Electron-Mediating Cu_A Centers in Proteins: A Comparative
High Field 1^H ENDOR Study

Boris Epel,† Claire S. Slutter,† Frank Neese,§ O Peter M. H. Kroneck,§ Walter G. Zumft,‖ Israel Pecht,‡ Ole Farver,† Yi Lu,§ and Daniella Goldfarb*,†

Contribution from the Departments of Chemical Physics and Immunology, Weizmann Institute of Science, Rehovot, Israel; Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany, Lehrstuhl für Mikrobiologie, Universität Fridericiana, D-76128 Karlsruhe, Germany, Royal Danish School of Pharmacy, Institute of Analytical & Pharmaceutical Chemistry, DK-2100 Copenhagen, Denmark, and Department of Chemistry, University of Illinois, Urbana, Illinois 61801

Received November 9, 2001. Revised Manuscript Received March 4, 2002.

Abstract: High field (W-band, 95 GHz) pulsed electron-nuclear double resonance (ENDOR) measurements were carried out on a number of proteins that contain the mixed-valence, binuclear electron-mediating Cu_A center. These include nitrous oxide reductase (N_2OR), the recombinant water-soluble fragment of subunit II of Thermus thermophilus cytochrome c oxidase (COX) ba_9 (M160T9), its M160QT0 mutant, where the weak axial methionine ligand has been replaced by a glutamine, and the engineered “purple” azurin (purpAz).

The three-dimensional (3-D) structures of these proteins, apart from the mutant, are known. The EPR spectra of all samples showed the presence of a mononuclear Cu(II) impurity with EPR characteristics of a type II copper. At W-band, the g_1 features of this center and of Cu_A are well resolved, thus allowing us to obtain a clean Cu_A ENDOR spectrum. The latter consists of two types of ENDOR signals. The first includes the signals of the four strongly coupled cysteine β-protons, with isotropic hyperfine couplings, A_{iso}, in the 7−15 MHz range. The second group consists of weakly coupled protons with a primarily anisotropic character with A_{zz} << 3 MHz. Orientation selective ENDOR spectra were collected for N_2OR, M160QT0, and purpAz, and simulations of the cysteine β-protons signals provided their isotropic and anisotropic hyperfine interactions. A linear correlation with a negative slope was found between the maximum A_{iso} value of the β-protons and the copper hyperfine interaction. Comparison of the best-fit anisotropic hyperfine parameters with those calculated from dipolar interactions extracted from the available 3-D structures sets limits to the sulfur spin densities. Similarly, the small coupling spectral region was simulated on the basis of the 3-D structures and compared with the experimental spectra. It was found that the width of the powder patterns of the weakly coupled protons recorded at g_1 is mainly determined by the histidine H_1 protons. Furthermore, the splitting in the outer wings of these powder patterns indicates differences in the positions of the imidazole rings relative to the Cu_S_S core. Comparison of the spectral features of the weakly coupled protons of M160QT0 with those of the other investigated proteins shows that they are very similar to those of purpAz, where the Cu_A center is the most symmetric, but the copper spin density and Cu distances are somewhat smaller. All proteins show the presence of a proton with a significantly negative A_{iso} value which is assigned to an amide proton of one of the cysteines. The simulations of both strongly and weakly coupled protons, along with the known copper hyperfine couplings, were used to estimate and compare the spin density distribution in the various Cu_A centers. The largest sulfur spin density was found in M160T9, and the lowest was found in purpAz. In addition, using the relation between the A_{iso} values of the four cysteine β-protons and the H−C−S−S dihedral angles, the relative contribution of the hyperconjugation mechanism to A_{iso} was determined. The largest contribution was found for M160T9, and the lowest was found for purpAz. Possible correlations between the spin density distribution, structural features, and electron-transfer functionality are finally suggested.

Introduction

Two types of copper centers in proteins are known to mediate electron transfer (ET): the abundant type I (T1), which is a mononuclear copper binding site, and the mixed-valence bi-

* To whom correspondence should be addressed. E-mail: daniella.goldfarb@weizmann.ac.il.
† Department of Chemical Physics, Weizmann Institute of Science.
‡ Department of Immunology, Weizmann Institute of Science.
§ University of Konstanz.
‖ University of Illinois.
⊥ Current address: Max-Planck Institut für Strahlenchemie, Stiftstrasse 34-36, D-45470 Mülheim an der Ruhr, Germany.

reductases, in all of which it serves as the initial electron-accepting site.\(^4\) While the structure of the T1 site has been known for a long time (the 3-D structure of plastocyanin has been determined already in 1977\(^5\)), Cu\(_A\) has been recognized as a binuclear center much later.\(^6,7\) It is the initial electron acceptor in cytochrome c oxidase (COX) and in nitrous oxide reductase (N\(_2\)OR).\(^8\) In addition, the quinol oxidase subunit II (CyoA)\(^9\) and azurin (purple azurin, purpAz)\(^10,12\) were converted by mutagenesis to form Cu\(_A\) centers. A recombinant water-soluble fragment of subunit II of Thermus thermophilus COX\(_9\) that contains the Cu\(_A\) site has been prepared (M160T0) as well.\(^13\) The 3-D structures of all of these proteins have been determined by X-ray crystallography.\(^9,12,14\)\(^\rightarrow\)\(^18,20,21\) Figure 1 shows the T1 copper site of azurin\(^15\) together with the Cu\(_A\) centers in M160T0,\(^20\) N\(_2\)OR,\(^20\) and purpAz.\(^12\) The copper ion in T1 is coordinated to a cysteine thiolate and two imidazole residues of histidine in a trigonal arrangement with rather short bonds (\(~2\) Å) and a fourth, weaker axial ligand (usually a thioether of methionine) at a distance of \(~3\) Å.\(^14,15,22,23\) The Cu\(_A\) center resembles two fused T1 centers, with thiocyanates of two cysteines, two histidine imidazoles, and two weak axial ligands, a methionine sulfur, and a main chain carbonyl oxygen of glutamine.\(^9,12,14\)\(^\rightarrow\)\(^21\)

Considerable efforts have focused on studying the relation between the 3-D structure of the Cu\(_A\) site, its spectroscopic properties, and electronic structure and ET characteristics such as redox potentials and reorganization energies.\(^24\)\(^\rightarrow\)\(^26\) The unusual electron mediators in Cu\(_A\) centers make them interesting model systems for studying ET processes in proteins such as photosynthetic reaction centers (RCs).\(^27\) The Cu\(_A\) site is involved in the redox communication between the reaction center antenna and RC, and the ET from the antenna to the reaction center is thought to influence the ET from the reaction center to NADPH.\(^27\) The study of ET processes in Cu\(_A\) centers is important for understanding the ET processes in RCs.\(^27\)

---

**Figure 1.** The 3-D structures of the nearest ligands of Cu\(_A\) sites in N\(_2\)OR (2.4 Å resolution),\(^20\) M160T0 (1.6 Å resolution),\(^21\) and purpAz (1.65 Å resolution)\(^12\) from a projection along the S–S direction of the Cu\(_S\) core, as compared to that of T1 of azurin.\(^15\) Black, gray, and white spheres represent atoms of Cu, S, and H, accordingly.

---


spectroscopic properties, the strong blue color of T1 and the dark purple color of Cu₄, stem from the S–Cu charge-transfer bands. The ⁶³⁶⁵Cu hyperfine interaction of T1 is unusually small⁵²,⁵³ because of the highly covalent nature of the Cu–S coordination and the large spin density on the sulfur. The Cu₄ center in the resting state has a total of one unpaired electron which is highly delocalized, with each copper having a formal oxidation state of 1.⁵⁸ The ⁶³⁶⁵Cu hyperfine coupling is in this case about one-half that of the T1 Cu(II) because of the delocalization of the unpaired electron over the two copperos.⁶ The highly covalent Cu–S bonds in both T1 and Cu₄ were found to be important for coupling into the ET pathways.²⁵,²⁹

One of the spectroscopic techniques that is most commonly used for the characterization of these ET-mediating centers is EPR spectroscopy. It provided the g- and ⁶³⁶⁵Cu hyperfine interactions that characterize the ground state and the spin delocalization. In addition, the EPR related methods, electron-nuclear double resonance (ENDOR)³⁰–³⁴ and electron-spin-echo envelope modulation (ESEEM)³⁵–³⁷ spectroscopies, have played an important role in the identification of the ligands constituting the coordination sphere of the copper ions and the spin delocalization over the site. X-band ESEEM spectroscopy has been most useful in the determination of the hyperfine and quadrupole couplings of weakly coupled ¹⁴N nuclei such as the remote nitrogens of the histidines’ imidazole and main chain amides.³⁶,³⁸,³⁹ The more strongly coupled nuclei, the cysteine β-protons and the directly bound histidine nitrogens, have been examined by X-band ENDOR.¹³,¹⁴ It has been shown that the weakly coupled nitrogens of azurin can also be detected by ENDOR at high fields in single crystals.⁴⁰

The isotropic hyperfine coupling of the cysteine β-protons is an important source of structural information because it depends on the H–C–S–Cu dihedral angle in T1⁴¹ and the H–C–S–S dihedral angle in Cu₄.¹³,¹⁴ Similarly, the hyperfine coupling of the histidine protons, specifically of the H₃ protons, can provide spin density and structural information. Moreover, the availability of experimental values of hyperfine interactions of ligand nuclei, which characterize the highly delocalized nature of the unpaired electron wave function, serves as important experimental constraints for the determination of the ground state by quantum chemical calculations.³⁶,⁴³ This may then provide the rationale for the unique properties of these ET sites.²⁵

Unfortunately, the ENDOR spectra of T1 and Cu₄ centers recorded at X-band frequencies often suffer from low resolution because of overlap of ¹H signals with those of the directly bound ¹⁴N.¹³,¹⁴ This problem can be alleviated by recording the spectra at Q-band,³⁴,⁴¹ but the reported ¹H spectra are often distorted and show only the high-frequency part of the symmetric ENDOR spectrum. Another problem of X-band ENDOR and ESEEM studies of the Cu₄ center is interferences from signals of a mononuclear Cu(II) impurity center.⁴⁴

We have now employed high field (95 GHz) ¹H ENDOR spectroscopy in a comparative study of the Cu₄ site present in four different proteins: N₉O₉, the soluble fragment of COX, M160T9, which is nine amino acids shorter than the original M160T0, the M160QT0 mutant, and purpAz. The spectra are free of ¹⁴N signals, well resolved, symmetric, and not distorted. Moreover, problems caused by contribution from mononuclear Cu(II), present in some of the proteins, were eliminated by the separation of the g₂ singularities of Cu₄ and T2. The larger spread of the EPR powder pattern also leads to better orientation selection that allows a more accurate determination of the isotropic and anisotropic hyperfine components. The study of several different Cu₄ sites allowed us to compare their sulfur and copper spin densities. This, combined with the available 3-D structures, permitted us to suggest some correlations between the hyperfine parameters of weakly and strongly coupled protons, the spin density distribution, and structural properties of these sites.

**Experimental Section**

**Sample Preparations.** Azurin from *Pseudomonas aeruginosa* was obtained as described.³⁷ The sample concentration was 2 mM in a 100 mM Hepes buffer pH = 7. M160T0 is a product of the original soluble fragment construct of cytochrome *ba₃* from *Thermus thermophilus* that encodes 135 amino acids of subunit II, omitting the transmembrane helix that anchors the domain in the membrane. In M160T9, nine C-terminal amino acids are missing, including one histidine,¹³ and M160QT0 is the mutant where the methionine axial ligand has been replaced with glutamine.⁴⁴,⁴⁵ Details of the protein preparation and purification are given in refs 13, 44, and 45. The concentrations of the M160T9 and M160QT0 samples were 1.5–3.0 mM Cu₄ in 50% glycerol and 50% 100 mM phosphate/200 mM NaCl, pH 7. Two N₂-OR samples, both from *Pseudomonas stutzeri*, were studied. The first, labeled with ³⁴S, was isolated from the wild-type strain (ATCC 14405) grown with 0.25 mM Na₂³⁴SO₄ at a final concentration of 90 mg/mL in a solution of 50 mM Tris-HCl, pH 7.5. The second was from MK (His-deficient strain) cells grown in L-histidine (40 mM) with a final concentration of 105 mg/mL in 50 mM Tris-HCl pH 7.5.⁴⁶ Both yielded proteins with the same properties as confirmed by the experimental results. The enzyme was studied in its resting state where the Cu₄ is 2622–2629.

oxidized, and the catalytic Cu_{II} is EPR silent. The purpAz sample was obtained as reported, and the protein concentration was 0.35 mM in 50 mM ammonium acetate, pH 5.1. The complexes of Cu(II) tetraimidozole (Cu–Imid) and Cu(II) bis-histidine (Cu–His) were prepared as described earlier, and the Cu(II) concentration was 2 mM in 50:50 water:glycerol. Samples of all proteins and model compounds (total volume of 1–2 μL) were placed in EPR quartz tubes (0.8 OD) for all measurements.

**Spectroscopic Measurements.** W-band pulsed EPR and ENDOR measurements were carried out at 94.9 GHz and 4.5 K using a home-built spectrometer described elsewhere. Field-sweep (FS) echo-detected (ED) EPR spectra were recorded using the two-pulse echo sequence \((\pi/2 – \tau – \pi – \tau – \pi – \tau – \text{echo})\), where the echo intensity was registered as a function of the magnetic field. Typically, microwave (MW) pulse lengths \((t_{\text{MW}})\) of 0.09 and 0.18 μs were used with \(\tau = 0.3\) μs. The magnetic field values were calibrated using the Larmor frequency of the protons, \(v_{L}\), as determined by the ENDOR measurements. The \(^1\text{H} \text{ENDOR spectra were measured using the Davies ENDOR pulse sequence (} \pi – T – \pi/2 – \tau – \pi – \tau – \pi – \tau – \text{echo}, with an RF pulse applied during the time interval } T_2\). The experimental conditions for the Davies ENDOR spectra were \(t_{\text{MW}} = 0.2, 0.1, 0.2\) μs, \(T = 0.35–0.5\) μs, \(t_{\text{RF}} = 25–40\) μs. In variable mixing time ENDOR experiments, an additional delay, \(t_{\text{mix}}\), is inserted between the RF pulse and the echo detection sequence. The frequency scale in the \(^1\text{H} \text{ENDOR spectra is given with respect to the } \text{H Larmor frequency. The repetition rate in these experiments, except for those stated otherwise, was } 150 \text{ Hz.}

**Simulations.** EPR simulations were carried out using a program developed by Neese. \(^{19,20}\) \(^1\text{H} \text{ENDOR spectra were simulated using a program developed in our laboratory according to Erickson et al.}^{51}\) In this program, the hyperfine coupling tensor \(A\) is not limited to axial symmetry. The anisotropic part, given by \(D\), was calculated for the CuA centers from the atomic coordinates using the point-dipole approximation as described by Neese.\(^{50}\) For each proton denoted by \(i\), \(D\) is obtained from contributions of six \(D_{ij}\) \((j = 1, 6)\) matrices, corresponding to the individual \(H–X_i\) interactions where \(X_i = \text{Cu}_1, \text{Cu}_2, S_1, S_2, N_1, N_2\). The principal values of each \(D_{ij}\) are \((-a_{ij}, -a_{ij}, 2a_{ij})\), where \(a_{ij} = (\mu_0/4\pi)\rho_j\delta_{i,0}g_j^2[3h_i^2]_{i,0}\), and \(\rho_j\) is the spin density on the \(X_i\) nucleus. The individual \(D_{ij}\)’s, expressed in the principal axis system of \(g\), are then obtained by summing over all six dipolar interactions according to

\[
D_i = \sum_{j=1}^{6} D_{ij} = \sum_{j=1}^{6} R(\theta_i, \phi_i)D_{ij}R^{-1}(\theta_i, \phi_i) \quad (1)
\]

where \(\theta_i\) and \(\phi_i\) are the polar and azimuthal angles representing the orientation of the \(X_i–H\) vector with respect to the \(g\) principal axis system, and \(R(\theta_i, \phi_i)\) is the corresponding rotation matrix. Finally, the \(D_{ij}\) were diagonalized to give the principal components of each coupled proton and the Euler angles, \(\alpha, \beta, \gamma\), relating it to the \(g\) frame. \(\theta_i\) and \(\phi_i\) describe the orientation of the external magnetic field, \(B\), with respect to the \(g\) principal axis system.

The orientation of \(g_{ij}\) was used along the bisector of the two vectors normal to the \(S–\text{Cu}_i–S\) and \(S–\text{Cu}_j–S\) planes and that of \(g_{ij}\), parallel with the \(S–S\) direction.\(^{50,52}\) The \(\theta_i, \phi_i, \text{and } t_{\text{RF}}\) were calculated from the atomic coordinates of the 3-D structures, and the protons were placed using InsightII by MSI.

In the simulations, the selected orientations were determined as follows: the EPR spectrum was simulated, and using the \(g\) and \(A\) values, the hyperfine coupling constants, a plot of the resonant magnetic fields as a function of the angles \(\theta_i\) and \(\phi_i = 90^\circ\), \(0^\circ\) was generated. Selected orientations were then determined graphically assuming a line width of 100 G.

**Results**

The W-band FS-ED EPR spectra of \(N_2\text{OR}, M160T9, M160QT0\), purpAz, and azurin are presented in Figure 2, and the \(g\)-values determined from these spectra are summarized in Table 1. All spectra, except that of azurin, show, in addition to the \(g_{ij}\) feature of CuA (marked on the figure), a peak at ~3.3 T which is assigned to the \(g_{ij}\) feature of a mononuclear Cu(II) center. Unlike X-band, at W-band the two centers are easily distinguished even at the \(g_{ij}\) region. In the case of M160T9, this feature was unambiguously assigned to a type II (T2) Cu(II) ion on the basis of X-band spectra measured at 160 K, where only the T2 spectrum is detected, and by simulations of the W-band spectrum using the CuA and T2 \(g\)-values.\(^{44}\) On the basis of the similar position of the above extra feature, its different relative intensity and relaxation characteristics, we assign it also in purpAz, M160QT0, and \(N_2\text{OR}\) to a mononuclear Cu(II) “impurity” center. The \(g\) singularity of the CuA center is clear in all spectra, whereas that of the T2 is outside the displayed magnetic field range. The dotted line shows the simulated spectrum of the CuA of \(N_2\text{OR}\) calculated with the parameters given in Table 1. The spin–lattice relaxation time, \(T_1\), of the

![Figure 2. W-band FS-ED EPR spectra](image)

### Table 1. \(g\) and \(A(Cu)\) Values of the Different CuA and T1 Centers Investigated

| Protein   | \(g_{xx}\) | \(g_{yy}\) | \(g_{zz}\) | \(A_{||}\) (mT) |
|-----------|-----------|-----------|-----------|----------------|
| \(N_2\text{OR}\) | 2.005     | 2.02      | 2.175     | 4.28         |
| M160T9    | 2.0       | 2.02      | 2.189     | 3.1           |
| M160QT0   | 2.02      | 2.02      | 2.176     | 4.2           |
| purple Az | 2.01      | 2.04      | 2.17      | 5.5           |
| Azurin    | 2.039     | 2.057     | 2.273     | 5.48         |

* The average value of the two copper (117 and 124 MHz) was taken.

mononuclear Cu(II) is significantly longer than that of the CuA center, and, therefore, its relative contribution to the spectrum can be reduced by partial saturation, achieved by increasing the repetition rate from 150 Hz to 0.5–1 kHz. This has been used while recording the ENDOR spectra of N2OR and purpAz. The only protein that contained a negligible amount of mononuclear Cu(II) is the M160QT0 mutant. This center may originate from the protein isolation process or from a time-dependent loss of copper from the CuA site to a nonspecific site where it is coordinated to a histidine residue (see below).44 In the case of N2OR, it may also originate from a partial reduction/decay of the EPR silent CuZ catalytic site which is a possibility supported by its different line shape. We rule out the possibility that the 3.3 T feature appears as a consequence of a line shape distortion because of echo detection because all spectra were recorded with the same MW power and pulse width, and yet this line is not observed in azurin and is very weak in M160QT0.

The 1H ENDOR spectra of N2OR, M160T9, M160QT0, and purpAz, recorded at the CuA gL position, where there are no interferences from the mononuclear Cu(II), are presented in Figure 3. Two types of protons are recognized. The first consists of protons with large, mostly isotropic hyperfine couplings in the range of 7–15 MHz attributed to the cysteine β-protons,31,44 whereas the second comprises the weakly coupled protons with couplings smaller than 3 MHz. The resolution of the β-protons signals of N2OR, M160T9, and M160QT0 allows the distinction of inequivalent protons (see next section). In contrast, the signals of the purpAz’s β-protons are unresolved, exhibiting an average splitting of 10.8 MHz. For comparison, the spectrum of azurin recorded at g3y is displayed as well. There, at gL, the two cysteine β-protons are not resolved and have a coupling of 22.2 MHz, which is about twice the value observed for these protons in purpAz.

The 1H ENDOR spectra of the weakly coupled protons of the CuA centers have, in general, similar line shapes. They differ mainly in the total width of the powder pattern, where the M160T9 spectrum exhibits the largest coupling. There, the outer edges exhibit two features with couplings of 2.8 and 2.3 MHz. In N2OR and purpAz, these are reduced to 2.6 and 2.1–2.2 MHz, whereas in M160QT0 they almost merge into one feature with a splitting of 2.5 MHz. As expected, the width of the powder patterns of the weakly coupled protons of azurin is almost twice as large, 4.2 MHz.

The availability of the 3-D structures of three of the investigated proteins combined with well-resolved orientation selective sets of ENDOR spectra provide a unique opportunity to analyze the spectra on the basis of the 3-D structure. This leads to a more accurate determination of the spin density distribution and to the direct correlations between spectral features and structural parameters. Although the X-ray determined 3-D structure of these proteins does not provide proton coordinates, except for water molecules and OH groups, a good estimate of their positions can be obtained using well-known bond angles and lengths. Accordingly, we have employed InsightII (by MSI) to place the protons of the ligands of CuA center in their 3-D structures. Some of these protons are shown in the structures depicted in Figure 1. The coordinates of these protons were then used to estimate the various Dk’s according to the procedure outlined in the Experimental Section. For example, Table 2 lists the \( D_{kk} \) (\( k = x, y, z \)) values of the β-protons of N2OR and the Euler angles relating them with the g-frame.

In the case of N2OR, the available 3-D structure has been determined on a crystal where the CuA is in the reduced state,20 while the EPR measurements were all carried out on the resting, that is, mixed-valence state. The use of the coordinates of the reduced state is justified by the subtle structural changes the site probably undergoes upon reduction. This is based on studies of the structure of bovine heart COX, determined in both the reduced and the oxidized states.19 The differences observed were rather limited, manifested mainly in a slight expansion of the reduced state with Cu–X bond length differences on the order of 0.05 Å and practically no change in the orientation of the ligand residues. Similarly, EXAFS measurements of reduced and mixed-valent states of the soluble CuA fragment of Thermus thermophilus and Bacillus subtilis resolved only minor changes in the core geometry.54 These changes are insignificant with respect the structural differences among the different proteins. In the next two sections, the analysis of the ENDOR spectra of the β-protons and the weakly coupled protons is presented.

Cysteine β-Protons. Orientation selective 1H ENDOR spectra of N2OR are shown in Figure 4. The spectral region of the β-protons is free from mononuclear Cu(II) interferences because their large couplings are unique to the cysteine ligands in T14 and CuA centers.31 We first attempted to reproduce the orientation dependence of the β-proton signals using the isotropic hyperfine values determined from earlier X-band ENDOR measurements31 (13.3, 11.7, 10.4, 8.6 MHz for C561_1HB, C565_1HB, C561_2HB, C565_2HB, respectively) and the \( D_{kk} \) components listed in Table 2, calculated with \( \rho_S \), \( \rho_C \), and \( \rho_N \) values of 22, 25, and 3% on each Cu, S, and directly coordinated N, respectively. The latter are within the range found experimentally and predicted theoretically.55,31,50 Earlier X-band EPR and 14N ENDOR results showed that the spin densities on both copper and two bound 14N nuclei are close,42,52 and we have, therefore, assumed the same \( \rho_S \) for both sulfurs. Changing the spin densities to 20, 27, and 3%, respectively, led to relatively insignificant variations in the range of ±0.05 MHz in the \( D_{kk} \) and ±0.5° in the angles. The simulated traces are displayed as dashed lines in the low-frequency part of Figure 4.

Table 2. Hyperfine Interaction Parameters of the Cysteine \( \beta \)-Protons of the Proteins Investigated As Determined from the Best-Fit Simulations of the Orientation Selective ENDOR Spectra, As Compared to Values Calculated from the 3-D Structures\(^{20} \) and \( \rho_S = 22\% \), \( \rho_C = 25\% \), \( \rho_N = 3\% \) (in Parentheses)\(^a\)

<table>
<thead>
<tr>
<th>protein</th>
<th>proton</th>
<th>( A_{iso} )</th>
<th>( D_{xx} )</th>
<th>( D_{yy} )</th>
<th>( D_{zz} )</th>
<th>( \alpha )</th>
<th>( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N(_2) OR</td>
<td>C561_1HB</td>
<td>13.8</td>
<td>-1.84(-2.14)</td>
<td>-1.36(-1.55)</td>
<td>3.2(3.69)</td>
<td>131(37.1)</td>
<td>52(32.9)</td>
</tr>
<tr>
<td></td>
<td>C561_2HB</td>
<td>11.0</td>
<td>-1.92(-2.01)</td>
<td>-1.46(-1.56)</td>
<td>3.38(3.66)</td>
<td>118(148.9)</td>
<td>55(47.5)</td>
</tr>
<tr>
<td></td>
<td>C565_1HB</td>
<td>12.2</td>
<td>-1.58(-1.93)</td>
<td>-1.04(-1.60)</td>
<td>3.38(3.72)</td>
<td>139(131.3)</td>
<td>49(26.3)</td>
</tr>
<tr>
<td></td>
<td>C565_2HB</td>
<td>9.1</td>
<td>-1.67(-1.84)</td>
<td>-1.48(-1.50)</td>
<td>3.11(3.34)</td>
<td>142(166.5)</td>
<td>49(50.9)</td>
</tr>
<tr>
<td>M160T9</td>
<td>C149_1HB</td>
<td>10.0</td>
<td>-1.92(-2.12)</td>
<td>-1.36(-1.60)</td>
<td>3.38(3.72)</td>
<td>139(131.3)</td>
<td>49(26.3)</td>
</tr>
<tr>
<td></td>
<td>C149_2HB</td>
<td>15.4</td>
<td>-1.84(-2.17)</td>
<td>-1.36(-1.52)</td>
<td>3.20(3.68)</td>
<td>-32(21.4)</td>
<td>58(44.3)</td>
</tr>
<tr>
<td></td>
<td>C153_1HB</td>
<td>6.8</td>
<td>-1.67(-2.18)</td>
<td>-1.44(-1.55)</td>
<td>3.11(3.72)</td>
<td>44(57.0)</td>
<td>131(169.0)</td>
</tr>
<tr>
<td></td>
<td>C153_2HB</td>
<td>12.2</td>
<td>-1.58(-2.08)</td>
<td>-1.23(-1.46)</td>
<td>2.82(3.54)</td>
<td>13(162.0)</td>
<td>128(138.0)</td>
</tr>
<tr>
<td>M160QT0</td>
<td>1</td>
<td>10.3</td>
<td>-1.92</td>
<td>-1.46</td>
<td>3.38</td>
<td>41</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.3</td>
<td>-1.84</td>
<td>-1.36</td>
<td>3.20</td>
<td>33</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.6</td>
<td>-1.58</td>
<td>-1.23</td>
<td>2.82</td>
<td>13</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.1</td>
<td>-1.67</td>
<td>-1.44</td>
<td>3.11</td>
<td>39</td>
<td>126</td>
</tr>
</tbody>
</table>

\(^a\) The angle \( \gamma \) was omitted as it does not affect the spectrum because of the axial symmetry of \( g \).

Figure 4. \(^1\)H W-band ENDOR spectra of a frozen solution of N\(_2\)OR recorded at different fields along the EPR powder pattern (4.5 K). The amplitude of the signals in the region of \((3-14) \text{ MHz}\) was scaled up for better visualization. The dashed traces were calculated with the parameters listed in Table 2 and the \( A_{iso} \) values determined by Neese et al.\(^31\) (see text). The dotted traces represent the best-fit simulated spectra calculated with the parameters listed in Table 2 for the cysteine \( \beta \)-protons, and in Table 6 for the weakly coupled protons. An individual line width of 0.9 MHz was used for the \( \beta \)-protons and 0.15 MHz for the others.

or nuclear relaxation, the ENDOR signals corresponding to the \( M_S = \frac{1}{2} \) manifold lose intensity and may even reach a negative amplitude.\(^{55,56}\) This allows the identification of the ENDOR signals belonging to the different \( M_S \) manifolds and thereby the determination of the sign of the coupling. The ENDOR spectrum of N\(_2\)OR recorded at the Cu\( _A \) \( g_{zz} \) position as a function of \( r_{max} \) exhibits a decrease in the relative intensity of the low-frequency components of the \( \beta \)-proton doublet and assigns them to the \( M_S = \frac{1}{2} \) manifold, yielding \( A_{iso} > 0.57 \). The positive shift of these protons in NMR spectra is consistent with this assignment.\(^{42,58}\)

Orientation selective ENDOR spectra of M160QT0 are shown in Figure 5. The spectra are, in general, similar to those of N\(_2\)OR; the resolution is lower, and \( \Delta V_{z}(\beta) \) is slightly larger. Because there is no 3-D structure available for this mutant, we used the \( D_{zz} \) values obtained from the best-fit simulations of N\(_2\)OR and fitted \( A_{iso} \) and the angles. This was done under the assumption that the \( D_{zz} \) are primarily determined by the S–H distance, which does not vary from structure to structure, although \( \rho_S \) may vary and change somewhat the \( D_{zz} \) values. In addition, the relatively large individual line width which is on


the order of $D_{yy}$ makes the simulations less sensitive to the $D_{zh}$ values. The simulated spectra are presented as dotted traces in Figure 5, and the simulation parameters are listed in Table 2. A comparison with the values obtained for N$_2$OR shows a reduction in $A_{iso}$ of some protons.

Unlike N$_2$OR, the orientation selective ENDOR spectra of the $\beta$-protons of purpAz did not show any features that could be used to distinguish the different protons (see Figure 6).

Moreover, the splitting remained practically constant at 10.8 MHz throughout the field range of the EPR spectrum. From the line width and by comparison with the M160QT0 spectra, we estimated the maximum and minimum $A_{iso}$ values as 12.5 and 9 MHz, respectively. Unfortunately, we were not able to obtain orientation selective spectra for M160T9 with a reasonable S/N, yet we have used the same strategy applied for M160QT0 to simulate the spectrum recorded at $g_L$ and we obtained the values of $A_{iso}$. The best-fit simulated trace is shown as a dotted trace in Figure 3, and the parameters used are listed in Table 2. This spectrum was simulated previously with similar $A_{iso}$ values, but on the basis of the N$_2$OR results described above, we concluded that the couplings are positive in all CuA centers.

**Weakly Coupled Protons.** The strategy we adopted in the analysis of the weakly coupled protons differed from the case of the $\beta$-protons, where best-fit simulations were carried out, for the following reasons: (i) in a significant number of spectra there are interferences from a mononuclear Cu(II) ion center, (ii) the number of protons contributing to this spectral region is relatively high, (iii) the relative intensities of peaks very close to the Larmor frequency are underestimated because of the limited selectivity of the MW pulses. We have, therefore, concentrated on reproducing the total width of the spectra with emphasis on the region of ±0.5 MHz and up, where the contribution of more distant protons and the effect of the MW pulse nonselectivity become negligible. In all cases, except M160QT0, we started with the dipolar interactions calculated from the 3-D structures, and, whenever required, the agreement with the experimental results was improved by changing the spin densities, adding isotropic coupling constants. If this did not yield satisfactory results, mild changes in the angles were introduced.

We begin the analysis of the weakly coupled protons with the ENDOR spectra of M160QT0 because it is the only protein in the series investigated where the spectra are practically free from “impurity” signals throughout the full EPR spectral range. In this case, the 3-D structure is not available, and we attempted to fit the spectral features with couplings larger than 1 MHz. Table 3 shows that in all proteins a relatively large number of protons are expected to contribute to this spectral region. In all cases, $D_{zz}$ ≤ 3.6 MHz, which can be used as an upper limit. In addition, we took into consideration that in all structures the histidine $H_{41}$ protons are expected to have relatively large couplings ($D_{zz}$ is in the range of 2.4–3.0 MHz and $\beta = 90 \pm 10^\circ$) and that they should have major contributions to the outer wings ($A_{max} = 2.6$ MHz) in spectra recorded close to $g_L$. Other protons that are close to the Cu$_2$S$_2$ core in all structures are the amide proton of one of the cysteines and the $\alpha$-proton of one of the histidines. These protons are characterized by $\beta < 70^\circ$, and, therefore, their maximum hyperfine coupling should be observed at $\theta_L < 90^\circ$.

We first fitted the $A_{max}$ features of the 3.377 and 3.355 T with a proton having $H_{41}$ characteristics. The experimental spectra (Figure 5) shows that a second proton, with $A_{max} = 2.6$ MHz that is reached toward the center of the EPR powder pattern, is present as well. The relatively high intensity of this feature indicates that it corresponds to an $A_{xx}$ or $A_{yy}$ singularity, rather than to $A_{zz}$. Such features can be obtained from a proton with an $A_{iso} < 0$ and $\beta \approx 25–45^\circ$. The fit was improved by adding three more protons. The final simulation with all five protons is compared with the experimental set in Figure 5, and the best-fit parameters are listed in Table 3. The total width of the spectra is reasonably reproduced, but the fit of the peaks’ position and relative intensity varies. This is expected because not all protons have been taken into account, and the sensitivity of Davies ENDOR to larger couplings has been neglected.

Protons 1 and 5 have the largest values of $\beta$ and are, therefore,
assigned to the two histidines’ H₄. The protons with the relatively large negative Aiso (no 2 and 4) can be assigned to (i) the amide proton C153_H because of the large spin density on S and the finite spin found on a backbone nitrogen¹⁴ and (ii) to the α-proton of H157 because of its angle β and its close distance to the Cu₄S₂ in M16OT0 (see Table 3). The possibility that it can also be one of the NH₂ protons of the replacing glutamine cannot be ruled out, however.

The spectra of purpAz and M16QT0 recorded within the range 3.37–3.31 T are almost identical. Attempts to simulate the M16OQT0 spectra using the calculated D obtained from the 3-D structure of purpAz (see Table 3) while varying the Aiso values did not produce the overall width of the spectra. Comparison of calculated values for purpAz with those obtained by fitting the M16OQT0 spectra shows that the dipolar couplings calculated for purpAz are too small to fit the experimental results. It was possible to obtain D values that agree better with the experimental results by changing ρs, ρcu, and ρN to 15, 32, and 3%, respectively. This gave larger Dzz values for the histidine H4 protons (increase to 2.90 and 2.38 MHz) without varying significantly those of the other protons.

The ENDOR spectra of the weakly coupled protons of N₂-OR, shown in Figure 4 and measured within the range B0 = 3.129–3.248 T, contain contributions from both Cu₄ and the mononuclear Cu(II) impurity. The most apparent contributions of the latter are the clear singularities at ±2.5 MHz in the spectra recorded at B0 = 3.167–3.248 T (see arrow in Figure 4). This assignment was substantiated by recording the spectra with a faster repetition rate, which leads to a partial saturation of the mononuclear Cu(II) EPR signal, while that of the Cu₄ is less affected because of the difference in spin–lattice relaxation times. Under these conditions, the intensities of the ENDOR peaks at ±2.5 MHz were reduced significantly. These signals correspond to an Azz value of ~5 MHz which suggests that they arise from the imidazole protons of a remote Cu(II) binding histidine residue. This assignment is supported by comparison with the ENDOR spectra of azurin and of the Cu–Imid and Cu–His complexes, that can serve as models for the coupling of the imidazole protons (see Figure 3). The Azz values of these model⁴⁷,⁵⁶ are about twice those observed for the Cu₄ centers, as expected considering the spin distribution over two copppers in Cu₄. These signals are also clear in purpAz (see Figure 6). They were also observed in earlier X-band ENDOR spectra of N₂OR where they have been assigned to unknown protons in the Cu₄ centers.³¹

The weak coupling regions of the ENDOR spectra of N₂OR were simulated using the calculated D parameters of the protons in the vicinity of the Cu₄S₂ core listed in Table 3 (except for H526_HA which has the smallest predicted Dzz value). The major discrepancy between the calculated and experimental spectra was the absence of an intensity at ±1.4 MHz in spectra recorded with field settings in the center of the EPR powder pattern. Comparison with the spectra of M16OQT0 (see above) strongly suggests that these features arise from a Cu₄ proton with a substantial negative Aiso and value of β ~ 45°. Consequently, finite Aiso values were tried for the amide protons C565_H and W563_H. Setting Aiso = −0.9 MHz for C565_H and changing β from 66° to 60° improved the fit significantly, whereas the introduction of Aiso for W563_H led to large deviations at 3.359 T, where mononuclear Cu(II) does not interfere. The simulated spectra are shown in Figure 4.

The simulations of the weakly coupled protons along with the dipolar couplings calculated from the 3-D structures show that the splitting of the outer wings in spectra recorded around gzz is determined primarily by the two H4 protons, although some contributions from a proton with Aiso ~ (−0.5)–(−0.7) MHz with a small β (like proton 4 in M16OQT0) may also be significant. The outer wings in the gzz ENDOR spectrum of M16OT9 appear at 2.8 and 2.3 MHz, which is slightly larger than that observed for N₂OR and M16OQT0. Simulations were carried out using the calculated D values and orientations determined from the structure of M16OT0, listed in Table 3, and splitting of the outer edges in the simulated spectrum was too small. Although the 3-D structure was determined for M16OT0, the similar spectroscopic properties of M16OT0 and M16OT9⁴⁴ justify the assumption that the structure of the Cu₄ center has not changed by the removal of the nine terminal amino acids. A better fit could be obtained by introducing Aiso = 0.2 MHz to H157_HE1. A similar quality fit could be obtained by setting Aiso = 0 and ρs, ρcu, and ρN to 18, 30, and 2%, respectively, but the higher Aiso(Cu) values of N₂OR make the first option more attractive. In addition, in light of the spectral analysis of M16OQT0 and N₂OR, we introduced Aiso = −1.3 MHz for the amide proton C153_H. This did not increase the maximum splitting but improved the fit of the relative intensity of the feature at ±1.15 MHz. An Aiso = −0.3 MHz was also taken for G151_H. A comparison between the simulated and experimental spectra is presented in Figure 3.

Discussion

Generally, the ENDOR spectrum reflects the spin density distribution in the paramagnetic center. An unambiguous determination of these densities does, however, require the knowledge of the 3-D structure. The simulations of the orientation selective ENDOR spectra of the cysteine β-protons of N₂OR showed that the calculated Dzz values based on the 3-D structures and using ρs = 22%, ρcu = 25%, and ρN = 3% are overestimated by 10–20% relative to the Dzz value obtained from best-fit simulations of the orientation selective ENDOR spectra (see Table 2). Moreover, the same smaller Dzz values reproduced the spectra of the β-protons of M16OQT0 and M16OT9. Because the major contribution to the anisotropic hyperfine interaction of these protons arises from the S–H distance, this discrepancy can be ascribed to an overestimation of ρs. However, reducing ρs means increasing ρcu or ρN, which would in turn lead to an increase of the hyperfine coupling of the H4 protons (see below) and to a disagreement with the experimental results. Consequently, we attribute the overestimate in the calculated Dzz values to the use of the point-dipole approximation which may not be fully appropriate for an S–H distance of 2.3 Å. Another possibility is an error in the proton coordinates because of the limited resolution of the X-ray diffraction data used to determine the structure (2.4 Å in the case of N₂OR). In the case of the β-protons, the deviation in Dzz means an error of about +0.1 Å in the S–H distance.

The simulation of the orientation selective spectra gave the Aiso values of the β-protons, which are related to the H–C=S–S dihedral angle, ψ, according to

$$A_{iso} = \rho_s (B \sin^2 \phi + C)$$  \hspace{1cm} (2)

where B represents the part of Aiso that results from hyperconjugation and is orientation dependent, whereas C is a measure
and fitting of the data to eq 2 yields

equation. Figure 7A shows the dependence of the

contribution of other mechanisms such as spin polariza-
tion. Figure 7A shows the dependence of the 

protons, \( A_{iso} \) for the various CuA centers

to estimate \( \rho_B \) and \( \rho_C \). Accordingly, the unresolved

values of \( N_2 \)-OR values. In the first, we

of the CuA centers, and the line width in this region looks similar in all proteins. Therefore, we have used the average value of the two copper ions, determined from simulations of the multifrequency EPR spectra of \( N_2 \)-OR,25

isotropic part of the hyperfine interaction and 3-D structure, provided a good estimate of the spin densities. However, because the resolution, purity, and availability of the orientation selective ENDOR spectra varied among the proteins examined, we have used the copper hyperfine interaction to refine their relative spin densities. A plot of the \( A_{iso} \) value of \( \beta \)-protons versus the observed \( A_{iso}(Cu) \) or \( A_{iso}(Cu) \) of the investigated proteins shows a linear dependence with a negative slope (see Figure 8). This correlation is consistent with \( A_{iso} \) being proportional to \( \rho_s \), and \( A_{iso}(Cu) \) and \( A_{iso}(Cu) \) to \( \rho_{Cu} \), where \( \rho_s + \rho_{Cu} = \) constant. This is also in agreement with recent density functional theory (DFT) calculations.26 Furthermore, it indicates that the \( B + C \) values of all proteins are rather close. The ENDOR data of \( N_2 \)-OR were the best in terms of resolution and completeness, and, therefore, spin densities of all other proteins were scaled relative to \( \rho_{Cu}(N_2 \text{-OR}) = 25\% \), as determined from the ENDOR simulations. Next, we calculated the relative \( \rho_{Cu} \) of all proteins from the copper hyperfine interaction, where \( A_{iso}(Cu) \) values were calculated from the \( A_{iso}(Cu) \) values listed in Table 1, and taking \( A_{iso}(Cu) = 2.0 \) mT for all proteins.59 Two approaches were employed to calculate the relative \( \rho_{Cu} \). In the first, we simply scaled \( \rho_{Cu} \) according to the \( A_{iso}(Cu) \) values:

\[
\rho_{Cu}(x) = \frac{A_{iso}(Cu)(x)\rho_{Cu}(N_2 \text{-OR})}{A_{iso}(Cu)(N_2 \text{-OR})}
\]

in Figure 7A by the dotted line and the \( A \) represent the fit to M160T9 and the dashed line and the \( A \) correspond to purpAz. The dashed—dotted line represents the fit to M160QT0 (O), where the values of \( \rho_B \) and \( \rho_C \) were determined using the minimum and maximum experimental values of \( A_{iso} \), assuming angles of 0° and 90°; whereas the other two angles where determined from the fit. (B) \( \rho_S \) values of the proteins investigated calculated using the two approaches described in the text. (The error bars were estimated by taking \( A_{iso}(Cu) = 1 \) mT rather than 2 mT.) (C) The values of \( B \) and \( C \) of the various proteins.

Figure 7. (A) Plot of \( A_{iso} \) of the cysteine \( \beta \)-protons as a function of the H–C–S–S dihedral angle. The solid line was obtained by fitting the experimental \( A_{iso} \) values of \( N_2 \)-OR, denoted by ■, to eq 2. All of the rest of the solid symbols are calculated \( A_{iso} \), obtained using the \( \rho_B \) and \( \rho_C \) values of \( N_2 \)-OR and the dihedral angles obtained from the 3-D structures of COX,18 CyOA,19 M160T9,21 purpAz.20 The dotted line and the □ represent the fit of M160T9, and the dashed line and the ▼ correspond to purpAz. The dashed—dotted line represents the fit to M160QT0 (O), where the values of \( \rho_B \) and \( \rho_C \) were determined using the minimum and maximum experimental values of \( A_{iso} \), assuming angles of 0° and 90°; whereas the other two angles where determined from the fit. (B) \( \rho_S \) values of the proteins investigated calculated using the two approaches described in the text. (The error bars were estimated by taking \( A_{iso}(Cu) = 1 \) mT rather than 2 mT.) (C) The values of \( B \) and \( C \) of the various proteins.

Figure 8. The correlation between the maximum \( A_{iso} \) value of the \( \beta \)-cysteine protons, \( (A_{iso})_{max} \) and \( A_{iso}(Cu) \), and \( A_{iso}(Cu) \) for the various CuA centers investigated.

(59) The \( A_{iso}(Cu) \) splittings are usually not resolved in the EPR spectra of the CuA centers, and the line width in this region looks similar in all proteins.
the relative spin density was scaled using the expressions given for \( \alpha^2 \); the spin density in the Cu atom \(-|A_1; x^2-y^2|>-|A_2; x^2-y^2|<\) molecular orbital, as outlined by Neese,\(^{20}\) that takes into account \((A_1(Cu)-A_1(Cu))\) and \(g\)-values. In this case, we set \(2\rho_{Cu}(N_2OR) = \alpha^2(t(Cu)) = 2^{0.25}. \) The \( \rho_s \) values of each protein, obtained using the two approaches and \( \rho_S = [0.50 - (\rho_{Cu} + \rho_{N})] \) with \( \rho_{N} = 3\% \), are presented in Figure 7B. The difference between the two sets is small, both showing the same trend. This is consistent with the trend observed in simulations of the weakly coupled protons, although the actual numbers may vary. Finally, the values of the \( B \) and \( C \) parameters were calculated using the average \( \rho_{Cu} \) value for each protein, and they are displayed Figure 7C. The observed trend indicates an inverse relation between \( B \) and \( C \). When the former increases, the latter decreases.

The \( \rho_{S} \) value obtained for purpAz, 14.5—18.3\%, is smaller than that reported recently for the same protein (23\%) using S-, K-edge XAS.\(^{25}\) Recent DFT calculations on an optimized cluster with a structure similar to that of CuA in bovine COX gave \( \rho_{Cu} = 0.21, 27\% \) and \( \rho_{Cu} = 0.18, 27\% \).\(^{26}\) Other DFT calculations, based on the structure of purpAz, report for \( \rho_{Cu} = 0.23, 25\%, 20, 22\%, \) and 21, 23\% and for \( \rho_{Cu} = 0.18, 26\%, 22, 30\%, \) and 20, 30\%, depending on the calculation method.\(^{25}\) The large difference in the \( \rho_{Cu} \) values of the two copper ions is also in disagreement with the EPR spectra of the presently investigated proteins where \( ^{63,65}Cu \) hyperfine couplings of two copper ions are practically equivalent (see Table 1).

Unlike the cysteine \( \beta \)-protons, analysis of the weakly coupled protons has been limited by the overlap of a relatively large number of protons with similar hyperfine coupling characteristics. In addition, interferences from mononuclear Cu(II) protons has been limited by the overlap of a relatively large number of protons with similar hyperfine coupling characteristics. (ii) The small spin density on the directly bound nitrogens was assumed to be the same for both imidazoles and set to be 3\% in all proteins. This was justified by calculations of \( D \) that showed that the effect of \( \rho_{S} \) on the proton couplings is insignificant. (iii) The same \( \rho_{S} \) values were taken for the two thiolates and justified by the good fit of the \( A_{iso} \) values of the \( \beta \)-protons to the relation given in eq 2. (iv) Similarly, the \( \rho_{Cu} \) on both copper ions was set to be the same on the basis of the EPR spectra. (v) The spin densities estimated from the ENDOR simulations were further scaled according to the copper hyperfine interaction, which was supported by the linear correlation of the \( \beta \)-protons (\( A_{iso,max} \) and \( A_{iso}(Cu) \) (and \( A_{iso}(Cu) \)). (vi) The 3-D structure used for N2OR is that of the reduced CuA center, in which at least the CuA center of COX was found to be highly similar to that of the mixed-valence state.\(^{19}\)

In the following, we attempt to correlate the observed trends in \( \rho_{S} \) with isolated structural features in the CuA sites such as Cu—Cu, Cu—S, and the Cu—weak ligand distance, the hinge angle of the CuS2 core, or the orientation of the imidazoles. DFT calculations gave a B_{iso} orbital ground state, which is \( \sigma^* \) with respect to the Cu—Cu axis, and \( \rho_{S} \) decreases with increasing Cu—Cu distance.\(^{26}\) The difference in the Cu—Cu distance (2.51 Å (M160T0))\(^{21}\) and 2.47 Å (N2OR))\(^{20}\) is, however, too small to account for the \( \rho_{S} \) difference.\(^{26}\) A close examination of the N2OR and M160T0 structures (see Figure 1) reveals that significant differences are the larger hinge angle in N2OR and a significantly shorter Cu—N bond in M160T0. The shorter Cu—N distance should attract spin density from the S, rather than increase it. Consequently, the lower \( \rho_{S} \) in N2OR could originate from the large hinge angle of the core. The purpAz structure exhibits more differences relative to the other two. The hinge
angle is the closest to 180° (similar to M160T0), but the orientation of one of the imidazoles is significantly different. Moreover, the distances between the copper ions and the weak ligands are significantly different. One of the copper ions has two weak ligands at a distance of 3.0–3.1 Å, and the other has one weak ligand at 2.2 Å. The distances in the case of M160T0 and N2OR are 2.46, 2.62 Å and 2.47, 2.60 Å, respectively. According to Randall et al.,24 an increase in the weak ligand interaction, caused by the reduced distance, should lead to an increase in the Cu–Cu distance and to a decrease in \( \rho_S \). Hence, in purpAz there are two opposite trends, the shorter distance to E114 should decrease \( \rho_S \) and increase the Cu–Cu distance, whereas the increase in the distance to M123 should increase \( \rho_S \). The lowest \( \rho_S \) in purpAz suggests that the effect of E114 dominates although the Cu–Cu distance in purpAz, 2.42 Å, is not significantly longer than in the others. The above discussion shows that there are actually many structural features in the CuA site that can be slightly modified and used to tune the ground state. These are complex, and understanding their explicit effect on the spin density distribution requires systematic quantum chemical calculations. The detailed information we obtained on the hyperfine couplings of the \( \beta \)-protons and on some of the weakly coupled protons for a series of CuA centers sets experimental constraints that can be used for further improvement of the DFT determined ground state.

We finally discuss the trends detected in the spin densities in terms of ET properties. DeBeer-George et al.25 have calculated possible ET pathways in COX and noted the highly anisotropic redox active orbital with the sulfur bonds being the most covalent. This is highly relevant because the metal–ligand covalency is important, and the electron-transfer rate must be weighted by the appropriate ligand character in the redox active site. These authors calculated three possible pathways for the CuA to heme-a ET, and showed that when the covalency factor is taken into account, the path through one of the cysteines becomes competitive with the shorter path going through one of the imidazole ligands. Therefore, on the basis of our results, the higher covalency factor is expected for M160T9, and the lower is expected for purpAz. This, however, is only one out of several factors that determines the ET rate, and comparative experimental results which isolate the various contributions in the different studied proteins are not available. Consequently, at present this correlation cannot be checked experimentally.

The ENDOR spectra of all investigated CuA centers showed the presence of a proton with a negative Aiso interaction. There are two candidate sites for this proton. One is the amide of one of the cysteines, which is closer to the Cu2S2 core, and its orientation is conserved in all structures, as shown in Figure 1. The second is the \( \alpha \)-proton of one of the histidines, which in all structures seems to be close to the Cu2S2 core. The first option is more likely because in all proteins a weakly coupled amide nitrogen with Aiso \( \approx \) (1–1.3) MHz has been detected. In M160T9, M160QT0,44 and purpAz,60 it was clearly observed in HYSCORE spectra, whereas in N2OR it was detected in the three-pulse ESEEM spectrum.36 This nitrogen was assigned to the amide of the cysteine that is bent over the core as opposed to the second one that is oriented away from the core.44 It is, therefore, expected that the amide proton will also exhibit some isotropic coupling. The cysteine residue that is bent over the core in COX is C200.17,18 A recently proposed ET pathway calculated for bovine COX suggests that C200 takes part in the input path of the electron from cytochrome c.25 Hence the Aiso values of the amide nitrogen and proton may be significant for the ET efficiency.

The finite isotropic hyperfine interaction observed for a number of rather remote protons and nitrogens shows that the spin distribution goes far beyond the Cu2S2N2 core. Although the latter holds the majority of the spin, and, therefore, provides a good approximation for the above calculation of the spin density distribution and the B and C values, detailed theoretical calculations should eventually account for the larger distribution which may be significant in terms of the electronic properties of the site.

Conclusions

W-band pulsed ENDOR measurements provided symmetric and resolved \(^1\text{H}\) spectra of the CuA centers in N2OR, M160T9, purpAz, and the M160QT0 mutant. Simulations of orientation selective spectra yielded the Aiso values of the four strongly coupled cysteine \( \beta \)-protons. The available 3-D structures show that the H–C–S–S dihedral angles are well conserved. Consequently, the spread of the Aiso values of the cysteines’ \( \beta \)-protons of a particular CuA center provides directly \( \rho_S B \) and \( \rho_S C \). A negative linear relation was found between the maximum Aiso value of the cysteine \( \beta \)-protons and Aiso(Cu) (or Aiso(Cu)). This allowed us to refine the relative \( \rho_S \) and \( \rho_{Cu} \) values of the investigated CuA centers, as determined from simulations of the ENDOR spectra using anisotropic hyperfine parameters extracted from the available 3-D structures. Within experimental error, the ENDOR data are consistent with the \( \rho_S \) and \( \rho_{Cu} \) values for the two Cu and S in the Cu2S2 core. The largest \( \rho_S \) was found for M160T9, and the lowest was found for purpAz. The \( \rho_S \) values were further employed to determine the B and C parameters which give the relative contributions of the hyperconjugation mechanism, manifested by the size of B, to the isotropic hyperfine coupling of the cysteine \( \beta \)-protons. Analysis of the ENDOR spectra of the weakly coupled protons showed that the width of their powder patterns at \( g_\perp \) is mainly determined by the histidine H1 protons and the splitting, often appearing in the outer wings, is a measure of differences in their position with respect to the Cu2S2 core. Comparison of the spectral features of M160QT0 with those of the other proteins investigated shows that its CuA center is highly symmetric, similar to that in purpAz. Still, it has a smaller spin density on the copper and smaller H1–Cu distances. All proteins show the presence of a proton with a relatively large negative Aiso value which is assigned to an amide proton of one of the cysteines.

Acknowledgment. This work has been supported by the German-Israel Foundation for Scientific Research (D.G., I.P., P.M.H.K.). W.G.Z. acknowledges the support of “Fonds der Chemischen Industrie”. We thank Dr. Igor Gromov for performing some of the W-band measurements and Stefan Stoll (ETH) for his help regarding the use of his EasySpin toolbox for MatLab (by MathWorks, Inc.).