W-band (95 GHz) pulsed EPR and electron–nuclear double resonance (ENDOR) spectroscopic techniques were used to determine the hyperfine couplings of different protons of Cu(II)–histidine complexes in frozen solutions. The results were then used to obtain the coordination mode of the tridentate histidine molecule and to serve as a reference for Cu(II)–histidine complexation in other, more complex systems. Cu(II) complexes with L-histidine and DL-histidine-α,β,β-δ were prepared in H2O and in D2O, and orientation-selective W-band 1H and 2H pulsed ENDOR spectra of these complexes were recorded at 4.5 K. These measurements lead to the unambiguous assignment of the signals of the Hα, Hβ, imidazole Hε, and the exchangeable amino, Hام, protons. The 14N superhyperfine splitting observed in the X-band EPR spectrum and the presence of only one type of Hα and Hβ protons in the W-band ENDOR spectra show that the complex is a symmetric bis complex. Its gD is along the molecular symmetry axis, perpendicular to the equatorial plane that consists of four coordinated nitrogens in histamine-like coordinations (NNNN). Simulations of orientation-selective ENDOR spectra provided the principal components of the protons’ hyperfine interaction and the orientation of their principal axes with respect to gD.

From the anisotropic part of the hyperfine interaction of Hα and Hβ and applying the point-dipole approximation, a structural model was derived. An unexpectedly large isotropic hyperfine coupling, 10.9 MHz, was found for Hα. In contrast, Hα of the Cu(II)−1-methyl-histidine complex, where only the amino nitrogen is coordinated, showed a much smaller coupling. Thus, the hyperfine coupling of Hα can serve as a signature for a histamine coordination where both the amino and imino nitrogens of the same molecule bind to the Cu(II), forming a six-membered chelating ring. Unlike Hα the hyperfine coupling of Hβ is not as sensitive to the presence of a coordinated amino nitrogen of the same histidine molecule.

Introduction

Copper–histidine coordination is highly abundant in biological systems. Histidine residues serve as ligands in most of the copper enzymes and proteins and play a role in the process of copper transport in biological systems.1 Recently, copper–histidine complexes were also identified as physiologically significant medicines in the treatment of Menkes and Wilson diseases.2 Inspired by the catalytic activity of type 2 Cu(II) centers in copper oxidases,3 Cu(II)–histidine complexes were encapsulated in zeolite Y and were found to catalyze the oxidation of alcohols, alkanes, and alkenes at mild temperatures.4 There are three potential coordination sites in the histidine molecule: the amino (Nα) nitrogen, the imidazole imido (Nε or Nβ) nitrogen, and the carboxylate oxygen. The mode of coordination, however, strongly depends on the solution pH, the composition, and the complex environment. This variability has led to numerous studies devoted to the determination of the structure of Cu(II)–histidine complexes in a variety of systems.5–9 In proteins the situation is simpler because the amino and the carboxylate groups are unavailable as they are involved in the creation of the peptide bond, thus leaving only one of the imidazole nitrogens (Nε or Nβ) as a potential coordination site.

Because of the importance of Cu(II)–histidine based complexes, the coordination geometry of the Cu(II) has been investigated in variety of model systems, such as doped crystals, frozen solutions, and zeolites, by X-ray diffraction, NMR, EPR, and diffuse reflectance spectroscopies.4,10,11 For example, the crystal structure of Cu(II) doped in L-histidine·HCl·H2O reveals that the Cu(II) ion is coordinated to amine and imino nitrogens, and a carboxylate oxygen from three different histidine molecules, along with two chloride ions.7 This structure is different from that of Cu(II) doped in a Cd(histidinate) complex in the solid state12 where it was proposed that the Cu(II) coordinates to amine and imino nitrogens, and a carboxylate oxygen of one histidine molecule, and to a carboxyl oxygen of a neighboring histidine molecule. In aqueous solutions, Cu(II)–histidine complexes assume various structures depending on the pH value.

and both mono- and bis-histidine complexes were identified.5,13–15
Nevertheless, studies based on different spectroscopic techniques vary in their conclusions with respect to the prevailing structure even under the same experimental conditions. For example, at physiological pH (≈7.3), Romanelli and Basosi16 proposed from electron-spin echo modulation (ESEEM) studies that the Cu-(II)—histidine complex has two histamine-like coordinations in the equatorial plane and two solvent molecules at the axial positions. In contrast, NMR studies of this complex indicated a mixture of two complexes: one with two histamine-like coordinations and another with one histamine and one glycine-like coordinations.11 Similarly, Goodman et al. and Basosi et al. support the presence of mixed complexes in the solutions but with different ratios.6,14,15

In principle, EPR measurements combined with electron nuclear double resonance (ENDOR) experiments can provide detailed information concerning the first, second, and even the third coordination sphere of the Cu(II)—histidine complex by following the 14N and 1H signals. The complete assignment of the 1H signals of Cu(II)—histidine in aqueous solution has not been reported so far due to the problem of overlapping 14N and 1H signals at X-band frequencies.17 With the advent of high-frequency/high-field ENDOR spectroscopy these problems are overcome because the signals of different types of nuclei are well separated because of their larger nuclear Zeeman energy.18–21 In this work we present an orientation-selective W-band (94.9 GHz) pulsed ENDOR investigation aimed at the assignment of the 1H ENDOR signals and the determination of the structure of the Cu(II)—bis-histidine complex at pH = 7.3. For this purpose, in addition to normal histidine, a histidine deuterated at the α and β positions and a histidine methylated at the Nα position were used. In addition, the complexes were prepared in H2O and D2O solutions to identify the exchangeable protons, and both 1H and 2H ENDOR spectra were recorded. This allowed the unambiguous assignment of the various ENDOR signals to specific protons in the histidine molecule. By measurement of orientation-selective ENDOR spectra, which were further analyzed by computer simulations, it was also possible to determine the magnitude and orientation of the 1H hyperfine interaction. For some of the protons these were further interpreted in terms of the orientation of the 1H–Cu(II) vector with respect to the molecular symmetry axis, providing the conformation of the histidine ligands in the complex. These results can serve as a basis for future studies requiring the structure of Cu(II)—histidine complexes in other systems such as complexes encapsulated in zeolite cages.22,23

Experimental Section

Sample Preparation. The Cu(II)—histidine complexes were prepared according to reported procedures. A typical preparation procedure involves mixing, in a molar ratio of 1:5, anhydrous CuCl2 and l-histidine·HCl·H2O (Merck) or dL-histidine·α-doped (CDN Isotopes) or 1-methyl-l-histidine (ICN Biochemicals Inc.) in H2O or D2O (Cambridge Isotope Laboratories, Inc.). The solution was stirred for an hour at room temperature. The pH of the solution was adjusted to 7.3 using 0.1 N NaOH/NaOD (ISOTEC, Inc.) and/or dilute H2SO4/D3SO4 (Sigma Co.) solutions. Glycerol or glycerol-d1 (Cambridge Isotope Laboratories, Inc.) were used to produce a good glass upon freezing. The [Cu(imidazole)2]Cl2 complex was prepared as reported earlier.23 The concentrations of all the Cu(II) complexes were 2 mM. All the chemicals were used without further purifications.

Spectroscopic Measurements. Continuous wave (CW) X-band EPR spectra were measured at 9.16 GHz and 150 K, using a Varian E-12 EPR spectrometer. 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.26 Field-sweep (FS) echo-detected (ED) EPR spectra were recorded using the two-pulse echo sequence (τ/2, −τ, −τ, −τ−echo) where the echo intensity was registered as a function of the magnetic field. Typically, microwave (MW) pulse lengths (τMW) of 0.05 and 0.1 MS were used with τ = 0.3 ms. The magnetic field values were calibrated using the Larmor frequency of the protons, νp, as determined by the ENDOR measurements. The 1H ENDOR spectra were measured using the Davies ENDOR pulse sequence (τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−τ−tau
**Results**

**Assignment of ENDOR Signals.** Schematic representations of the histidine-based ligands used in this work and possible molecular structures of their Cu(II) complexes are given in Figure 1. The nomenclature used in this work for the various molecular structures of their Cu(II) complexes are given in Table 1. The spectra of the Cu(II) complexes exhibit a powder pattern characteristic of an axial tensor where the 63,65-Cu hyperfine splitting is unresolved. The g values obtained are listed in Table 1. The 1H ENDOR spectrum of Cu–His in H2O, recorded at the g⊥ position, is shown in Figure 3a. The spectrum is rather complicated and consists of contributions from a number of protons. Assuming that in the Cu–His complexes the Cu(II) coordinates to two symmetrically situated histidine ligands with a histamine-like coordination, the ENDOR spectrum should consist of the following contributions from each histidine: (i) three imidazole protons (Hα, Hβ, and Hε), (ii) one proton at the Cβ position (Hγ), (iii) two protons on Cγ (Hδ), and (iv) two exchangeable protons on the amino nitrogen, Hαam. In addition, water protons (H2O), from weak axial coordination, cannot be excluded. Hence, the assignment of the signals to the various protons is not straightforward. The signals of the exchangeable protons on the amino nitrogen, Hαam, can be identified by comparing the spectra of Cu–His in H2O and D2O solutions. The W-band FS-ED EPR spectra of these complexes are depicted in Figure 2b. All spectra exhibit a powder pattern characteristic of an axial tensor where the 63,65-Cu hyperfine splitting is unresolved. The g values obtained are listed in Table 1.

The 1H ENDOR spectrum of Cu–His in H2O, recorded at the g⊥ position, is shown in Figure 3a. The spectrum is rather complicated and consists of contributions from a number of protons. Assuming that in the Cu–His complexes the Cu(II) coordinates to two symmetrically situated histidine ligands with a histamine-like coordination, the ENDOR spectrum should consist of the following contributions from each histidine: (i) three imidazole protons (Hα, Hβ, and Hε), (ii) one proton at the Cβ position (Hγ), (iii) two protons on Cγ (Hδ), and (iv) two exchangeable protons on the amino nitrogen, Hαam. In addition, water protons (H2O), from weak axial coordination, cannot be excluded. Hence, the assignment of the signals to the various protons is not straightforward. The signals of the exchangeable protons can be identified by comparing the spectra of Cu–His prepared in H2O and D2O solutions. The 3H and 1H ENDOR spectra of Cu–His in D2O (traces b and c of Figure 3) consist of signals of only exchangeable and nonexchangeable protons, respectively. The broad signals appearing at about ±3.4(0.1) and ±6.5(0.2) MHz in the 1H spectrum are missing in the 3H ENDOR spectrum and are therefore assigned to Hαam. The number in brackets represents the uncertainty of the measurement. The signals at ±2.2(0.1) MHz and ±1.1(0.05) MHz in the 3H ENDOR spectrum (Figure 3b) are assigned to Hε because of methylation. The W-band FS-ED EPR spectra of these complexes are depicted in Figure 2b. All spectra exhibit a powder pattern characteristic of an axial tensor where the 63,65-Cu hyperfine splitting is unresolved. The g values obtained are listed in Table 1.

![Figure 1](image1.png)

**Figure 1.** Schematic representations of (a) histidine, (b) histidine-α-d3β-d7, (c) 1-methyl-histidine, (d) Cu(II)–histidine complex with NNNN coordination, (e) Cu(II)–histidine complex with NNNO coordination and a seven-membered chelating ring, (f) Cu(II)–1-methyl-histidine complex with NNNO coordination and a five-membered chelating ring, (g) Cu(II)–1-methyl-histidine complex with NNOO coordination. Solvent molecules are not drawn in the figure.

**Table 1.** EPR Parameters of the Cu(II)–Histidine Complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>g0</th>
<th>g⊥</th>
<th>A63Cu</th>
<th>A14N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu–His in D2O</td>
<td>2.24</td>
<td>2.058</td>
<td>17.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Cu–His–d5 in D2O</td>
<td>2.24</td>
<td>2.058</td>
<td>17.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Cu–MeHis in D2O</td>
<td>2.23</td>
<td>2.063</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Cu–Imid in H2O</td>
<td>2.26</td>
<td>2.062</td>
<td>18.3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* g0 was determined from the CW X-band EPR spectra at 150 K, whereas g⊥ was obtained from W-band FS-ED EPR at 4.5 K.
Figure 3. ENDOR spectra of Cu(II)–histidine complexes measured at the $g_\perp$ position (4.5 K): (a) $^1$H ENDOR spectrum of the Cu–His complex in H$_2$O; (b) $^2$H ENDOR and (c) $^3$H ENDOR spectra of the Cu–His complex in D$_2$O; (d) $^1$H ENDOR and (e) $^3$H ENDOR spectra of Cu–His-d$_3$ complex in H$_2$O; (f) $^1$H ENDOR spectrum of the Cu–His-d$_3$ complex in D$_2$O. The $^3$H ENDOR spectra are plotted in the $^1$H frequency scale.

Table 2. Assignment of the ENDOR Signals of the Various Protons in the Cu(II)–Histidine Complexes As Measured from Cu–l-Histidine and Cu–dL-Histidine.α,β,δ Complexes in H$_2$O/Glycerol and D$_2$O/Glycerol-d$_6$ Measured near the $g_\perp$ Position

<table>
<thead>
<tr>
<th>Proton position of signals, MHz</th>
<th>Proton position of signals, MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_a$</td>
<td>$H_\perp$</td>
</tr>
<tr>
<td>$H_b$</td>
<td>$H_\parallel$</td>
</tr>
<tr>
<td>$H_c$</td>
<td>$H_\parallel$</td>
</tr>
</tbody>
</table>

$^a$ The number in parentheses indicate the uncertainty.

To evaluate the sensitivity of the hyperfine coupling constants of various protons of histidine to the coordination mode of the Cu(II), the Cu–MeHis complex was prepared in H$_2$O and D$_2$O. Because of the methylation at the N$_e$ position, the imidazole nitrogen is no longer a coordination site in this complex. As a result, a complex with two MeHis ligands coordinated in a glycine-like mode at the equatorial positions, or with four MeHis coordinated via amine nitrogens, is expected. The $g$ and $A(Cu)$ values of this complex are very close to that of Cu–His (see Table 1), thus favoring the four-amine coordination geometry. The $^1$H and $^2$H ENDOR spectra measured at the $g_\perp$ position are shown in Figure 4. The absence of the strong signals at $\pm 5.2(0.2)$ MHz assigned earlier to H$_e$, H$_d$, and most probably the features giving the larger doublets are due to H$_c$. The signals of these two protons do not exceed $\pm 3.7$ MHz, and most probably the features giving the larger doublets are due to H$_b$.

Orientation-Selective ENDOR Measurements. Having identified the signals of the various protons/deuterons, we proceeded with orientation-selective experiments designed to provide the principal components of the hyperfine interaction and the relative orientation of its principal axes with respect to the direction of $g_\perp$. These, in turn, yield the conformation of the ligands in the metal complex. We started with the $^1$H ENDOR signals of H$_a$ in the spectra of Cu–His in D$_2$O, shown in Figure 5a, where they are well-resolved. As the magnetic field approaches $g_\parallel$, the spectra become more and more asymmetric. This is not due to instrumental problems or baseline drift but originates from partial saturation of the NMR transi-
The line shape and field dependence of the $H_R$ signals were simulated, as shown in Figure 5b, and the simulation parameters are listed in Table 3. The integration range used for $\theta_0$ was $0^{\circ}$ to $180^{\circ}$ for all spectra and that for $\theta_0$ is listed in the figure. $H_R$ exhibits an axially symmetric hyperfine interaction ($A_\perp = 9.6$ MHz and $A_\parallel = 13.5$ MHz) with a relatively large isotropic hyperfine coupling and a small anisotropic component. The latter corresponds to a Cu-H$_R$ distance of 4.0 Å assuming that the point-dipole approximation is valid. The simulations also yield $\beta = 60^{\circ}$, where $\beta$ is the angle between the direction of the major principal axis of the hyperfine direction and the $g_\parallel$ axis.

The hyperfine parameters of H$_am$ were obtained from orientation-selective 2H ENDOR spectra of Cu-His in D$_2$O (Figure 6) and 1H ENDOR spectra of Cu-His-d$_3$ in H$_2$O (Figure 7a). The 2H ENDOR spectra consist primarily of H$_am$ signals, which exhibit large hyperfine couplings because the amine protons are close to the Cu(II) (see Discussion), and of the imino proton of the imidazole ring (H$_{im}$), which is expected to have a considerably smaller hyperfine coupling. In addition, the presence of weakly coupled protons from an axial water ligand cannot be excluded at this point. Accordingly, the broad but intense 2H ENDOR signals appearing at $\pm 3.4(0.1)$ and $\pm 6.5(0.2)$ MHz in the spectrum recorded at $g_\perp$ (3.295 T) (Figure 6) are assigned to H$_am$. These 2H ENDOR singularities merge as the magnetic field decreases and then spread apart again at $g_\parallel$. While the 2H ENDOR signals allowed us to identify the H$_am$ singularities and their field dependence, their spectral resolution is relatively poor because of quadrupolar splitting. A better resolution of the H$_am$ signals is obtained from the 1H ENDOR spectra of Cu-His-d$_3$ in H$_2$O as shown in Figure 7a. The spectral features observed could not be reproduced by a single nucleus or two different

Table 3. Principal Components of the Hyperfine Tensor and the Euler Angles Relating Its Orientation to the $g$ Tensor As Obtained from the Simulation of the Orientation-Selective ENDOR Spectra

<table>
<thead>
<tr>
<th>protons</th>
<th>$A_{xx}$ MHz</th>
<th>$A_{yy}$ MHz</th>
<th>$A_{zz}$ MHz</th>
<th>$\alpha$ deg</th>
<th>$\beta$ deg</th>
<th>$\gamma$ deg</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_R$</td>
<td>$\pm 9.6$</td>
<td>$\pm 9.6$</td>
<td>$\pm 13.5$</td>
<td>0</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>H$_R$</td>
<td>$\mp 1.0$</td>
<td>$\mp 2.4$</td>
<td>$\pm 5.4$</td>
<td>0</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>H$_{im}$</td>
<td>$\mp 6.0$</td>
<td>$\pm 7.0$</td>
<td>$\pm 14.0$</td>
<td>0</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>H$_{im}$</td>
<td>$\mp 6.0$</td>
<td>$\pm 10.0$</td>
<td>$\pm 14.0$</td>
<td>0</td>
<td>84</td>
<td>90</td>
</tr>
</tbody>
</table>

nuclei with axially symmetric tensors. Hence, the $^1$H ENDOR signals of H$_{am}$ were simulated using two nuclei with different nonaxial hyperfine interactions. The simulated spectra of H$_{am}$ are given in Figure 7b. The hyperfine coupling parameters and the Euler angles used for the simulation are listed in Table 3.

The $^1$H ENDOR spectra of Cu—His$_{d}$ in D$_2$O is relatively simple and consist of the signals of imidazole protons (H$_{am}$, H$_{e}$) only. Orientation-dependent $^1$H ENDOR spectra are presented in Figure 8a. The quality of the spectra recorded at fields close to $g_0$ fields is poor because of low S/N and the large asymmetry; nevertheless, it was possible to simulate the H$_{e}$ features in the range 3.3036—3.1775 T as shown in Figure 8b. The spectra were simulated with a nonaxial hyperfine tensor as listed in Table 3.

Finally, for the sake of reference and completeness, orientation-selective $^1$H ENDOR measurements were also carried out on Cu—Imid in H$_2$O, and ENDOR spectra at a few selected magnetic fields are given in Figure 9. X-band ENDOR measurements of this system, showing $^{15}$N and $^1$H signals, were reported. In addition X-band $^1$H ENDOR spectra recorded at $g_1$ and $g_2$ positions were simulated by using the crystal structure parameters. Here we present the $^1$H W-band ENDOR spectra of Cu—Imid in H$_2$O to show that in the histidine complex the hyperfine coupling of the imidazole proton, H$_e$, is considerably less susceptible than H$_{am}$ to the coordination mode.

**Discussion**

The resolved $^{14}$N superhyperfine splittings at the $m_I = 3/2$ $^{63,65}$Cu hyperfine component of the CW X-band EPR spectra show that the Cu(II)—histidine complex in a frozen aqueous solution (pH = 7.3) must be a bis complex. Further details about the specific coordination mode and conformation of the ligands were obtained from orientation-selective W-band $^1$H and $^2$H ENDOR measurements. In these measurements we concentrated on the ENDOR signals of three types of protons: H$_{am}$, H$_e$, and H$_n$. The first provides evidence for the coordination of the amino nitrogen, N$_{am}$, whereas the second reports on the imino nitrogen, N$_{imid}$—coordination. The most significant and useful is the hyperfine coupling of H$_n$, which was found to be highly sensitive to the coordination mode of the histidine. When both N$_{am}$ and N$_{imid}$ of a particular histidine molecule are coordinated to the Cu(II) ion (histamine coordination), forming a six-membered chelating ring, H$_n$ assumes a large isotropic hyperfine coupling, 10.9 MHz. In contrast, when the coordination is only through one nitrogen, N$_{am}$, its isotropic hyperfine coupling is significantly smaller (<3 MHz). Accordingly, the hyperfine coupling of H$_n$ can be used as a signature for the coordination mode of Cu(II)—histidine complexes. H$_e$, on the other hand, is rather insensitive to these structural changes. Only one type of H$_n$ signal was identified in the ENDOR spectra of all the Cu—His complexes investigated (d,L natural abundance and deuterated in the $\alpha$ and $\beta$ positions). Therefore, we conclude that the complex present in frozen aqueous solutions at pH = 7.3, prepared with excess histidine, is Cu(II)(histidine)$_2$, where N$_{am}$ and N$_{imid}$ of both histidines are coordinated at the equatorial positions, forming two chelating rings, thus confirming the NNNN equatorial coordination.

Through computer simulations of the orientation-selective ENDOR signals of H$_{am}$, H$_e$, and H$_n$, the principal components of the hyperfine tensor, and the relative orientation of its $z''$ axis with respect to the $g_0$ direction, $z'$, given by $\beta$, was determined. In principle, if $z'$ and $z''$ can be associated with easily identified geometrical characteristics of the molecule, then $\beta$ provides straightforward conformational information. Since we did not detect different ENDOR signals corresponding to the same type of proton in the two histidine ligands, we conclude that the complex is symmetric and the direction of $g_0$ is along the molecular symmetry axis normal to the equatorial plane. The anisotropic hyperfine interaction of H$_n$ is axially symmetric and relatively small. It is therefore reasonable to assume that it can be described by the point-dipole approximation where $z''$ is along the Cu—H$_n$ direction. Consequently, we obtained from the anisotropic hyperfine value a distance of 4.0 Å with $\beta =$
and cannot be readily calculated. Table 4 lists the values of Cu–
resulted in avoid any other axial coordination. The H
(31) Cerius2

A computer model of the complex has been constructed as
follows: two histidine molecules (D configuration) were placed
in the equatorial positions, and two water molecules were fixed
at a rather long Cu–O distance (2.2 Å) in the axial positions
to avoid any other axial coordination. The Hα protons were fixed
at 4.0 Å with β = 60° with respect to the normal to the NNNN
coordinated plane, along the Cu–O (water) axis. The position
and geometry of the rest of the molecules were optimized freely
using the software Cerius2 of MSI.31 Thus, the Cu(II) is in an
elongated octahedral environment with the four coordinating
nitrogens of two histidines in the equatorial plane as shown in
Figure 10.

When the point-dipole approximation applies, the correlation
between the anisotropic hyperfine and the geometrical param-
eters of the molecules is straightforward and the structural
information is easily extracted. This is not the case when this
approximation is not valid, and one has to take into account
the spin density on nuclei other than the Cu(II). The ENDOR
simulations showed that the hyperfine interaction of Hα and Hαm
protons is nonaxial, indicating that the point-dipole approxima-
tion is not appropriate and that therefore the Cu–H distance
cannot be readily calculated. Table 4 lists the values of Cu–H
and β for these protons as obtained from the model complex
discussed above (Figure 10). These distances would have resulted in $a_\perp$ values of 2.4(0.1), 4.5(0.1), and 5.2(0.4) MHz
for Hα, Hαm(1), and Hαm(2), respectively. The experimental $a_\perp$

<table>
<thead>
<tr>
<th>proton</th>
<th>Cu–H, Å</th>
<th>β, deg</th>
<th>$a_\perp$, MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hα</td>
<td>4.0</td>
<td>60</td>
<td>1.3</td>
</tr>
<tr>
<td>Hαm</td>
<td>3.26 (0.02)</td>
<td>66 (8)</td>
<td>2.4 (0.1)</td>
</tr>
<tr>
<td>Hαm</td>
<td>2.64 (0.02)</td>
<td>85 (5)</td>
<td>4.5 (0.1)</td>
</tr>
<tr>
<td>Hαm</td>
<td>2.51 (0.05)</td>
<td>77 (7)</td>
<td>5.2 (0.4)</td>
</tr>
</tbody>
</table>

*Geometrical parameters for the two histidine ligands were slightly different, and the listed values are the average. The difference is noted in parentheses. †Calculated from the Cu–H distance using the point-dipole approximation. Positions of this proton have been fixed according to the experimental results.

value, 2.37 MHz, of Hα (calculated from $\frac{1}{6}[2A_{zz} – (A_{xy} + A_{x\alpha})]$) compares reasonably with the geometry of the model. Consequently, we can assume that the direction of $z''$ is approximately along Cu–H, and that β can be used to obtain information regarding the conformation. Indeed the β value obtained from the model is 66°, close to the experimental value of 70°. The values calculated for Hαm(1) and Hαm(2) deviate significantly from the experimental values because the point-dipole approximation is far from being valid for these protons.

Conclusions

W-band 1H and 2H ENDOR spectra of the Cu(II) complexes with L-histidine, DL-histidine-α-d,β-d, and 1-methyl-histidine in frozen solutions of H2O and D2O (pH = 7.3) enabled us to assign the ENDOR signals of the different protons in the Cu(II)–histidine complex. This information was then used to determine the coordination mode of the complex, which was found to consist of two histidine ligands symmetrically placed in the equatorial positions with NNNN coordination. Orientation-selective ENDOR measurements and their computer simulations gave the magnitude of the principal components of the hyperfine interaction and its orientation with respect to the g tensor principal axis system. This provided information regarding the conformation of the histidine ligands. The hyperfine coupling of Hα is highly sensitive to the coordination mode. It has a large isotropic hyperfine coupling when both nitrogens of one histidine molecule are coordinated, generating a six-

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