity occurs at the fourth line centered at 3254 G) due to three nitrogen atoms (A_N^{O} = 13.4 G). The interpretation of the rest of the complex perpendicular region first involves locating the fourth parallel line (centered at 3128 G) partially resolved on the low-field side of the perpendicular envelope. Three of the potential seven lines are evident ($A_N^{\parallel}$ = 12.1 G). Careful examination of the remaining perpendicular region leaves only seven well-resolved intense lines ($A_N^{\perp}$ = 15.7 G) which again can be assigned to the coupling of three equivalent nitrogen atoms. As expected, the magnitude of the nitrogen coupling on the overshoot line is intermediate between that associated with the parallel and perpendicular regions. However, what is most significant is that this interpretation implies that the endogenous and exodogenous nitrogen atoms are equivalent chemically and thus that the endogenous nitrogen structure

Table I.[a] Spin Hamiltonian Parameters for Cu(II)

Galactose Oxidase		
$g_\parallel = 2.277$	$A_\parallel = 175.0$	$A_N^\parallel = 14.5$
$g_\perp = 2.055$	$A_\perp = small$	$A_N^\perp = 15.1$
		$A_N^O = 14.8$
Galactose Oxidase-$^{19}F^-$ Complex		
$g_\parallel = 2.305$	$A_\parallel = 159.7$	$A_N^\parallel = 11.2$
$g_\perp = 2.050$	$A_\perp = small$	$A_N^\perp = 14.3$
		$A_N^O = 13.3$
		$A_F^\parallel = 41.0$
		$A_F^\perp = 175.4$
		$A_F^O = 128.1$
Galactose Oxidase-Imidazole Complex		
$g_\parallel = 2.254$	$A_\parallel = 167.5$	$A_N^\parallel = 12.1$
$g_\perp = 2.041$	$A_\perp = small$	$A_N^\perp = 15.7$
		$A_N^O = 13.4$

[a] A values are given in gauss; no second-order correction terms are included in the g values. A_N^O and A_F^O represent ligand hyperfine splittings on the "overshoot" line.

is due to two histidine imidazole ligands.[17] Any other amines considered to date (pyridine and methylimidazole) give splittings of a more complex nature, probably characteristic of an N_2N' pattern.

By considering the magnitude and the direction of the g-value shifts[6] upon F^- and imidazole binding (Table I summarizes all the pertinent data for these experiments) it is possible to bracket the nature of the replaceable endogenous ligand in the spectrochemical series.[18] Water falls in the spectrochemical series between these limits and is a prime candidate for one ligand site.[10-21]

Taken together these results suggest that the copper(II) site in galactose oxidase consists of two histidine imidazoles and an exchangeable water molecule. The axially symmetric spectrum implies a pseudo-square-planar structure. Based upon comparative values of $g_\parallel$ and $A_\perp$,[6] coordination by sulfur appears unlikely.[22] Previous model studies which suggested another oxygen ligand, perhaps a carboxylate group, are still consistent with these data.[4]

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