Cu(II) Coordination in Arthropod and Mollusk Green Half-Methemocyanins Analyzed by Electron Spin−Echo Envelope Modulation Spectroscopy†

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ABSTRACT: Hemocyanin (Hc) is a dinuclear copper protein that binds oxygen reversibly. The structure of the Cu(II) site in a derivative of hemocyanin known as green half-met (GHM) has been analyzed using the pulsed EPR technique of electron spin−echo envelope modulation (ESEEM) spectroscopy. The derivative, prepared by treating the native protein with nitrite at low pH, contains a mixed-valent binuclear copper center. It was shown through chemical assays and the ligand exchange reaction products identified by EPR spectroscopy to contain a nitrite ligand bound to Cu(II). The ESEEM spectra of green half-methemocyanins from mollusks and arthropods indicated that three imidazole ligands are coordinated to Cu(II). Therefore, a tetragonal N_3O ligand structure (O is an oxygen of nitrite) is proposed. For GHM Hc from the mollusks *Octopus vulgaris* and *Rapana thomasiana*, the isotropic nitrogen nuclear hyperfine coupling constant, a_{iso}, for the Nδ (or remote) nitrogen of two imidazoles was approximately 1.4 MHz, while for the third, $a_{iso} \simeq 2.2$ MHz. The difference between the two weaker nitrogens and the single, more strongly coupled nitrogen was smaller by 0.2 MHz in the GHM Hcs from the arthropods *Carcinus maenas*, *Homarus americanus* and *Panulirus interruptus*. The nitrogen nuclear quadrupole coupling constants and asymmetry parameters, e^2Qq and η, for the Nδ nitrogens in nearly all cases were near 1.4 MHz and 0.8, respectively, although *Rapana thomasiana* GHM Hc exhibited a reduction in η that may indicate weaker hydrogen bonding in the active site of this protein. The g and A_{Cu} (copper nuclear hyperfine coupling) values for the derivatives, and the finding of three similar nuclear hyperfine coupling constants for the Nδ sites of imidazole ligands, when considered with the orientation-specific information obtained using angle-selection methods for simulation of ESEEM spectra, suggest a distorted tetragonal Cu(II) structure in which three imidazoles and a nitrite ligand are bound near the equatorial plane. The finding that the two molluscan GHM Hcs exhibit differences associated with the remote nitrogen of imidazoles bound to Cu(II) may be related to a structural variability in the active sites of these proteins not found in the arthropodan GHM Hcs examined.

In hemocyanin, reversible oxygen binding occurs at a binuclear copper site (Ellerton et al., 1983). A model for the active site in oxyHc[1] has recently been presented for subunit II of *Limulus polyphemus* Hc (Magnus et al., 1993), while structures of deoxyHcs have been reported for both *L. polyphemus* (Hazes et al., 1993) and *Panulirus interruptus* Hcs (Gaykema et al., 1984; Volbeda & Hol, 1989a). The crystallographic studies of these arthropodan Hcs show that three imidazole ligands are bound to each copper atom in both the oxy and deoxy forms of the protein. The geometry of copper in deoxyHc is distorted trigonal (Hazes et al., 1993; Gaykema et al., 1984; Volbeda & Hol, 1989a); in the oxy form, the ligand (chemically equivalent to peroxide dianion) (Van Holde, 1967; Loehr et al., 1974; Freedman et al., 1976) binds to copper in a plane containing two imidazoles, with a third imidazole bound to copper in a longer "axial" bond (Magnus et al., 1993), giving a square pyramidal geometry.

In addition to the oxygen binding property common to the binuclear sites of Hcs and the analogous protein tyrosinase (Robb, 1984), a variety of enzymatic activities has been documented for these proteins, including catalase activity, which is efficient in molluscan Hcs but absent from the arthropodan Hcs (Ghiretti, 1956; Santamaria et al., 1990), phenolase and catecholase activity in arthropodan and molluscan Hcs (Santamaria et al., 1990) and tyrosinase (Robb, 1984), and monooxygenase activity carried out by tyrosinase (Lerch, 1981) and molluscan Hcs (Santamaria et al., 1990). The origin of the specific structural and electronic changes that modify the chemistry of binuclear centers in these different proteins has not yet been described in detail, although accessibility to the active site may be one important contributing factor (Himmelwright et al., 1980).

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[1] Abbreviations: cw, continuous wave; ESEEM, electron spin−echo envelope modulation; GHM, green half-met; Hc, hemocyanin; LEFE, linear electric field effect; nqi, nuclear quadrupole interaction; PAS, principal axis system; tbp, trigonal bipyramidal; TEPA, tris[2-(2-pyridyl)ethyl]amine; SOD, superoxide dismutase.

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Since there are few X-ray crystallographic structural models of Hcs or other multinuclear copper proteins [see Messerschmidt et al. (1992) for ascorbate oxidase], approaches to understanding the structure and function of binuclear copper sites remain in the realm of spectroscopic investigation. Oxidized and metal-substituted Hc derivatives have often been used to allow the application of EPR techniques to studies of the metal site structure. One such derivative is known as green half-met (GHM) or half-met nitrite Hc (Himmelwright et al., 1979). According to most reports, this derivative, prepared in a variety of ways (using sodium nitrite or NO, for example), contains a binuclear Cu(I)−Cu(II) center to which an exogenous nitrogen oxide ligand is bound (Schoot Uiterkamp, 1972, 1974; Van der Deen & Hoving, 1977; Himmelwright et al., 1979; Verplaetse et al., 1979; Tahon et al., 1990). The copper center is formulated as half-oxidized because the EPR signal intensity of GHM Hc corresponds to 1 Cu(II)/mol binuclear site (Schoot Uiterkamp & Mason, 1973; Van der Deen & Hoving, 1977; Salvato et al., 1989). The relationship between the structure of this center and the structure of oxy- and deoxyHc active sites, however, has not been adequately addressed.

In a previous report from these laboratories, it was concluded that the copper center in GHM Hc from *Octopus vulgaris* does not contain an exogenous nitrogen oxide ligand derived from the preparative reaction (Salvato et al., 1989). This conclusion was based on the low yield of chromophore found using a Griess reagent assay sensitive to nitrite (Vogel, 1961). According to another report, nitrite was said to be bound to the binuclear copper center in *Busycon canaliculatum* GHM Hc on the basis of ligand exchange reactions (Westmoreland et al., 1989). The EPR signal characteristic of Cu(II) in GHM Hc could be produced by adding nitrite to an aquo half-metHc prepared in a series of dialyses, whereby the nitrite assumed to be originally bound was sequentially replaced by azide, acetate, and finally water ligands. Other authors reporting the results of a nitrite assay for GHM Hc from the mollusk *Helix pomatia* found a stoichiometry of 0.65 nitrite/mol of protein (Tahon et al., 1990). These results prompted the present reevaluation of the stoichiometry of nitrite tightly bound in *O. vulgaris* GHM Hc using new assay procedures. Here, nitrite stoichiometries of around 0.7 equiv/mol of cupric copper are found. Some of the details concerning potential sources of interference in the assays are presented.

In a study reporting the ESEEM spectrum of *O. vulgaris* GHM Hc, it was again suggested either that nitrite was not bound to cupric copper or that it was bound in such a way that the interaction between the nitrogen of nitrite and the unpaired electron of copper was too weak to be detected, as might be expected for axial NO_2^- in a tetragonal Cu(II) complex (Jiang et al., 1993a). We have now extended the ESEEM experiments to address the assignment of nitrogen nuclear hyperfine and quadrupole coupling parameters for imidazole ligands to Cu(II) and to enumerate these ligands in several GHM Hcs. The proteins used here include two from mollusks (*O. vulgaris* and *Rapana thomasiana*) and three from arthropods (*Carcinus maenas*, *Homarus americanus*, and *P. interruptus*), with results in all cases consistent with three imidazole ligands bound to cupric copper. The finding that coupling parameters for several arthropodan Hcs were nearly identical while those for two molluscan proteins exhibited significant alterations in their respective coupling characteristics may be related to structural variability in molluscan Hc active sites.

MATERIALS AND METHODS

O. vulgaris hemocyanin was purified by ultracentrifugation according to Salvato et al. (1979). Its concentration and that of the GHM derivative were calculated using $\epsilon_{280} = 70.5$ mM^{-1} cm^{-1} (Salvato et al., 1979). *C. maenas* Hc was purified according to a published procedure (Bubacco et al., 1992), and for this protein $\epsilon_{280} = 93$ mM^{-1} cm^{-1} (Tamburro et al., 1977). *R. thomasiana* Hc was collected by bleeding live animals and was purified according to the procedure used for *O. vulgaris* Hc. *H. americanus* Hc was collected by syringe puncture of the dorsal lacuna of live animals and was purified following the procedure used for *O. vulgaris* Hc. *P. interruptus* Hc, obtained from live animals, was purchased from Pacific Biomarine Laboratories, Inc. (Venice, CA). The hemolymph was dialyzed overnight against 10 mM Ca^{2+} in Tris-HCl buffer (pH 7.5) and then against 20 mM phosphate buffer (pH 7.5). The protein solution was brown in color due to contamination by an orange-pigmented material, which could be partially removed by precipitation in 25% saturated ammonium sulfate. The remaining contaminant was removed by DEAE chromatography in 20 mM phosphate buffer containing 20 mM NaCl (pH 6). Hc was eluted using 200 mM NaCl in the same buffer.

The green half-met derivatives were prepared by dialysis of 1 mM native Hc for ≈18 h against 200 vol of 5 mM sodium nitrite plus 5 mM ascorbic acid in 20 mM phosphate buffer (pH 5.5), followed by dialysis against three changes (≈200 vol) of 20 mM phosphate buffer (pH 7.0). This method, previously shown to give complete conversion of *O. vulgaris* Hc to GHM Hc (1 mol of Cu(II)/mol binuclear site) (Salvato et al., 1989), functioned similarly for all Hcs used.

Quantitation of Cu(II) was achieved by the double integration of EPR signal intensity, using computer programs provided by the ESR Center of the University of Illinois (Morse, 1987). The Cu(II) released from acidified Hc itself was used as a standard: for example, the addition of 1 vol of 4% H_3PO_4 to a sample of GHM Hc released an amount of Cu(II) that gave a signal intensity (corrected for dilution) approximately double that observed before acidification.

The green half-met derivative was converted to a purple, cyanide form either by dialysis overnight against 10 mM CN^- in phosphate buffer (pH 8.0) followed by dialysis against phosphate buffer alone (pH 7.0) or by using $^{13}CN^-$ or $C^{15}N^-$ by the direct addition of phosphate-buffered potassium cyanide to give 1 or 6 mM (excess) CN^-.

Spectroscopy. Continuous wave EPR spectra were recorded at 77 K using a Varian E-112 spectrometer equipped with a Systron-Donner frequency counter and a Varian NMR Gaussmeter. They were simulated using QPOWA programs (Belford & Nilges, 1979; Nilges, 1979; Maurice, 1981) running on a MicroVAX II computer. The 35 GHz spectra of *O. vulgaris* and *C. maenas* GHM Hcs, kindly provided by Dr. N. Dennis Chasteen (University of New Hampshire), showed resolution of g_y and g_x features not achieved at X-band (not shown).

X-band ESEEM spectra were recorded at 1.4 K on an instrument previously described (McCracken et al., 1987), using either a strip-line transmission cavity (Mims, 1974)

or a folded strip-line cavity that accommodates standard EPR tubes (Britt & Klein, 1987). Three-pulse (stimulated echo) envelope modulation data were recorded as previously described (Peisach et al., 1979; Mims & Peisach, 1979) and were Fourier cosine transformed (Shimizu et al., 1979) to give the spectra presented here, after a dead-time reconstruction procedure was applied (Mims, 1984). Simulation of spectra was accomplished according to methods previously described (Cornelius et al., 1990; Magliozzo & Peisach, 1992), using an angle-selection approach based on **g** tensor anisotropy and the copper nuclear hyperfine coupling (Henderson et al., 1985; Hurst et al., 1985). This approach allows for the evaluation of orientation-dependent parameters that are more directly obtained in single-crystal experiments. The spin Hamiltonian for the $S = {}^1/_2$, $I = 1$ coupling system is given in previously published material (Jiang et al., 1990).

The *isotropic* nitrogen nuclear hyperfine coupling is reported as $a_{iso} = {}^1/_3[A_1 + A_2 + A_3]$. The A terms correspond to the hyperfine interaction along three axes that are rotated into the frame of the **g** tensor by Euler rotation angles α, β, and γ, also evaluated in the process of simulation. The frequency as well as the shape of the $\Delta m_I = 2$ transition in spectra recorded at any magnetic field setting within the EPR absorption strongly depends on a_{iso} and the anisotropy in the coupling, such that good estimates for all hyperfine coupling parameters can be obtained by the simulation of one spectrum. The shape of the $\Delta m_I = 2$ component is also sensitive to the values of the Euler rotation angle β for both the nitrogen hyperfine PAS and nqi PAS. This angle gives the displacement of A_3 (A_{max}) or Q_{zz} (Q_{max}) from the g_z axis. The intensities of resolved $\Delta m_I = 2$ transitions, relative to each other and to the low-frequency features, were used to quantitate the number of nitrogen atoms coupled to Cu(II).

Composite time domain envelopes, which were formed as the product of two or three simulated time domain functions according to the product rule for multiple nuclei coupled to an electron spin (Mims & Peisach, 1981; Dikanov & Tsvetkov, 1992), were Fourier transformed to produce ESEEM simulations. Other procedures, such as the application of decay functions to time domain functions, have been discussed previously (Magliozzo & Peisach, 1992).

Nitrite Analysis. The following methods are based on the diazotization of sulfanilic acid (or sulfanilamide) by nitrous acid formed *in situ* from nitrite (or NO), followed by coupling to α-naphthylamine (or α-naphthylethylenediamine) to give a pink azo dye (Griess−Ilosvay test): (IA) this method uses sulfanilic acid and α-naphthylamine (Vogel, 1961) as in a previously published report (Salvato et al., 1989); (IB) the same procedure using sulfanilamide and α-naphthylethylenediamine in place of sulfanilic acid and α-naphthylamine gave improved results; (II) the same reagents as used for IA, following a different protocol (Fries & Getrost, 1977) using 1% sodium acetate and 40% acetic acid in the final assay solution; (III) a premixed reagent solution containing 1% sulfanilamide plus 0.1% α-naphthylethylenediamine dihydrochloride in 4% H_3PO_4 (Green et al., 1982) was used, with optical measurements performed at 545 nm; (IV) the method reported by Tahon et al. (1990).

RESULTS

The green half-met derivatives used for spectroscopic studies in this paper were prepared by dialysis of native Hc against nitrite plus ascorbate, according to a previously published method (Salvato et al., 1989). This method gives complete conversion of native Hc to GHM Hc, which, according to EPR quantitation, contains 1 mol of cupric copper/mol of active site. All nitrite assays, including the one previously described, gave nitrite stoichiometries ranging from 50% to 70% of 1 equiv of cupric copper.

In a previous report, the stoichiometry of nitrogen oxide ligand bound to GHM Hc was found to be much less than 1 equiv of the cupric copper content (Salvato et al., 1989). The latter conclusion was based on the low yield of chromophore in a Griess−Ilosvay reagent assay using method IA. Protein precipitation occurred under the conditions used in this method. In methods II−IV, the same reagents or sulfanilamide and α-naphthylethylenediamine were used in acidic medium in which Hc remained soluble.

Several interferences in the assays that could lead to underestimates of nitrite were identified, the most serious of these being potential ascorbate contamination in the Hc derivative. Interference caused by the protein itself and instability of the chromophore in the absence of strongly acidic conditions can also lead to low apparent yields of nitrite; for example: (1) the addition of 0.05 mM ascorbate to a sample of 1 mM GHM Hc caused a 20% reduction in the yield of pink chromophore (method III); (2) in method IA (but not in the other methods), increasing the aliquot of 1 mM GHM Hc from 20 to 50 μL in a 1 mL assay mixture caused a 7% *decrease* in the yield of pink chromophore; (3) incubation of the final assay solution of method IA led to fading of the pink color on a time scale of minutes. The simultaneous occurrence of these interferences is believed to account for the low levels of nitrite previously reported (Salvato et al., 1989).

Even after we ensured that these problems were no longer a potential source of error (for example, by extending dialysis time and exchange volumes), the nitrite determined analytically was not equimolar with cupric copper. Although the diazotized intermediate is stable under the acidic assay conditions (in 4% H_3PO_4 or 60% acetic acid), an interference due to an *in situ* reaction probably involving Cu(I) and liberated nitrite (nitrous acid) could not be eliminated. The presence of Cu(II) does not cause any interference, as demonstrated by the analysis of nitrite in a small molecule complex Cu(II)TEPA(NO_2) (Jiang et al., 1993a), which yields the expected stoichiometry of 1 mol of nitrite/mol of Cu(II) (method III, data not shown). It then seems that a fraction of the nitrite bound in GHM Hc remains undetectable in the Griess−Ilosvay assays, if, as has been assumed, a unique Cu(II) site is present.[2]

cw-EPR Spectroscopy. The cw-EPR spectrum of GHM Hc from several species has appeared in the literature and is characterized by a rhombic **g** tensor (Schoot Uiterkamp et al., 1974; Van der Deen & Hoving, 1977; Himmelwright et al., 1979; Verplaetse et al., 1979). The g and A_{Cu} values are consistent with a ground state wave function of $d_{(x^2-y^2)}$ character (Abragam & Bleaney, 1970) containing a small contribution from d_{z^2} to account for the rhombicity in the **g**

[2] Though the EPR signal of *O. vulgaris* GHM Hc does not change upon extensive dialysis or in the presence of excess nitrite, it remains possible that samples of GHM Hc contain a mixture of Cu(II) species, a fraction of which lacks nitrite. No additional features appeared in the Q-band EPR spectra, however, making this an unlikely possibility.

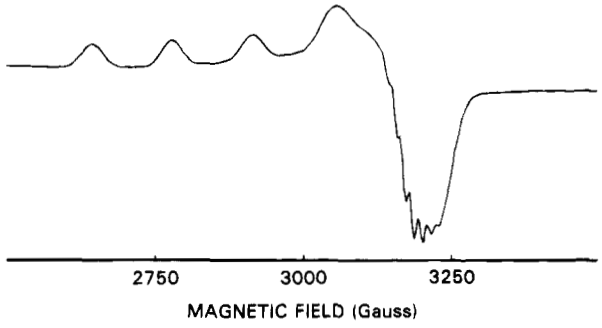

FIGURE 1: EPR spectrum of *H. americanus* green half-methemocyanin in 20 mM potassium phosphate in D_2O (pH 7.0) (uncorrected meter reading). Spectrometer conditions: $\nu = 9.108$ GHz, power = 2.0 mW, modulation amplitude = 6.3 G.

tensor (Westmoreland et al., 1989). The g values calculated directly from Q-band EPR spectra of the GHM Hc derivatives from *O. vulgaris* (g = 2.294, 2.090, 2.043) and *C. maenas* (g = 2.295, 2.095, 2.049) differed by less than 0.5% from those evaluated by simulation of X-band spectra (not shown). Copper nuclear hyperfine couplings at $g_{\|}$ were 425 MHz for *O. vulgaris* and 410 MHz for *C. maenas* GHM Hcs, but could not be evaluated at $g_{\perp}$.

The correlation between the EPR spectrum characteristic of arthropodan GHM Hc and a nitrite ligand bound to Cu(II) in the active site is presented in the accompanying paper concerning a mononuclear Cu(II) derivative of *C. maenas* Hc (Bubacco et al., 1995). This Cu(II)-Hc was shown, after the addition of nitrite, to exhibit EPR and ESEEM spectra closely related to those of GHM Hc prepared from the native protein. Similar g and A_{Cu} values were found, and the ESEEM spectra for both Hc derivatives were nearly superimposable. For molluscan GHM Hc, a series of ligand exchange reactions demonstrated that, in *O. vulgaris* GHM Hc, nitrite is an exchangeable ligand bound to Cu(II). In these reactions, previously reported for the binuclear GHM Hc from *B. canaliculatum* (Westmoreland et al., 1989), nitrite is replaced by dialysis against azide, then acetate, and then H_2O, after which nitrite is reintroduced. The added nitrite immediately produces the EPR spectrum of GHM Hc (data not shown).

In general, the EPR spectra of molluscan GHM Hcs do not exhibit superhyperfine splittings. The spectra of the arthropodan GHM Hcs from *C. maenas*, *H. americanus*, and *P. interruptus* contain superhyperfine splittings of ≈14 G in the $g_{\perp}$ regions. For *C. maenas* and *P. interruptus* GHM Hc, six or seven superhyperfine lines are resolved (data not shown). Better resolution was found in the EPR spectrum of *H. americanus* GHM Hc in D_2O buffer (Figure 1). At least eight (possibly nine) lines in the $g_{\perp}$ region arise from superhyperfine coupling to nitrogen ligand nuclei and are not due to copper hyperfine splittings.[3] The spectrum of *R. thomasiana* GHM Hc (not shown) closely resembles that of *O. vulgaris* GHM Hc, although it exhibits an interesting difference from other Hcs in its ESEEM spectrum (see the following).

The EPR spectrum of GHM Hc from *O. vulgaris* is altered upon the binding of CN^- (Figure 2b), in analogy to the

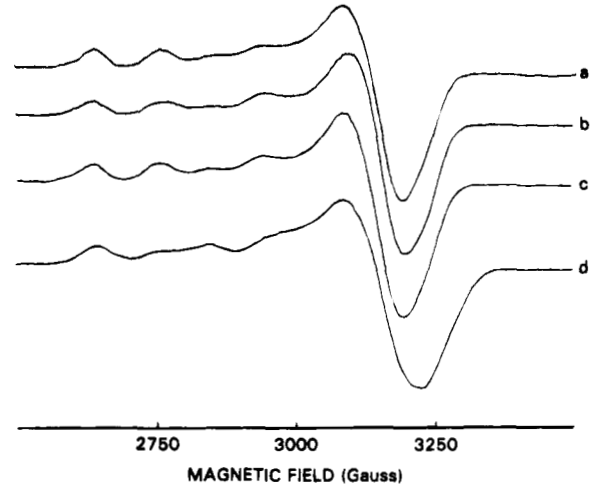

FIGURE 2: EPR spectra of *O. vulgaris* green half-metHc (1 mM) after the addition of cyanide: (a) $C^{15}N^-$; (b) CN^-; (c) $^{13}CN^-$; (d) excess $^{13}CN^-$. Spectrometer conditions: (a) $\nu = 9.107$ GHz, power = 2.0 mW, modulation amplitude = 6.3 G; (b) $\nu = 9.117$ GHz, power = 2.0 mW, modulation amplitude = 10 G; (c) $\nu = 9.117$ GHz, power = 2.0 mW, modulation amplitude = 12.5 G; (d) $\nu = 9.117$ GHz, power = 2.0 mW, modulation amplitude = 10 G.

behavior of other GHM Hcs (Himmelwright et al., 1979). [This ligand has been used to probe the structure of imidazole-containing metal binding sites in enzymes such as carbonic anhydrase (Haffner & Coleman, 1975) and SOD (Rotilio et al., 1972).] The half-met Hc-CN^- spectrum is axial, does not exhibit any ligand superhyperfine splittings, and has $g_{\|}$ and $A_{\|}$ values similar to those of GHM Hc. The spectrum in Figure 2b was recorded from a sample that had been incubated overnight in 10 mM buffered CN^- and then dialyzed against phosphate buffer to remove excess CN^-. A sample of GHM Hc was also incubated with a small excess and then a larger excess of $^{13}CN^-$ (Figure 2c,d). [No loss of Cu(II) signal intensity occurs under these conditions.] No new superhyperfine splittings at $g_{\|}$ appear, although in excess $^{13}CN^-$ a broadening occurred at $g_{\perp}$.[4] Also, as can be seen in Figure 2a compared to Figure 2b, no new superhyperfine features or even increased linewidths appear when $C^{15}N^-$ is used; thus, there is no clear evidence for a binding mode in which the ^{15}N of $C^{15}N^-$ is bound to Cu(II), as was suggested by Pavlosky and Larabee (1988).

The lack of superhyperfine coupling in the EPR spectrum of CN^--bound forms is informative, in that it suggests that the cupric site in *O. vulgaris* half-metHc does not achieve a square or square pyramidal geometry of the type documented for the metal binding site in cyanide-treated Cu(II)-carbonic anhydrase or SOD (Haffner & Coleman, 1975; Rotilio et al., 1972).

ESEEM Spectroscopy. Our aims in applying the ESEEM technique were to determine the number of imidazole ligands bound to cupric copper in both molluscan and arthropodan GHM Hc derivatives and to elucidate structural or electronic differences between the active sites in the Hcs from these phyla.

The ESEEM spectra for the GHM Hcs from both mollusks and arthropods reveal small but significant differences in the nitrogen nuclear coupling parameters for the Nδ of imidazole

[3] The evenly spaced splitting pattern could arise from sets of copper nuclear hyperfine lines only for $g_{\perp}$ values split by approximately 56 G, which is 24 G smaller than the actual splitting between g_y and g_x at X-band.

[4] This spectrum may indicate the presence of a mixture of species, as evidenced by the additional copper nuclear hyperfine splittings at $g_{\|}$.

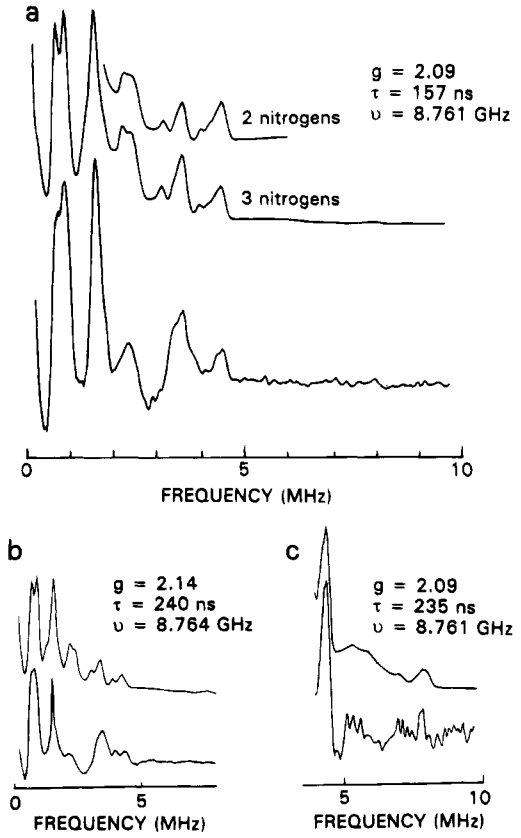

FIGURE 3: ESEEM spectra and simulations for *O. vulgaris* GHM Hc. The spectra are offset on an arbitrary intensity scale. Microwave frequencies, experimental g values, and τ values are given in the figure: (a) (bottom) spectrum recorded at a magnetic field of 3000 G; (top) ESEEM simulations incorporating coupling interactions for two or three nitrogen sites. For the two-nitrogen case, one weak and one strongly coupled nitrogen were assumed to contribute (N1 and N3 in Table 1); for the three-nitrogen case, two weak (N1, N2) plus one strongly coupled nitrogen (N3) were assumed. (b) (bottom) ESEEM spectrum recorded at 2932 G; (top) three-nitrogen ESEEM simulation (N1, N2, and N3, Table 1). (c) 5-fold enlargement of the 5−10 MHz region for data (bottom) and simulation (top) in (a), except $\tau = 235$ ns. (The small features in simulations near 3.0 and 3.85 MHz are combination frequency components that did not appear in the data.)

ligands. Figure 3a shows an ESEEM spectrum of *O. vulgaris* GHM Hc and simulations of the data (discussed later) assuming two or three nitrogen ligands. The data pattern is typical of X-band ESEEM spectra arising from the remote (amino) nitrogen of imidazole bound to Cu(II) (Mims & Peisach, 1978), for which exact cancellation (Flanagan & Singel, 1987) of nitrogen nuclear Zeeman and hyperfine coupling interactions occurs. The low-frequency components at 0.64, 0.81, and 1.53 MHz are assigned to this nitrogen on the basis of their similarity to the zero-field nqi transition frequencies, ν_+, ν_-, ν_0 (Mims & Peisach, 1978), of the amino nitrogen of histidyl imidazole (Edmonds & Sommers, 1973; Hunt et al., 1975). The characteristics of these components are simulated by appropriate evaluation of e^2Qq, the nitrogen nuclear quadrupole coupling constant, η, the asymmetry parameter for this interaction, and a set of Euler rotation angles. The line at 2.3 MHz arises from a combination of lower frequency components (see the following) and indicates multiple nitrogen coupling in a single electron spin system (Kosman et al., 1980; Lin et al., 1986; McCracken et al., 1988).

Table 1: Nuclear Hyperfine and Quadrupole Coupling Constants for the Remote Nitrogen of Imidazole Ligands Bound to Cu(II) in GHM Hcs Evaluated by Simulation of ESEEM Spectra[a]

protein source		hyperfine coupling				quadrupole coupling	
		A_1	A_2	A_3	a_{iso}[b]	e^2Qq	η[c]
O. vulgaris (mollusk)	N1	1.1	1.44	1.49 (0°, 75°, 0°)[d]	1.34	1.52 (0°, 50°, 0°)[e]	0.8
	N2	1.1	1.52	1.56 (0°, 75°, 0°)	1.39	1.52 (0°, 50°, 0°)	0.8
	N3	1.73	2.46	2.46 (0°, 70°, 0°)	2.22	1.55 (0°, 45°, 0°)	0.85
R. thomasiana (mollusk)	N1, N2	1.1	1.42	1.46 (0°, 85°, 0°)	1.33	1.55 (0°, 40°, 0°)	0.73
	N3	1.8	2.46	2.46 (0°, 80°, 0°)	2.24	1.52 (0°, 45°, 0°)	0.76
C. maenas (arthropod)	N1, N2	1.0	1.58	1.60 (0°, 70°, 0°)	1.39	1.62 (0°, 45°, 0°)	0.85
	N3	1.6	2.2	2.2 (0°, 78°, 0°)	2.0	1.42 (0°, 40°, 0°)	0.85

[a] Uncertainties in a_{iso}, e^2Qq, and η are less than 10%; uncertainties in the values assigned for Euler rotation angle β are less than 10°. Values for α and γ were fixed at 0°. [b] $a_{iso} = {}^1/_3[A_1 + A_2 + A_3]$. [c] $\eta = (q_{xx} - q_{yy})/q_{zz}$. [d] Euler rotation angles α, β, and γ, relative to the **g** tensor. [e] Euler rotation angles for the nqi principal axis system relative to the **g** tensor.

The broad features near 4 MHz are $\Delta m_I = 2$ (double-quantum) transition components arising from the electron spin manifold in which nitrogen nuclear hyperfine and Zeeman interactions are additive (Mims & Peisach, 1978; Jiang et al., 1990). The double-quantum transition frequency is approximated by

$$\nu_d = 2[(\nu_I + a_{iso}/2)^2 + (e^2Qq/4)^2(3 + \eta^2)]^{1/2}$$

where ν_I is the nitrogen nuclear Zeeman frequency (Dikanov & Tsvetkov, 1992). The resolution of components at 3.5 and 4.4 MHz, considered along with the appearance of the combination frequency components at 2.3 MHz, suggested the presence of contributions from at least two imidazoles.

A simulation assuming two nitrogens is shown in Figure 3a. In this simulation, $a_{iso}(1) = 1.35$ MHz and $a_{iso}(2) = 2.2$ MHz (i.e., the parameters corresponding to N1 and N3 of *O. vulgaris* given in Table 1). The width and intensity of the feature at 3.5 MHz suggested, however, that two closely spaced $\Delta m_I = 2$ transitions were present, along with the component from the more strongly coupled nitrogen (N3). Therefore, sets of parameters for three nitrogens were finally used to best simulate the data,[5] and these are summarized in Table 1. The nuclear quadrupole coupling constants were approximately 1.5 MHz for all nitrogens, with η values of 0.8 or 0.85. In all cases, the value for the Euler rotation angle β was near 50° for the nqi and 80° for the nuclear hyperfine principal axis systems. The structural implications of the values for these angles are discussed in the following.

The $\Delta m_I = 2$ component at 4.4 MHz, which appears as a single broad peak in the spectrum recorded at a magnetic field of 3000 G (Figure 3a), splits into two components (3.9

[5] We note here that, in all simulations for more than one nitrogen, the combination component at 2.3 MHz (McCracken et al., 1988) appears more intense than it does in the data. For this reason, our approach to the assignment of imidazole nitrogen multiplicity relied more heavily on the relative intensities of the $\Delta m_I = 2$ components than on the intensity of the 2.3 MHz combination line.

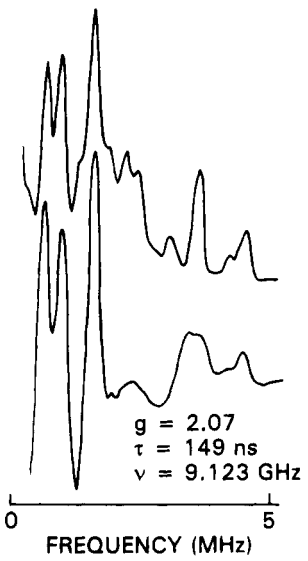

FIGURE 4: ESEEM spectra and simulation for *R. thomasiana* GHM Hc: (bottom) spectrum recorded at a magnetic field setting of 3150 G; (top) ESEEM simulation incorporating coupling interactions for three nitrogen sites. Parameters used in the simulation are given in Table 1, and other conditions are given in the figure.

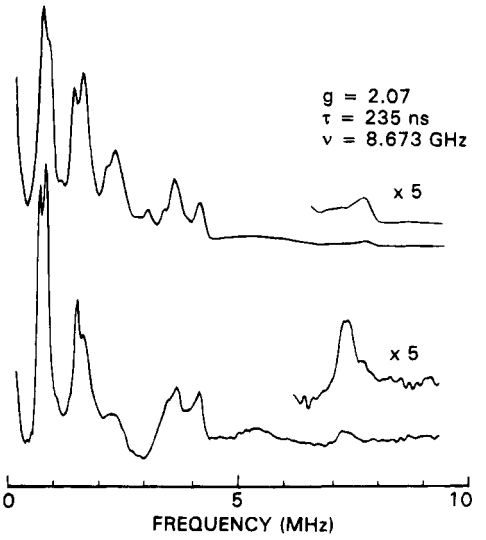

FIGURE 5: ESEEM spectra and simulation for *C. maenas* GHM Hc: (bottom) spectrum recorded at a magnetic field setting of 3000 G; (top) ESEEM simulation incorporating coupling interactions for three nitrogen sites. Parameters used in the simulation are given in Table 1, and other conditions are given in the figure.

and 4.35 MHz) in the spectrum recorded at 2932 G (Figure 3b). This change is achieved in simulations by assigning appropriate values for $A_{1,2,3}$. An additional feature arising from multiple nitrogen coordination in *O. vulgaris* GHM Hc is a high-frequency combination line appearing at approximately 7.9 MHz. This component, apparent in Figure 3c at the sum of the frequencies of the $\Delta m_I = 2$ transitions (3.5 + 4.4 MHz), demonstrates that both the weaker and the stronger nitrogen couplings are part of the same electron spin system, i.e., all three imidazole ligands are bound to Cu(II) in the active site. An analogous high-frequency combination feature was documented in the analysis of ESEEM spectra of a Cu(II)-doped zinc(II) bis(1,2-dimethylimidazole) dichloride complex (Colaneri et al., 1990) and in Cu(II) bis- and tris(imidazole) ligand complexes (Goldfarb et al., 1991).

The simulation of ESEEM spectra for other Hcs was performed only for data recorded near g_x.[6] The results (Table 1) for *R. thomasiana* GHM Hc (Figure 4) indicate hyperfine coupling parameters quite close to those for *O. vulgaris* GHM Hc, but a shift in the position of the lowest frequency components indicated a reduction in the value of η. Note that the linewidth at 3.5 MHz in the simulation is too narrow and apparently too intense compared to the data because two equivalent nitrogen couplings were assumed for the weaker set.

For the GHM Hc from *C. maenas*, the assignment of multiple imidazole nitrogen components based on the $\Delta m_I = 2$ features is similar to that described earlier (Table 1). The $\Delta m_I = 2$ component (Figure 5) for the more strongly coupled nitrogen appears at 4.1 MHz ($a_{iso} = 2.0$ MHz), while the less strongly coupled nitrogens, which give rise to the 3.6 MHz component(s), were treated as a *pair* of equivalent sites having $a_{iso} = 1.39$ MHz. [Note that, due to τ suppression effects (Peisach et al., 1979), the relative intensities of the $\Delta m_I = 2$ components are nearly equal in the data presented in the figure, although in spectra recorded at other τ values (not shown), the 3.6 MHz peak is nearly twice as intense as the 4.1 MHz line, more obviously illustrating the nitrogen multiplicity (two weaker, one stronger). Simulations generally exhibit weaker suppression effects than those encountered in sets of ESEEM data.]

The simulation of spectra for the GHM Hc from *C. maenas* involved an additional difficulty because two components are partially resolved near 1.5 MHz in Figure 5 (bottom). These components were considered to correspond to two different ν_+ transitions in the sets of nqi lines arising from different nitrogen sites. The ratio of intensities of the components near 1.5 MHz to those at 0.7 MHz was consistent with the assignment of a larger e^2Qq value to the nitrogen having the smaller a_{iso} value. These features, however, are difficult to simulate in the three-nitrogen composite, and it is possible that the e^2Qq values are somewhat over- and underestimated for these nitrogens.

The nitrogen nuclear quadrupole coupling parameters for *C. maenas* GHM Hc (Table 1) were closely related to those discussed earlier for *O. vulgaris* GHM Hc.

The high-frequency sum feature (Figure 5, inset) in spectra from *C. maenas* GHM Hc does not appear as a single peak, as was described earlier for *O. vulgaris* GHM Hc. A feature is seen at 7.2 MHz, which corresponds to twice the frequency of the $\Delta m_I = 2$ line for the weaker nitrogen components, along with a shoulder at 7.8 MHz (the sum of 3.6 + 4.1 MHz in analogy to the 7.9 MHz line discussed earlier for *O. vulgaris* GHM Hc). Both of these components appear in the three-nitrogen simulation, although their intensities are opposite those in the data. The interpretation of the behavior of the intensity of these components is not

[6] In our approach to the evaluation of electron–nuclear coupling parameters, electron spin–echo data may be collected at several magnetic field settings and also at several microwave frequencies. The simulation of the body of data then allows for a better evaluation of the anisotropic nitrogen nuclear hyperfine coupling parameters. No large errors in the evaluation of a_{iso} are incurred, however, when a more limited approach to data collection and simulation is taken (Jiang et al., 1993b). For the case of GHM Hc from *O. vulgaris*, the simulation of data collected at more than one magnetic field has allowed us to understand that the appropriate simulation of data recorded at $g_\perp$ only, when the shape of the $\Delta m_I = 2$ line is carefully considered, does not lead to large errors in a_{iso}.

thoroughly understood, although the presence of the highest frequency combination line is consistent with both weak and stronger imidazole nitrogen couplings contributing to the ESEEM frequency pattern.

The ESEEM spectra of GHM Hcs from the arthropods *P. interruptus* and *H. americanus* are nearly identical to those of *C. maenas*, and coupling parameters for three imidazole nitrogens similar to those found for the latter are expected.

No evidence for a contribution from the ^{14}N of nitrite is found in the spectra presented here for any GHM Hc, in agreement with results previously reported for *O. vulgaris* GHM Hc (Jiang et al., 1993a).

DISCUSSION

In the interest of proposing structural models for the cupric site in GHM Hc, we have reevaluated the stoichiometry of nitrite tightly bound to the derivative prepared from *O. vulgaris* Hc and have also examined the quantitative aspects of electron−nuclear coupling using ESEEM spectroscopy.

Nitrite in GHM Hcs. The stoichiometry of nitrite in various preparations of *O. vulgaris* GHM Hc determined using Griess−Ilosvay assays under a variety of conditions was closer to 1 equiv/mol of cupric copper than had been reported previously (Salvato et al., 1989). An improvement in analytical methodology involved the use of strongly acidic conditions in the assay. Also, greater care in the removal of ascorbate used in the preparation of the derivative may account for higher yields of chromophore. A suspected interference that consumes nitrite upon acidification of the protein, due to an *in situ* reaction involving Cu(I), cannot be eliminated. According to the analytical findings and other spectroscopic evidence presented in the following, the presence of a tightly bound nitrite ligand, as originally suggested by Himmelwright et al. (1979), clearly is substantiated.

Since the Griess−Ilosvay assay is also sensitive to NO, and this ligand has been suggested by other authors to be bound to copper in GHM Hc (Tahon et al., 1990), we attempted to demonstrate that *nitrite anion* is in fact the exogenous ligand bound to Cu(II). Two experimental approaches have addressed this issue: In one, previously described for *B. canaliculatum* Hc (Westmoreland et al., 1989), a series of ligand exchange reactions starting from GHM Hc and proceeding through azide, acetate, and finally the aquo form of the half-met protein showed that the EPR spectrum of the nitrite form (the starting material) could be restored when nitrite was added to the aquo half-met form. This approach gave similar results when applied to *O. vulgaris* GHM Hc (not shown).

In another approach, presented in the following paper (Bubacco et al., 1995), we show that a *mononuclear* Cu(II) derivative of *C. maenas* Hc prepared in the absence of nitrite exhibited EPR and ESEEM spectra very closely related to those of GHM Hc, after the addition of nitrite. The similarities include a_{iso} values for two weak nitrogens and a more strongly coupled imidazole nitrogen in the nitrite form of the mononuclear Cu(II)-Hc derivative nearly equivalent to those in *C. maenas* GHM Hc. One important conclusion that may be drawn from the latter result is that the structure of Cu(II) in the GHM Hc binuclear site does not strongly depend on the presence of the second copper atom, a result that argues against the bridging mode for bound nitrite suggested by Westmoreland et al. (1989).

Electron−Nuclear Couplings in GHM Hcs. The nitrogen nuclear hyperfine and quadrupole coupling parameters calculated in the analysis of ESEEM spectra of the GHM Hcs (Table 1) are similar to those reported for the remote nitrogen of imidazole in other copper proteins (Jiang et al., 1990). Three imidazole ligands were identified in each GHM Hc, with a small phylum-specific difference between the molluscan and arthropodan proteins: The arthropodan GHM Hc has a smaller difference between the single stronger and two weaker nitrogen nuclear hyperfine interactions.

The nitrogen nuclear hyperfine coupling constants for both the directly coordinated and the remote nitrogens have been evaluated in some model complexes, with the former approximately 22-fold stronger than the latter in, for example, copper(II) tetrakis(imidazole) (Van Camp et al., 1982) and Cu(II)-doped *l*-histidine dihydrochloride (McDowell et al., 1989; Colaneri & Peisach, 1992). If the remote nitrogen coupling values found for *C. maenas* GHM Hc are scaled up by 22-fold, the a_{iso} values for the first coordination sphere nitrogens will be 31 and 44 MHz. For *O. vulgaris* GHM Hc, the remote nitrogen couplings would scale to 31 and 49 MHz for the directly coordinated nitrogens. These coupling estimates provide evidence for a more distorted (less tetragonal) first coordination sphere in the molluscan Cu(II) site, according to the trends in coupling vs geometry reported by Iwaizumi et al. (1986). The hyperfine coupling values for *C. maenas* translate into splitting factors of 11 G for two nitrogens and 15 G for the third, consistent with the splitting estimated from the cw-EPR spectrum of this protein. For the molluscan case, the splittings would be 11 and 17 G, although no resolution of superhyperfine features is apparent in the X-band cw-EPR spectrum of *O. vulgaris* GHM Hc. The lack of resolution may arise from line broadening due to *g* strain and/or unresolved Cu hyperfine splittings.

The inequivalence in hyperfine couplings for imidazoles in the arthropodan Cu(II) site is not likely to be due to the chemical difference between Nδ and Nϵ of the substituents since the Nϵ nitrogen of each histidyl imidazole ligand is expected to be the directly coordinated atom, as reported for *P. interruptus* Hc (Hazes et al., 1993; Gaykema et al., 1984; Volbeda & Hol, 1989a). In the molluscan GHM Hcs, however, the larger difference in hyperfine couplings among the ligands could reflect a change in the identity of the remote nitrogen for the strongly coupled site. Interestingly, the remote nitrogen in one of the six imidazole ligands in the binuclear or type 3 copper pair within the trinuclear copper site of ascorbate oxidase is the Nϵ site (Messerschmidt et al., 1992).

It is also unlikely that the small difference in couplings among the imidazoles of the Cu(II) site arises from a subunit heterogeneity in the Hcs examined, since all ESEEM spectra had the same pattern of two weaker and one stronger nitrogen couplings, yet the Hcs studied are expected to have quite different subunit compositions (Herskovitz, 1988; Miller et al., 1990). The presence of high-frequency combination lines (near 7 MHz) also argues against heterogeneity contributing to the multiplicity of ESEEM components because it indicates that three imidazoles are coupled to the same Cu(II).

A final structural idea related to the hyperfine coupling parameters is that the finding of equal a_{iso} values for a pair

of nitrogens along with stronger coupling for the third may indicate a *trans* influence in the nearly tetragonal Cu(II), site such that the different nitrogen (the more strongly coupled one) occupies a position opposite the non-imidazole ligand site (assumed to be an oxygen of nitrite). A related effect has been noted for the imidazole couplings to Cu(II) in SOD cyanide (Van Camp et al., 1982).

The nuclear quadrupole coupling parameters for the amino nitrogen of imidazole ligands in copper proteins do not vary much from the value found for histidyl imidazole, since the remote nitrogen valence shell remains essentially unchanged when the *imino* nitrogen coordinates to a metal. The values of these parameters depend on the orbital populations in the two N−C σ bonds, the p−π bond, and the N−H bond of the amino nitrogen. Differences for imidazole ligands in different proteins are suggested to arise from changes in hydrogen bonding (Jiang et al., 1990; Colaneri & Peisach, 1992), since the N−C σ bond orders (and presumably the p−π bond occupancy) are not expected to vary; differences in the polarization of the N−H bond then account for the origin of the shifts. The relatively small η values reported for amine oxidase (McCracken et al., 1987), phenylalanine hydroxylase (McCracken et al., 1988), and galactose oxidase [calculated in Jiang et al. (1990) from data in Kosman et al. (1980)] suggest that weak hydrogen bonds exist at the imidazole *amino* nitrogens in the metal sites of these copper proteins. For several other copper proteins, η values close to 0.9 are characteristic of strong hydrogen bonding (Jiang et al., 1990). The crystal structures of both *L. polyphemus* and *P. interruptus* Hcs reveal that all *amino* nitrogens of the active site imidazole ligands are hydrogen bonded to water molecules or main chain carbonyl groups (Volbeda & Hol, 1989a; Hazes et al., 1993). The large η values found for *C. maenas* GHM Hc therefore were not unexpected. The values found for *O. vulgaris* GHM Hc would predict strong hydrogen bonding for the remote N of imidazole ligands in the Cu(II) site of this molluscan protein as well.

The reduced η value for *R. thomasiana* GHM Hc compared to those for the other Hcs may be an indication of weaker hydrogen bonds in the active site of this Hc, although the differences are smaller than the maximum difference noted so far for a collection of copper proteins (Jiang et al., 1990). Hydrogen-bonding interactions (and van der Waals contacts) in the arthropodan Hc active site are suggested to be important in fixing the geometry of the imidazoles (Hazes et al., 1993) and may be important in allosteric mechanisms involving subunit cooperativity, since they can provide a link between the active site and more remote regions. Whether differences in nqi parameters are related to functional differences between this Hc and others remains to be demonstrated, although we note here that *R. thomasiana* Hc exhibits a reverse Bohr effect compared to the other Hcs examined in this study as well as to nearly all other Hcs (Ellerton et al., 1983; Boteva et al., 1991).

Structural Implications of ESEEM Results. Since the approach to simulation of ESEEM spectra utilizes an angle-selection methodology based on **g** tensor anisotropy, the Euler rotation angles for the nitrogen nuclear hyperfine PAS and the nqi PAS can be related to certain structural features of the Cu(II) sites. For example, considering first the nitrogen hyperfine interaction, the large value for the Euler rotation angle β used in all simulations presented here is consistent with the axis orientations expected for the **g** tensor of tetragonal copper and the **A** tensor for a dipolar-coupled amino nitrogen of imidazole. If the nitrogens directly coordinated to Cu(II) in GHM Hc are considered to lie in or near an equatorial plane (the $d_{x^2-y^2}$ orbital plane), then g_{max} (g_z) will lie perpendicular to this plane (Boas, 1984). For the remote nitrogen of imidazole, it may be expected that the largest term in the hyperfine coupling to the unpaired electron will occur in the direction of the Cu(II)−N vector, i.e., A_3 (A_{max}) will lie near the Cu−Nδ direction. An orientation of hyperfine axes such as this was found in an ESEEM study of the Cu(II) imidazole moiety in single crystals of Cu(II)-doped histidine dihydrochloride (Colaneri & Peisach, 1992).[7] Thus, the large value for β is consistent with imidazole bound in or near the equatorial plane containing the unpaired electron orbital.

The nqi PAS for the amino nitrogen of metal-bound imidazole in nearly all cases is such that Q_{zz}, the largest coupling component, lies perpendicular to the imidazole ring plane (Ashby et al., 1978, 1980) [for a discussion of possible exceptions, see Jiang et al. (1990); Colaneri & Peisach, 1992]. Therefore, the values near 50° for the nqi Euler rotation angle β reported for all GHM Hcs examined here suggest a rotation of imidazole planes away from the equatorial plane of the copper site. This arrangement seems reasonable for multi-imidazole ligation given that it minimizes steric crowding. A propeller-like structure has also been described for a tripodal tris(imidazole) Cu(II) complex (Goldfarb et al., 1991) and for the type II copper site in the enzyme nitrite reductase (Godden et al., 1991), and such a feature is also found in the structures reported for deoxy (Cu(I)) arthropod Hcs (Hazes et al., 1993; Gaykema et al., 1984; Volbeda & Hol, 1989a).

Further details concerning the structure of the Cu(II) site in the binuclear center of GHM Hc are discussed in the following. None of the reports on this derivative has proposed a ligand composition for Cu(II) that would now be considered consistent with the structure of the oxy- or deoxyHc binuclear site presented in X-ray crystallographic studies (Hazes et al., 1993; Gaykema et al., 1984; Volbeda & Hol, 1989a). Since three imidazole ligands contribute to the ESEEM spectra of GHM Hcs, and a nitrite ligand is also bound, we can rule out a three-coordinate, deoxyHc-like structure for the Cu(II) site. Any close analogy to the structure of Cu(II) in the oxyHc site (Magnus et al., 1993), i.e., five-coordinate with three imidazole ligands and two atoms of nitrite bound to copper, can also be ruled out according to the following. The coupling pattern found in ESEEM spectra clearly is different from what is expected for a square pyramidal geometry with two *equatorial* and one *axial* imidazole ligand, since we find two equivalent and a third more strongly coupled nitrogen. In square pyramidal geometries, an axial nitrogen would be very weakly coupled to the copper unpaired electron spin (a_{iso} < 1 MHz) (Cornelius et al., 1990). No evidence for components

[7] In Cu(II)-doped crystalline zinc(II) bis(1,2-dimethylimidazole) dichloride, a reorientation of hyperfine axes for the substituted imidazole was found (Colaneri et al., 1990). Although these two examples illustrate that there can be an ambiguity in structural implications based on Euler rotation angles for the hyperfine axes relative to the **g** tensor axes, the Cu(II) site in GHM Hc is expected to be more closely related to the model having an imidazole rather than a dimethylimidazole ligand.

consistent with a coupling of this small magnitude was found in ESEEM spectra of GHM Hcs. This reasoning may then also rule out the equatorial binding of nitrite to copper as an oxygen surrogate. The absence of hyperfine interactions between either ^{14}N or ^{15}N of NO_2^- in EPR and ESEEM spectra of GHM Hcs (Van der Deen & Hoving, 1977; Jiang et al., 1993a) is consistent with this reasoning, given that equatorial ligands in a regular square pyramidal complex are expected to be strongly coupled to Cu(II).

Further evidence that the Cu(II) site in GHM Hc does not seem to easily accommodate *regular* five-coordinate square pyramidal (or four-coordinate square planar) structures comes from the absence of new features from EPR spectra due to either ^{13}C, ^{14}N, or ^{15}N, when cyanide containing these isotopes is bound to Cu(II). In Cu(II) carbonic anhydrase, for example, $^{13}CN^-$ binding to the three-imidazole metal binding site leads to extensive line broadening due to unresolved ^{13}C hyperfine interactions (Haffner & Coleman, 1975). Furthermore, when a second cyanide anion binds (in the presence of excess cyanide), superhyperfine splittings arising from two directly coordinated nitrogen ligands appear in the EPR spectrum, characteristic of square pyramidal geometry (Haffner & Coleman 1975). Although the binuclear GHM Hc of *O. vulgaris* did not exhibit analogous behavior with cyanide, *mononuclear* Cu(II) or Co(II) derivatives of *C. maenas* Hc do show the effects reported for carbonic anhydrase (Salvato et al., 1986; Bubacco et al., 1995). The fact that Cu(II) in the binuclear site could not accommodate square pyramidal structures suggests that the second metal ion imposes constraints on the geometry of the Cu(II) site.

An arrangement of ligands that could accommodate three imidazoles in equal positions around Cu(II) is a regular tbp structure. This geometry, however, may be ruled out on the basis of the tetragonal-type cw-EPR spectra of GHM Hcs.[8]

The EPR spectra of some five-coordinate model complexes with distorted geometries between idealized tbp and square pyramidal have been presented by Bencini et al. (1978). These have rhombic **g** tensors and A_{Cu} values similar to those of tetragonal Cu(II) complexes. One of these, Cu(II)-doped bis(*N*-methylsalicylaldiminato)zinc(II), contains two oxygen and three nitrogen ligands and duplicates extremely well the EPR parameters (both g and A values) of the GHM Hcs. [The copper nuclear hyperfine coupling at $g_\perp$ for GHM Hc cannot be evaluated at X-band, although the g and $A_\parallel$ Cu values for both molluscan and arthropodan GHM Hcs fall within the range calculated by Bencini et al. (1978).] If it is assumed that the GHM Cu(II) site is distorted five-coordinate, the three nearly equivalent imidazole remote nitrogen couplings indicate that these ligands should be placed in similar positions relative to the orbital containing unpaired spin, as stated earlier. The only arrangement to achieve this and maintain the $d_{(x^2-y^2)}$ ground state places three imidazoles close to equatorial positions with a fourth ligand (water or hydroxide) and an apical ligand being an oxygen of nitrite. Alternatively, one nitrite oxygen could be placed equatorially with water or hydroxide as an apical ligand to complete a hypothetical five-coordinate site. An arrangement in which two-point nitrite binding occurs with one oxygen equatorial and the second oxygen apical is also conceivable. The EXAFS results presented in the following paper (Bubacco et al., 1995) strongly suggest, however, that a four-coordinate mononuclear Cu(II) site in *C. maenas* Hc could reproduce well the cw-EPR as well as the ESEEM spectra of GHM Hc. Furthermore, both two- and three-pulse ESEEM spectra (not shown) of *O. vulgaris* GHM Hc in D_2O elicited only solvent deuterium interactions with Cu(II) and provided no evidence for a water or hydroxide ligand. Four-coordinate models therefore were sought in order to help establish the coordination number of Cu(II) in GHM Hcs.

The Cu(II) site in Ag_2Cu_2 SOD, which contains Cu(II) in the Zn(II) binding site (Gurbiel et al., 1993), serves as an interesting example. The Zn(II) site is known from X-ray crystallography of native SOD to be approximately tetrahedral in geometry, with three imidazole ligands and an oxygen ligand from a peptide carbonyl oxygen (Tainer et al., 1983). The Ag_2Cu_2 derivative exhibits a rhombic EPR signal (Pantoliano et al., 1982; Gurbiel et al., 1993) with g and A_{Cu} values similar to those of GHM Hc (although A_{Cu} is smaller by approximately 70 MHz, indicating larger distortions from tetragonal geometry than are found in GHM Hc). The ESEEM spectra of the Cu(II) site also have strong analogies to those of the GHM Hcs presented here. The patterns observed using LEFE in EPR for Ag_2Cu_2 SOD (Gurbiel et al., 1993) are strikingly similar to those of the GHM Hc from *B. canaliculatum* (Westmoreland et al., 1989), both in the magnitude of the g-shifts and in the general shape of the E vs H_o curves. The LEFE data were interpreted to be consistent with low symmetry (C_s) for the Cu(II) site in this mollusk GHM Hc (Westmoreland et al., 1989). Although the site was thought to be five-coordinate, the assignment of C_s symmetry is also reasonable for a four-coordinate Cu(II) site containing two equivalent ligands and two inequivalent ligands different from these. This symmetry is also consistent with the *trans* influence mentioned earlier, whereby two equivalent ligands and two inequivalent ligands are considered to lie near the equatorial plane of Cu(II).

Therefore, on the basis of these analogies, we suggest a four-coordinate N_3O structure for the GHM Cu(II) site, containing three imidazole ligands confirmed by ESEEM spectroscopy plus the exogenous nitrite ligand to complete the first coordination sphere in distorted tetragonal geometry. A small molecule complex that provides some additional evidence to confirm this structure is a GlyGlyGlyCu(II) complex, which at pH 6.5 has N_3O ligation (Crawford & Dalton, 1969) and $g_\parallel$ and $A_{\parallel Cu}$ values (Peisach & Blumberg, 1974) equal to those of GHM Hc.

[8] We can also rule out a structure analogous to a nitrite-containing Cu(II) complex proposed as a model for the active site of nitrite reductase (Tolman et al., 1991). This model, although it contains a ligand composition potentially analogous to that in GHM Hc (three pyrazolyl nitrogen ligands with two equatorial and one apical nitrogen and a bidentate nitrite ligand with one apical and one equatorial oxygen), has an EPR spectrum quite different from that of GHM Hc. The inverted $g_\parallel$ amd $g_\perp$ and large A_{Cu} splittings distinctly different from those for GHM Hc are characteristic of tbp Cu(II) complexes having a d_{z^2} ground state. Another potential analogue of GHM Hc that contains a nitrite ligand is the square pyramidal complex $Cu^{II}TEPA(NO_2^-)$ presented in the text. This model, the ESEEM spectra of which exhibit coupling to the ^{14}N of equatorially bound nitrite (Jiang et al., 1993a), structurally differs from what we propose for the Cu(II) site in GHM Hc. The Cu(II) in a binuclear copper mixed-valence model containing a bridging nitrite (Halfen et al., 1994), also a potential model for GHM Hc, contains two equatorial and one axial nitrogen in a square pyramidal geometry. We have already ruled out this arrangement of ligands for GHM Hc on the basis of the coupling pattern observed in ESEEM spectra.

Cu_A Sites in Molluscan and Arthropodan Hcs. In the arthropodan Hc from *P. interruptus*, Cu_B of the binuclear site is ligated by two histidyl imidazoles from one helical region (His 194 and His 198), with the third coming from the adjacent helical region (His 224) (Volbeda & Hol, 1989b). This sequence pattern is closely related to the sequence at the Cu_A binding site in this Hc, and in fact, there is a pseudo-2-fold rotational symmetry for these regions (Hazes et al., 1993). Sequence information now available for several proteins from both mollusks and arthropods indicates conserved histidines and other strong homologies for the Cu_B sites, but for the Cu_A site in molluscan Hcs, only two histidines are conserved (Lang & Van Holde, 1991). It has been suggested that Cu_A in molluscan Hcs may have only two histidyl imidazole ligands, with a different amino acid providing the third ligand, or that perhaps a total of only two endogenous protein ligands binds Cu_A (Lang & Van Holde, 1991). The findings that three arthropodan GHM Hcs exhibit nearly identical ESEEM spectra and that two molluscan examples are different from those and also different from one another in their quadrupole coupling parameters for the amino nitrogens of imidazoles lead to the suggestion that it is the Cu_A site in the molluscan cases that contains Cu(II), as this is the copper site in the variable region of these proteins. This hypothesis also leads to the proposal that Cu_A in molluscan Hcs has *three* imidazole ligands, despite the lack of conserved histidines (Lang & Van Holde, 1991).

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