Comparative ENDOR Study of Six Blue Copper Proteins

James E. Roberts,[1] John F. Cline,[1] Vanessa Lum,[2] Hans Freeman,[3] Harry B. Gray,[2] Jack Peisach,[4] Bengt Reinhammar,[5] and Brian M. Hoffman*[1]

Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois 60201, Arthur Amos Noyes Laboratory of Chemical Physics,† California Institute of Technology, Pasadena, California 91125, Department of Inorganic Chemistry, University of Sydney, Sydney 2006, Australia, Departments of Molecular Pharmacology and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461, and Department of Biochemistry and Biophysics, University of Göteborg and Chalmers Institute of Technology, S-412 96 Gothenburg, Sweden. *Received November 25, 1983*

Abstract: A comparative study of the magnetic properties of six representative type 1 Cu(II) blue copper centers was carried out by using electron nuclear double resonance (ENDOR). Four centers are EPR single-site proteins: plastocyanins from bean (*Phaseolus vulgaris*) and poplar leaves (*Populus nigra* italica), azurin from *Pseudomonas aeruginosa*, and stellacyanin from *Rhus vernicifera*. Two are in laccases, fungal (*Polyporous versicolor*) and tree (*R. vernicifera*), that have had their type two centers reduced. In each case the low-frequency ($\nu \leq 35$ MHz) region of the spectrum is dominated by the resonances from strongly coupled ($A^H > 20$ MHz) methylene protons of a coordinated cysteinyl mercaptide. In all cases but one, experiments at two microwave frequencies (9.6 and 11.6 GHz) also permitted detection of resonances from two, inequivalent, nitrogenous ligands. The coordination environments in the type 1 Cu(II) sites of the six proteins are broadly similar, but detailed analysis suggests that the stereoelectronic structure of the single-site proteins, as a group, differs in subtle but significant ways from that of the type 1 Cu(II) center of the laccases. The reduction potentials of the single-site type 1 Cu(II) centers correlate well with the bonding within a center, as reflected in the ligand ENDOR parameters: Reduction potentials decrease with decreasing bonding to (or ligand field at) the type 1 Cu(II). This correlation does not hold for the laccase type 1 sites, which appear to have enhanced π-bonding to mercaptide sulfur. Analysis of these results suggests that fine tuning of the reduction potentials of a type 1 Cu center is primarily achieved by altering the properties of the reduced, Cu(I), state.

The mononuclear Cu(II) site of the blue copper (type 1) proteins has unusual optical and magnetic properties.[6] The electronic spectrum is characterized by an intense absorption near 600 nm ($\epsilon \sim 10^3$-10^4), in contrast to the weak absorptions ($\epsilon \leq 10^2$) exhibited by mononuclear copper complexes of low molecular weight. Typically, the EPR spectrum of blue copper also is quite different from that observed for "normal" copper; the values of $g_\parallel$, A_Z^{Cu}, and A_{iso}^{Cu}, the isotropic coupling, all show unusually small values.[7]

Near infrared, visible absorption, circular dichroism, and pulsed EPR spectroscopy[7] are consistent with a distorted tetrahedral coordination geometry for blue copper site. It also has been suggested, in part from model compound studies,[8] that the intense blue color is due to RS → Cu charge-transfer excitation[6,9] and that the unpaired electron is substantially delocalized onto a sulfur ligand, accounting in part for the low values of A_Z^{Cu}.[10] For two blue copper proteins, poplar leaf plastocyanin[11] and *Pseudomonas aeruginosa* azurin,[12] the spectroscopically derived structural model has been substantiated by crystal structure analyses. In each case, the copper is coordinated by two histidine nitrogens and cysteinyl sulfur, with another sulfur, from methionine, nearby. The coordination environment must be somewhat different for the blue copper of stellacyanin, however, because the protein does not contain methionine.[13]

More detailed information about the coordination sphere in stellacyanin has been obtained from electron-nuclear double resonance (ENDOR) measurements.[14] We have now extended the ENDOR studies of blue copper centers to include measurements on stellacyanin, bean and poplar plastocyanins, azurin, and tree and fungal laccases. In each case the low-frequency ($\nu \leq$ 35 MHz) region of the spectrum is dominated by resonances from strongly coupled methylene protons of a coordinated cysteinyl mercaptide. Nevertheless, in all cases but one, experiments at two microwave frequencies have made it possible to detect the resonances from two highly inequivalent nitrogenous ligands of the Cu[II]. Their ^{14}N hyperfine couplings have been determined, as have the copper hyperfine and quadrupole coupling tensors.

These measurements thus constitute an extensive, comparative characterization of the magnetic properties of six representative blue copper centers. They confirm that the coordination environments in the type 1 Cu(II) sites on the six proteins are broadly similar. However, a more detailed analysis suggests that the stereoelectronic structure of these single-site proteins, as a group, differs in subtle but significant ways from that of the type 1 center of the laccases. Moreover, the variations in ENDOR parameters between the single-site proteins and the laccases, and within each of these two classes, correlate quite well with reported redox properties,[6,15] thereby leading to a fuller understanding of the means by which the reduction potentials of the copper are controlled by its coordination environment.

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(1) Northwestern University.
(2) California Institute of Technology.
(3) Albert Einstein College of Medicine of Yeshiva University.
(4) University of Göteborg and Chalmers Institute of Technology.
(5) University of Sydney.

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Experimental Section

Materials. Poplar[16] and bean[17] plastocyanins, stellacyanin,[14] azurin,[18] and type 2 reduced tree and fungal laccases[19] were prepared by the published procedures. Samples of final concentration ~2 mM were prepared in a 0.05 M potassium phosphate buffer, pH 7.0, containing 40% glycerol (v/v).

Deuterium-exchanged azurin samples were prepared by two procedures. The first involved diluting a 0.2-mL, ~1.5 mM sample 40-fold with D_2O at pD ~7.0 and room temperature for 24 h. The sample was reconcentrated by using a UM2 membrane (cutoff ~1000 daltons) in an Amicon ultrafiltration cell Model 12 and diluted 30% with glycerol immediately before freezing in liquid nitrogen. The second azurin reconstitution procedure involved: (i) removal of the copper; (ii) exchange with D_2O; and (iii) reconstitution with ^{63}Cu. All reagents used were obtained from standard commercial sources and used without special precautions except as identified below. ^{63}CuO (99% ^{63}Cu) was obtained from Union Carbide (Oak Ridge, TN) and converted to $^{63}CuCl_2$ by reaction with dilute hydrochloric acid. Distilled water was passed through an ion exchange column before use. All buffers were passed through a column of Chelex 100 (Bio-Rad Laboratories) exchange resin to remove trace metals before use; all labware was Nalgene, Teflon, or Lucite. Native and reconstituted proteins gave the same EPR and optical spectra.

To remove the copper from oxidized azurin,[20] approximately 2 mL of 0.4 mM protein was dialyzed against 1 L of 0.05 M NaCN, in 0.05 M Tris buffer, pH 9 at 0 °C. The buffer was changed five times over 32 h. The colorless apoprotein was then dialyzed against 1 L of 0.05 M Tris buffer, pH 9, to eliminate traces of cyanide. At this stage the apoprotein can scavenge minute quantities of divalent metal ions from buffers and labware.

Deuterium exchange was performed at 33 (±2) °C, by using 20 mL of 0.05 M potassium phosphate buffer in D_2O (pD <<equ7.4) with 3 M urea. The urea was lyophilized twice from a 1 g/mL D_2O solution. The buffered urea solution was changed three times over 6 h.

The urea was removed by dialyzing the 2-mL sample against 0.05 M potassium phosphate buffer in D_2O, pD ≅7.0 at 33 (±2) °C. The dialysate volume was changed five times in 24 h. The apoprotein was reconstituted with a 3-fold molar excess of $^{63}CuCl_2$ dissolved in D_2O (pD 7), turning it an intense blue color within minutes. Excess Cu^{II} was removed with a small Chelex column; the sample for study was prepared by concentrating to ~3 mM, followed by 40% dilution with glycerol followed by immediate freezing. Optical spectra showed that 70% of the protein reconstituted properly with Cu^{II}, on the basis of $A^{625}/A^{285} = 0.40$.

ENDOR. Experiments were performed at 2.0 K with a spectrometer discussed elsewhere.[21] The ENDOR signals were observed as a decrease in the dispersion mode EPR signal. Typical conditions were as follows: 100-kHz field modulation, 3–4 G; microwave power, 2–200 μW; radio-frequency sweep rates, >1 MHz/s (vide infra). All ENDOR signals were observed with both increasing and decreasing frequency sweeps; parameters reported are the average of these measurements. Using 20-W radio-frequency power gave ~1 G in the rotating frame. EPR spectral simulations were calculated by using program SIM 14, available from the Quantum Chemistry Program Exchange, Indiana University.

ENDOR is performed by monitoring the EPR signal intensity at fixed external magnetic field, H_0, while inducing nuclear transitions with a frequency swept radio-frequency field.[22] Therefore, an ENDOR signal arises from that subset of molecules with orientation such that EPR occurs in the vicinity of H_0.[23] For a paramagnetic center having a **g** tensor with approximately axial symmetry and $g_\parallel > g_\perp$, as is the case for the five proteins newly studied here, when H_0 is set to the low-field edge of the EPR spectrum, single-crystal-like ENDOR is observed; only copper centers having essentially a single orientation, with H_0 along $g_\parallel$, contribute to the ENDOR signal, and these ENDOR spectra can be directly interpreted in terms of the appropriate projection of the hfs tensor (i.e., $A_\parallel^{Cu}$). With H_0 set to the high-field edge of the spectrum, all molecules with H_0 lying in the basal plane of the **g** tensor can contribute. In this case the ENDOR spectrum of a given nucleus will exhibit maxima at frequencies corresponding to the in-plane extrema of the hyperfine tensor. With the magnetic field set at any intermediate field of the EPR spectrum, the observed ENDOR spectrum is a superposition from the set of all orientations having the appropriate intermediate g value.[24]

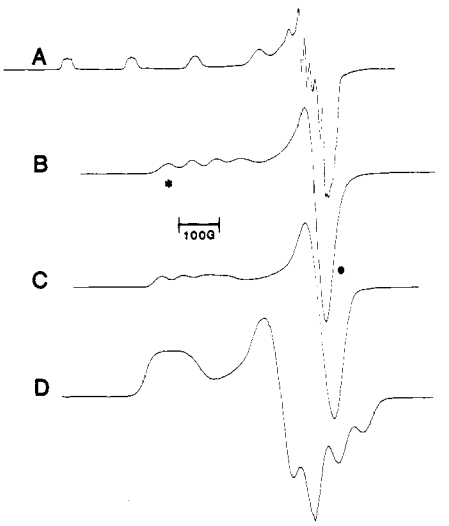

Figure 1. EPR spectra at 77 K of (A) ^{65}Cu transferrin, (B) bean plastocyanin, (C) azurin, and (D) stellacyanin. The value of $A_\parallel^{Cu}$ decreases from A to D. Note the lack of resolution in the $g_\perp$ region of B–D, although the stellacyanin spectrum has resolved copper hyperfine lines at the high-field edge. The EPR spectra of polar leaf plastocyanin and the type 2 reduced tree and fungal laccases are very similar to that of bean plastocyanin (part B). Typical field positions for collecting ENDOR spectra associated with $g_\parallel$ (*) and $g_\perp$ (●) are indicated on B. Conditions: microwave power, ~20 mW; 100-kHz modulation amplitude, 5 G; microwave frequency, ~9.13 GHz.

The normal ENDOR pattern for a set of magnetically equivalent protons is a pair of lines separated in frequency by the angle-dependent hyperfine coupling constants, A^H, and mirrored about the free-proton Larmor frequency, $\nu_H = g_H\beta_n H_0/h$ (13.62 MHz at 3200 G), according to eq 1.[22] This formula gives no information about the sign of A^H, which

$$\nu_\pm^H = |\nu_H \pm A^H/2| \quad (1)$$

therefore will be treated as the absolute value. A resonance may be assigned to protons by exploiting the linear dependence of ν_H, the center of the ENDOR pattern, on H_0 and hence the microwave frequency, ν(M): Where H_0 corresponds to a particular g value within the EPR envelope, then $H_0 = h\nu(M)/g\beta$ and $\nu_H = (g_H\beta_n/g\beta)\nu(M)$.

A nucleus, K, having spin greater than one-half ($I \geq 1$), such as ^{14}N, ^{63}Cu, and ^{65}Cu, can exhibit an angle-dependent quadrupole interaction term, p^K, as well as the nuclear Zeeman and hyperfine terms, and its possible ENDOR frequencies are

$$\nu_m^K = |A^K/2 \pm \nu_K \pm (2m-1)p^K| \quad (2)$$

where $-I^K + 1 \leq m \leq I^K$. The result is a pattern centered at the frequency $A^K/2$, which is independent of the spectrometer microwave frequency, and having as many as $4I^K$ lines. The conditions for observing copper endor are discussed in detail elsewhere.[14b,25] For K = ^{14}N, ν_N = 0.984 MHz at 3200 G, and eq 2 predicts a pattern of as many as four lines, centered at $A^N/2$, independent of the spectrometer microwave frequency. Further splitting by the quadrupole interaction has not been resolved in a biological copper center; in its absence the pattern reduces to a Larmor split doublet centered at $A^N/2$.

Results

EPR. Figure 1 presents EPR spectra from several copper-containing proteins. Figure 1A shows a 77 K spectrum of cop-

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Table I. ^1H and ^{14}N ENDOR Coupling Constants (MHz) of Blue Copper Centers[a]

	plastocyanin				laccase		
nucleus	bean	poplar leaf	azurin	stellacyanin	fungal	tree	$Cu(Im)_4$[b]
^{14}N (s)	49	49	(44)	32[c]	38		40
(w)	20	23	20	16	24	20	
^1H (1)[d]	27	27	24	21[c]	30	25	
(2)					25	20	

[a] Measured in $g_\parallel$ region of EPR spectrum. Within error, ^{14}N couplings are isotropic; ^1H couplings show as much as 10–20% anisotropy (see text). Estimated errors: A^N, ±2 MHz; A^H, ±1 MHz. [b] Reference 28. [c] From ref 14. [d] Coupling constant determined by twice the separation between the high-frequency, ν_+, proton peak (i.e., 30 MHz) and ν_H, except for tree laccase (see text).

per-substituted transferrin.[26] It exhibits features characteristic of near tetragonal (type 2) copper complexes: a large value for $A_\parallel^{Cu}$ (502 MHz), **g** tensor of nearly axial symmetry with $g_\parallel$ large, and resolved hfs from copper and/or nitrogen in the perpendicular region of the spectrum. The X-band EPR spectra of the type 1, or blue copper, proteins, bean plastocyanin and azurin (Figure 1B,C), again appear to have axial symmetry, but with $A_\parallel$ being the only resolvable hyperfine coupling, and with both $g_\parallel$ and $A_\parallel$ being much smaller than in type 2 copper complexes.[6,7,9,19] A small rhombic splitting is observed in the Q-band spectrum of azurin. The resting-state EPR spectra of poplar leaf plastocyanin and the type 2 depleted laccases all resemble that of bean plastocyanin (Figure 1B). The 77 K X-band EPR spectrum of stellacyanin is shown in Figure 1D. On the basis of Q-band measurements, the **g** tensor deviates slightly from axial symmetry.[13] The copper hyperfine couplings are similar in magnitude to those of the other type 1 centers, but note, however, that the largest principal hyperfine coupling does not occur along the same magnetic axis as the maximum g value, as in the other five proteins.[14,27]

^1H and ^{14}N ENDOR. Figure 2 presents representative single-crystal-like ENDOR spectra from type 1 copper proteins. The spectra from bean plastocyanin (Figure 2A), azurin (Figure 2B), stellacyanin (Figure 2C), and tree laccase (Figure 2D) all were taken at a microwave frequency of ~9.5 GHz with H_0 set to the low-field ($g_\parallel$) edge of the EPR envelope. The corresponding spectrum from fungal laccase has a similar appearance to that obtained from tree laccase. Spectra from poplar leaf plastocyanin are similar in all respects to the bean protein, although samples showed signs of slight deterioration due to prolonged storage, and the spectra were less well resolved.

The differing ENDOR patterns in Figure 2 display the variability among the type 1 copper(II) sites. Under the conditions employed, all proteins but stellacyanin show features near 14 MHz that are associated with weakly coupled protons; under different conditions, stellacyanin shows the same features. In all cases it is possible to resolve the resonance into numerous doublets, centered at ν_H (eq 1), associated with individual protons that are weakly coupled to the odd electron. Presumably, these protons are on nearby residues or on coordinating groups, such as imidazole[28] of histidine; they will not be considered further at this time.

Bean (Figure 2A) and poplar (not shown) plastocyanins and azurin (Figure 2B) are similar in that spectra taken with a microwave frequency of ~9.5 GHz show a single feature in the frequency range of ~18–30 MHz, whereas stellacyanin (Figure 2C) and tree (Figure 2D) and fungal (not shown) laccases show two peaks. The plastocyanins show no peaks to low frequency of the weakly coupled protons, whereas azurin, stellacyanin, and the laccases show features at differing levels of resolution.

Interpretation of these ENDOR spectra is difficult because they represent a complicated overlap of resonances from protons and nitrogens. Thus, we first present a detailed analysis of the most readily interpreted data set, that obtained from bean plastocyanin, and use this as a framework in developing the interpretation of the ENDOR patterns from the other proteins.

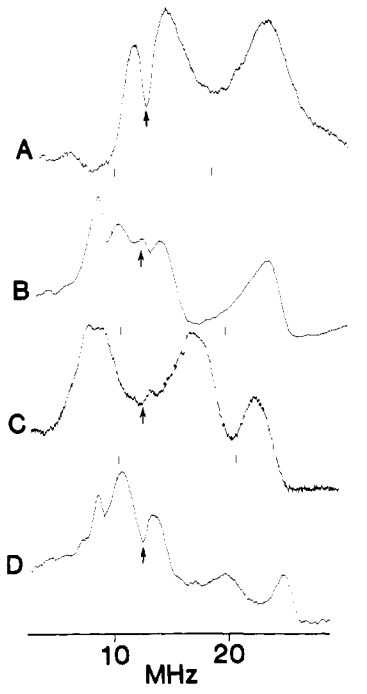

Figure 2. Representative ^1H and ^{14}N ENDOR spectra near $g_\parallel$ for (A) bean plastocyanin, (B) azurin, (C) stellacyanin, and (D) tree laccase. Detailed support for assignments are discussed in the text and the following figures. Arrows indicate ν_H, the free proton resonance frequency. Conditions: T = 2 K; microwave power, 2 μW; 100-kHz field modulation, 3G; radio-frequency sweep rate, ~2.5 MHz/s; microwave frequencies, 9.2 (B and C) and 9.6 GHz (A and D).

Plastocyanins. Figures 3A,B shows ENDOR spectra of bean plastocyanin with H_0 set at the low-field edge ($g_\parallel$) of the EPR envelope. The following analysis holds also for poplar plastocyanin, which will not be discussed independently. The features in Figure 3A near 14 MHz are associated with weakly coupled protons. The broad peak above 20 MHz can be assigned by comparing spectra taken in EPR spectrometers operating at ca. 9 and 12 GHz (Figure 3A,B and Figure 4). The peak at ca. 25 MHz in Figures 3A and 4A is split in Figures 3B and 4B, with the intensity that shifts to ca. 31 MHz representing the ν_+ transition (eq 1) of a proton with large coupling constant, $A^H \sim 27$ MHz (Table I). The feature that remains centered at ~25 MHz represents the unresolved pattern from a coordinating ^{14}N ligand; the resonance frequency and the center of the pattern corresponds to $A^N/2$ (eq 2). In addition, a signal newly apparent in the 9–13-MHz range of Figure 3B represents the unresolved ENDOR pattern from a second, more weakly coupled, ^{14}N ligand. The resonance from this ligand is completely masked at lower microwave frequency (Figure 3A) by the proton resonances centered about ν_H. The ^{14}N hyperfine couplings for the two coordinated nitrogens are given in Table I.

When H_0 is set to the high-field edge of the EPR spectrum, the ENDOR pattern of bean plastocyanin (Figure 3C) resembles that taken near $g_\parallel$, and the ENDOR spectra taken at numerous intermediate field positions are not appreciably different. In particular, ^{14}N resonances corresponding to $A^N/2 \sim$ 10–15 and

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Table II. Cu ENDOR Results for Blue Copper Centers

		plastocyanin				laccase	
interaction	direction	bean	poplar leaf	azurin	stellacyanin[a]	fungal	tree
A_i^{Cu}, MHz[b]	∥	185	186	161	96	270	129
	⊥	60	68	66	87 (y)		
					167 (x)		
P_i^{Cu}, MHz[c]	∥	5.5	5.0	5.5	167 (x)		7–8
					0.7[d] (y)		
					5.0[d] (x)		

[a] From ref 14. Note that the hyperfine tensor is roughly axial, but with the unique axis (largest value) corresponding not to the largest g value, $g_z = g_\parallel$, but to the smallest, g_x. [b] Estimated error ±4 MHz. [c] See text for discussion. Estimated error ±1 MHz. [d] P_y^{Cu} and P_x^{Cu} are the opposite sign from P_z^{Cu}.

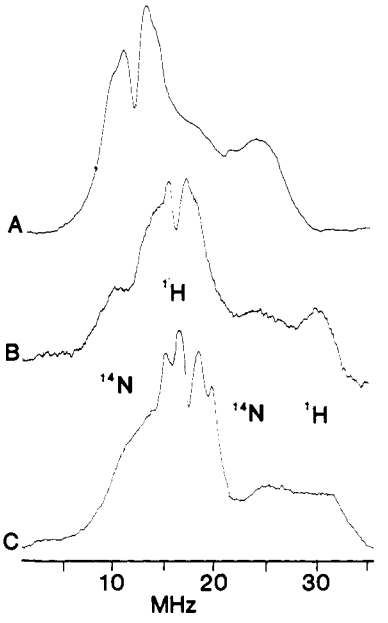

Figure 3. Representative ^{1}H and ^{14}N ENDOR spectra near $g_\parallel$ (A and B) and $g_\perp$ (C) for bean plastocyanin. Assignment of the resonances to protons and nitrogen are indicated. See text for complete discussion. Conditions: microwave frequencies, 8.7 (A) and 11.7 GHz (B and C); other conditions as in Figure 2.

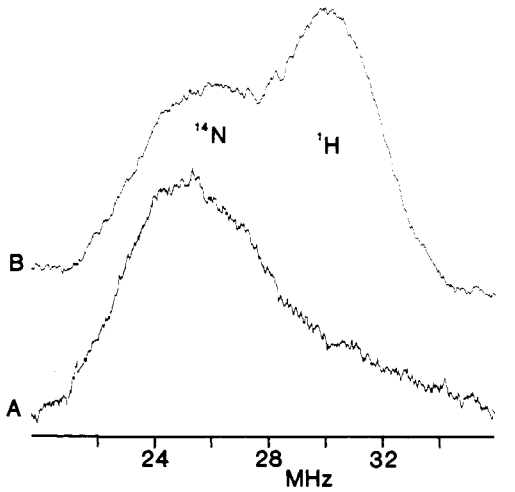

Figure 4. Bean plastocyanin ^{1}H and ^{14}N ENDOR spectra at $g_\parallel$, from 20 to 35 MHz, with microwave frequency of (A) 9.21 and (B) 11.66 GHz. The splitting in B leads to the indicated assignment in terms of ^{14}N and ^{1}H resonances. Conditions as in Figure 2.

20–25 MHz and proton resonances corresponding to $A^H \sim$ 20–30 and 4–5 MHz are revealed by the spectra taken at the available EPR microwave frequencies; the latter ^{1}H couplings are somewhat more resolved in Figure 3C.

The observation that the ENDOR spectra of bean plastocyanin does not change substantially with field corresponds to an invariance with respect to the magnetic field direction in the molecular frame. Namely, the hyperfine couplings for the two ^{14}N ligands and for the protons identified in the single-crystal-like spectra (of Figure 2) are effectively isotropic, as is the case with the ^{14}N couplings of imidazole bonded to Cu(II) in $[Cu(Im)_4]^{2+}$.[28] Detailed comparisons between spectra taken near $g_\parallel$, $g_\perp$, and at intermediate fields indicate that ^{1}H and ^{14}N coupling constants in all six proteins show no more than ca. 10% anisotropy. Thus, when discussing the other proteins, reference is made to anisotropy and field dependence only when it is useful to do so, and the tables in general give only isotropic values.

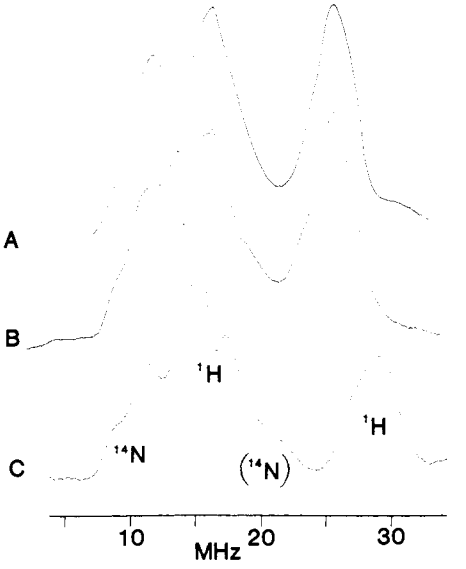

Figure 5. ^{1}H and ^{14}N ENDOR of azurin, near $g_\parallel$ showing effects of D_2O exchange (see Experimental Section) and variation in microwave frequency: (A) 9.6 GHz, H_2O; (B) 9.6 GHz, D_2O; (C) 11.7 GHz, D_2O. The invariance with deuterium exchange of the peak near 25 MHz (A and B), and its shift to 28 MHz at 11.7 GHz (C), shows that the resonance is associated with a strongly coupled, nonexchangeable proton, as discussed in the text. The features in C near 10–12 MHz arise from a weakly coupled ^{14}N ligand; the shoulder in C at ~22 MHz may be from a second ^{14}N. Conditions as in Figure 2.

Azurin. The ENDOR spectrum of azurin (Figure 2B) differs in appearance from that of bean plastocyanin (Figure 2A) through the presence of a feature near 10 MHz. This is associated with weakly coupled nitrogen (Table I), since it does not change position in spectra taken at 11.7 GHz (Figure 5), and even shows the splitting of $2\nu_N$ predicted by eq 2 for ^{14}N with an unresolvably small quadrupole coupling. The feature in Figure 2B at ca. 25 MHz is associated with a strongly coupled proton(s), since it does shift to higher frequency (Figure 5) in spectra taken at 11.7 GHz. The ^{14}N and ^{1}H couplings are given in Table I. Spectra of azurin do not give the clear evidence for a strongly coupled ^{14}N that is obtained with plastocyanin (Figure 3), but a shoulder near 20 MHz in Figure 5 appears to suggest such a nitrogen; the corresponding coupling is listed in Table I.

Stellacyanin. The ENDOR pattern of stellacyanin (Figure 2C) shows two features at frequencies above the free proton frequency. That at ~16 MHz was assigned by Rist and Hyde to a coordi-

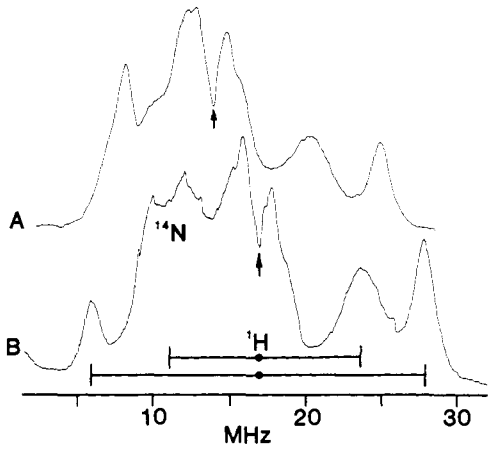

Figure 6. ENDOR spectra observed near $g_\perp$ for the type 1 Cu(II) site of type 2 reduced tree laccase at (A) 9.59 and (B) 11.66 GHz. The large shifts of the high-frequency resonances positively identify them as arising from protons. The expected lower frequency partner peaks are also observed as marked. The intensity near 10 MHz in both spectra is indicative of a weakly coordinating nitrogen. Arrow indicates ν_H, the free proton resonance frequency. Conditions as in Figure 2.

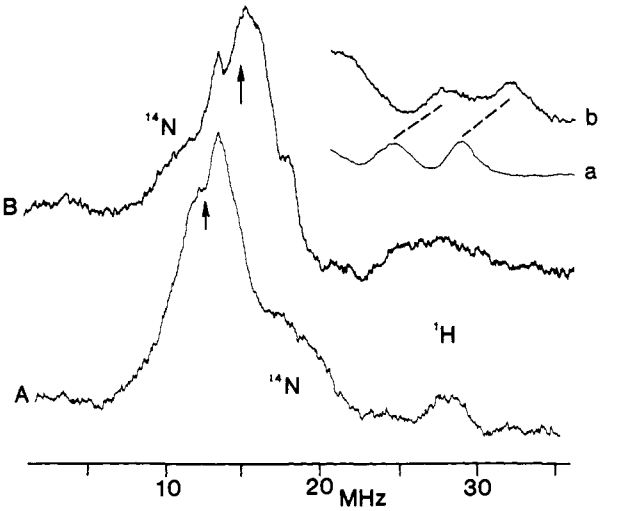

Figure 7. ENDOR spectra taken with H_0 near $g_\parallel$ for the type 1 Cu(II) site of type 2 reduced fungal laccase at (A) 9.60 and (B) 11.66 GHz. Although these spectra have a lower signal-to-noise ratio than those presented above, they indicate the presence of two types of strongly coupled protons and two nitrogen ligands. The assignment of the proton resonances is confirmed in the better spectra taken at $g_\perp$ (inset), in which two high-frequency features are shown to be proton resonances by their shift in frequency as the microwave frequency changes from (a) 9.60 to (b) 11.66 GHz. Arrows indicate ν_H, the free proton resonance frequency. Conditions as in Figure 2.

nating ^{14}N ligand;[14a] the coupling constant is given in Table II. They showed that the peak at ~25 MHz was primarily associated with strongly coupled proton(s) (Table I), but drew a tentative inference that the peak also involved the resonance of a second ^{14}N. We further suggested that the ^{14}N resonances were strongly anisotropic.[14b]

The differences between these conclusions and the present results for other type 1 centers led us to take new spectra of stellacyanin, represented by that of Figure 2C. In addition to the peaks at ν ~16 and 25 MHz, a feature centered at ~8 MHz, and showing a splitting of $2\nu_N$, is clearly visible under the present conditions of very low temperature (2 K) and relatively rapid radio-frequency scan. The feature, which is quite comparable to that seen with azurin, clearly is associated with a weakly coupled ^{14}N ligand with coupling as given (Table I). Since the 16-MHz feature definitely is associated with ^{14}N,[14a] acceptance of the tentative suggestion that the 25-MHz peak is associated in part with a nitrogenous ligand would mean that stellacyanin has three such ligands. It seems more reasonable to assign the 25-MHz peak solely to ^{1}H. In addition, the ENDOR of stellacyanin at intermediate fields also was reexamined. The spectra are in fact best interpreted in terms of two nitrogens having unequal, but approximately isotropic, couplings (Table I).

Laccases. The ENDOR spectrum in Figure 2 of the type 1 copper center of type 2 reduced tree laccase appears to be most like that of stellacyanin in that it shows two features at frequencies above the free proton region and one below. However, comparison of tree laccase spectra recorded at 9.6 and 11.7 GHz (Figure 6) shows that both higher frequency features shift with microwave frequency and thus are associated with strongly coupled protons, as indicated. Indeed, at the higher microwave frequency, both ν_+^H and ν_-^H peaks (eq 1) are observed for both types of protons. The coupling constants are given in Table I.

The intensity near 10 MHz in the laccase spectrum (Figure 2D) does not shift with microwave frequency and is therefore assigned to the weakly coordinating nitrogen characteristic of type 1 copper centers; its coupling is also in Table I. No ENDOR evidence for the second, more strongly coupled, nitrogen ligand in tree laccase has been obtained to date. Presumably, such a nitrogenous ligand exists, but its resonance is obscured by those of the protons. Support for this view is given by electron spin echo studies that indicate the presence of a histidine that is strongly coupled to Cu(II).[7d]

Spectra of the type 1 Cu(II) of type 2 reduced fungal laccase (Figure 7) strongly resemble those observed with tree laccase. In particular, resonances from two strongly coupled protons are observed, as confirmed by their shift with changes in the microwave frequency (see Figure 7, inset). Thus, the two laccases are similar in exhibiting two large proton couplings, in contrast to the single coupling of the mononuclear centers. For fungal laccase, however, the several coupling constants are such that a feature assigned to a strongly coupled ^{14}N is observable at 9.7 GHz, whereas the shift of the proton resonances at 11.7 GHz uncovers a shoulder assignable as the resonance from a weakly coupled nitrogen. The resulting coupling constants are given in Table I.

Identification and Analysis of Large Proton Couplings. All six type 1 centers contain one or more protons that exhibit unusually large hyperfine couplings (Table II). Deuterium exchange experiments with azurin were performed to assist in chemically identifying these protons. Simply exchanging the solvent with D_2O causes no change in the proton ENDOR, demonstrating that the signals do not arise from H_2O or OH^- at or near the copper site. An intriguing possibility was that the proton is from an amide engaged in hydrogen bonding with the copper-bound mercaptide sulfur, and that structure-dependent hydrogen bonding might influence redox properties.[29] This alternative was examined by using samples subjected to rigorous D_2O exchange conditions subsequent to removal of copper, as described in the Experimental Section. Proton NMR results on apoazurin indicate that all the amide nitrogen protons are fully replaced with deuterium during this procedure.[30] In contrast, ENDOR spectra of azurin (Figure 5) taken before exchange and after Cu(II) reconstitution following this D_2O exchange, show only the minor differences that can be associated with sample variability.

This experiment proves that the proton(s) associated with the peak at ~29 MHz in azurin, and by inference the strongly coupled protons in all cases, is not exchangeable and thus must be bonded to carbon. They are not associated with the coordinated histidyl residues, as the protons on imidazole bound to Cu(II) give small couplings, $A^H < 5.5$ MHz.[28] Given the X-ray crystallographic identification in poplar plastocyanin[11] and azurin[12] of the ligands bound to copper, the only plausible assignment of these protons is that they are the methylene protons of the strongly coordinating, mercaptide sulfur ligand of cysteine.

The chief mechanism giving rise to such a large, isotropic coupling is likely to be spin–polarization of the S–C and C–H

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bonds by σ-orbital spin density on sulfur. As a consequence of metal–ligand covalency, the unpaired electron of the blue copper site is in a σ^* antibonding orbital which may be written

$$\Psi(\sigma^*) = c_{Cu}d_{x^2-y^2}{}^{Cu} - c_s\sigma^s - \ldots \quad (3)$$

where σ^s represents the interacting σ-orbital of the cysteinyl sulfur. Since σ^s represents a $4s - 4p_\sigma$ hybrid, the relative s/p proportion, and thus the proton coupling, would be sensitive to the Cu–S–C_β angle, as well as to the net covalency (c_s).

The odd electron also can acquire sulfur $4p_\pi$ character, depending on the extent that the mercaptide sulfur is out of the plane of the $d_{x^2-y^2}{}^{Cu}$ orbital and on the dihedral angle between the d-orbital plane and the Cu–S–C_β plane. Spin density in a sulfur p_π orbital, ρ_π, would give an additional contribution to the β-proton isotropic hyperfine coupling, one which depends on the dihedral angle (θ_i) between the $4p_\pi$ orbital on sulfur and the C–H_i bond for the individual methylene proton[31,32]

$$A_i{}^H = \rho_\pi B \cos^2 \theta_i \quad (4)$$

where $B \sim 100$ MHz. This contribution would in general be different for the two protons, as would any effects of direct overlap between proton and Cu 3d orbital. In the Discussion section, the difference between proton ENDOR results for the type 1 centers of the four single-site proteins on the one hand, and for the two laccases on the other, will be examined in terms of the two coupling mechanisms.

Cu ENDOR. Resonances from copper are readily observed at frequencies above those for ^{14}N and ^1H, although they are much broader and often weaker than those for the ligand atoms. The ENDOR signals from copper strongly depend on the rate with which the radio-frequency field is swept and are seen most easily with rapid scans ($\gtrsim$20 MHz s^{-1}) that presumably cause rapid passage effects in the nuclear spin system; the details of the copper ENDOR measurements are presented elsewhere.[14b,25] The values of $A_\parallel{}^{Cu}$ and $P_\parallel{}^{Cu}$ obtained for the blue copper proteins are listed in Table II. The in-plane quadrupole tensor elements are not independently obtained in these measurements because the spectra are not resolved, but the average of the in-plane elements, $P_\perp$, is obtained from the measured values of $P_\parallel$ (Table II), by using the condition that the quadrupole tensor be traceless: $P_\parallel + 2P_\perp = 0$.

The nuclear quadrupole tensor for the copper nucleus reflects an interaction of the copper nucleus with the total nearby electron density, not merely with the odd electron.[33] The quadrupole tensor for stellacyanin (Table II) is very nearly completely rhombic: two elements are of roughly equal and opposite values and one is near zero. This indicates that pseudoaxial character of the **g** and hyperfine tensors reflects only an effective symmetry and that the total electron density around the copper deviates appreciably from any idealized symmetry. Unfortunately, the data for the other proteins do not permit a determination of the quadrupole tensor symmetry, but $P_\parallel$, the element of the Cu quadrupole tensor corresponding to the $A_\parallel$ direction, can be measured.

In each of the four single-site proteins, the same quadrupole coupling is observed: $P_\parallel \sim 5$–6 MHz. This is intermediate among the values reported for various copper complexes. For example, $P_\parallel{}^{Cu} \simeq 2$–5 MHz for CuS_4 coordination, but $P_\parallel{}^{Cu} \simeq 7$–9 MHz for CuO_4 coordination.[37] Examination of the results for CuS_4, CuS_2O_2, and CuO_4 square-planar complexes shows that the covalent character of the Cu–S bonds greatly reduces $P_{max}{}^{Cu}$,[34] and this is presumably reflected in the values for the Cu(II) site of blue copper proteins. In contrast, the quadrupole coupling in tree laccase is significantly larger, $P_\parallel \sim 7$–8 MHz.

Discussion

The magnetic properties of the six type 1 copper centers are broadly similar. In each protein, except tree laccase, we have assigned two nonequivalent nitrogenous ligands that have ^{14}N hyperfine interaction constants of markedly different magnitude. These observations can be nicely correlated with the results from X-ray and previous EPR studies, in particular a single-crystal study, of poplar plastocyanin.[35] To first approximation, the odd electron in a blue copper site resides in a copper, $3d_{x^2-y^2}$ orbital. In plastocyanin, a methionine sulfur, S(Met-92), lies nearly normal to the orbital plane, with the $g_\parallel$ axis pointing toward it. The other three ligands, two histidyl nitrogens and a cysteinyl sulfur, are near the orbital plane: N(His-37) lies approximately in the plane; N(His-87) is below the plane by ca. 10–20° and has a somewhat longer Cu–N bond length; S(Cys-84) is strongly coordinated at a distance of 2.13 Å and also is below the plane. Presumably, the larger ^{14}N hfs ($A^N \sim 50$ MHz) is associated with N(His-37), which interacts strongly with the odd electron of the copper because of its favorable orientation and bond length; note that the coupling constant is even larger than that in $[Cu(Im)_4]^{2+}$ (Table I). The other, less favorably coordinated nitrogen has a correspondingly lower coupling constant ($\sim$20 MHz). By application of these ideas to the set of six proteins, the variations in A^N could reflect reorientation of two histidyl ligands with respect to the $3d_{x^2-y^2}$ orbital and/or differences in bond length.

Perhaps the most striking aspect of the present study is the observation of protons with very large ($A^H \sim 20$–30 MHz), roughly isotropic hyperfine couplings. The invariance of the spectra with exhaustive D_2O exchange leaves as the only plausible interpretation of these signals that they arise from the β-protons of the strongly coordinating, mercaptide sulfur ligand of cysteine. Such large couplings indicate that the sulfur bears a large spin density in the consequence of strong Cu–S covalency. Thus, the ENDOR results correlate well with the interpretation of the 600-nm absorption band in terms of S $\rightarrow$ Cu charge transfer,[7] both signalling the importance of mercaptide sulfur in determining the characteristics of the type 1 Cu(II) center.

However, the proton ENDOR data also indicate significant differences among the type 1 Cu(II) centers. The single-site proteins show but a single large proton coupling, presumably associated with both β-protons of cysteine. It is likely that the coupling results from σ-polarization, and that differences among the mononuclear centers, arise from differences in Cu–S covalency and/or the conformation of the Cu–S–Cβ linkage. In contrast, each laccase exhibits two large proton couplings. Neither coupling can be associated with bound water, because A^H would then be strongly anisotropic and could be no larger than half those observed.[36] Therefore, *assuming* the same endogenous ligand set, the individual β-protons of cysteine must have different hyperfine constants. Such would occur if the coordination geometry around Cu causes the mercaptide sulfur to acquire a significant π-spin density, giving rise to a contribution to the hyperfine coupling that depends on the twist angle of the S–C_β bond (eq 4). Thus, the type 1 centers in the four mononuclear proteins appear to exhibit variations on one type of stereoelectronic structure, whereas the two laccases appear to represent a somewhat different structure.

Considering the single-site proteins in more detail, the two plastocyanins have indistinguishable ligand (and Cu) hyperfine couplings, and thus it is clear that the copper sites of the two proteins are virtually identical. The ligand hyperfine couplings for azurin are minimally smaller than the plastocyanin values, which indicates a small difference in coordination geometry. Such a difference is not ruled out by the crystallographic results. Stellacyanin shows the lowest ^{14}N and ^1H couplings of any of the

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Table III. Redox Properties of Blue Copper Centers

	plasto-cyanin[a]	azurin	stella-cyanin	laccase	
				fungal	tree
reduction potential[b]	360[c]	308[c]	191[c]	785[d]	394[d]
enthalpy of reduction[e]	-13.7	-16.6	-10.3	-22.1	

[a] Bean plastocyanin. [b] mV as NHE. [c] Reference 15. [d] Reference 19. [e] kcal/mol; ref 15.

systems studied, although interpretation of this difference, and the unusual properties of the copper hyperfine tensor of stellacyanin, as well, must await identification of the full set of ligands for the Cu(II) in this protein.[37]

A particularly interesting feature of these considerations is the emergence of a correlation between the reduction potentials of the single-site type 1 Cu(II) centers (Table III) and the bonding within a center, as reflected in the ligand ENDOR parameters (Table I): Reduction potentials decrease directly with decreasing hyperfine couplings to each of the two coordinating ^{14}N and with decreasing coupling to the cysteine protons.

The ligand hyperfine couplings, most especially the nitrogenous ligands, should vary directly with the strength of the bonding to (or ligand field at) the copper(II), which thus decreases in the order, plastocyanins > azurin > stellacyanin. Surprisingly, this is also the order of decreasing reduction potentials; in addition, stellacyanin also has the least exothermic enthalpy of reduction. We conclude from this that a variation in coordination environment that reduces the binding to Cu(II) must be accompanied by a similar but greater effect on the Cu(I) state. For example, the low reduction potential and small ligand hyperfine couplings of stellacyanin, as compared to plastocyanin, together indicate that a decreased stabilization of Cu(II) in stellacyanin is accompanied by an even greater destabilization of the Cu(I). In short, these results suggest that fine tuning of the reduction potential of a single-site type 1 Cu center is primarily achieved by altering the properties of the reduced, Cu(I), state.

In contrast, the blue copper site of fungal laccase has a rather low value for its larger ^{14}N coupling but the highest reduction potential and the most exothermic reduction enthalpy of the centers considered. In this case any weakening of the Cu(II)-N(His) interactions must be accompanied by strengthening in the Cu(I) state. This interpretation focusses attention on the inference, drawn from the proton ENDOR results, that the laccase coordination geometry increases Cu-S π-bonding. Since this is expected to be more influential in stabilizing Cu(I) than Cu(II), it may be that π-bonding is the factor that determines the reduction potential of the laccases. In support of this possibility, the proton couplings of the type 1 Cu(II) in tree laccase are less than those in the fungal protein, and the reduction potential is also less, although it is still greater than for any of the single-site proteins.

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^1H NMR Nuclear Overhauser Enhancement and Paramagnetic Relaxation Determination of Peak Assignment and the Orientation of Ile-99 FG5 in Metcyanomyoglobin

S. Ramaprasad, Robert D. Johnson, and Gerd N. La Mar*

Contribution from the Department of Chemistry, University of California, Davis, California 95616. *Received February 27, 1984*

Abstract: The interproton nuclear Overhauser effect (NOE) and paramagnetic dipolar relaxation rates for hyperfine-shifted resonances in the upfield portions of the 360-MHz NMR spectrum of sperm whale metcyanomyoglobin has led to the location and assignment of all six nonequivalent proton signals of an isoleucine in the heme pocket. NOEs between previously assigned heme methyls and Ile protons establish it as Ile-99 (FG5). Differential dipolar relaxation rates indicate that the orientation of Ile-99 is essentially the same as in single crystals. The time dependence of the interproton NOE for the γ-geminal protons indicates limited internal mobility for this side chain. The nature of the direction and relative magnitudes of the necessarily dipolar origin of the Ile-99 hyperfine shifts clearly demonstrate that the heme iron exhibits substantial rhombic as well as axial magnetic anisotropy and that the effective in-plane magnetic axes are not the same as observed in single crystals at cryogenic temperature.

It has been long recognized that van der Waals interactions between the folded polypeptide side chains and the heme play a crucial control function in hemoproteins.[1] Such interactions can involve imposing steric strain on the porphyrin skeleton,[2] modulation of the iron reactivity via π-π interaction with aromatic side chains,[3] steric blocking at the distal binding site,[4] and/or electronic, steric, and hydrogen-bonding interaction with a bound ligand.[5,6] While high-resolution X-ray diffraction studies have provided a wealth of structural details of both myoglobin[7,8] and hemoglobin[9,10] in single crystals, it has also become obvious that only through

* Present address: Department of Chemistry, University of California, Davis, CA 95616.

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