the blood have been nicely discussed by Harris et al.[9a]

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Complexation of Copper(II) with a Macrocyclic Peptide Containing Histidyl Residues: Novel Observation of NMR Spectra of Paramagnetic Copper(II) Compounds

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Interaction of Cu(II) and *cyclo*-(Gly-His-Gly-His-Gly-His-Gly), a synthetic macrocyclic peptide, was investigated by electronic absorption, EPR, and ^{1}H and ^{13}C NMR spectroscopy. These experiments have been conducted in aqueous solution as a function of pH and for different ligand:Cu(II) ratios. The results indicate the presence of two species. The species found under neutral conditions is described as one involving the three N(1) nitrogens of imidazole residues, while under basic conditions, the complexation occurs via four GlyNH deprotonated peptide nitrogens. Both systems are found to be in slow exchange over the wide pH range, with dissociation constants of 1.25×10^3 s^{-1} and $\ll 100$ s^{-1}, respectively. An unexpected behavior was observed at basic pH by ^{1}H and ^{13}C NMR techniques. The data are interpreted as Cu(II) being firmly trapped inside the cyclic cavity. The results revealed for the first time some important properties that may have significance in studying the paramagnetic behavior of biologically relevant Cu(II) compounds by NMR spectroscopy.

Interest in synthetic multidentate macrocyclic compounds is continually increasing because of their unique properties. Among these compounds, cyclic peptides, which owe their functional properties to their macrocyclic structure, have attracted much attention in recent years.[1] Cyclic peptides belong to a special class of peptides well-known for their biological functions as hormones, antibiotics, toxins, and regulators of ion transport.[2–4] The reduced flexibility of the peptide backbone and the absence of both N- and C-terminal functional groups make the cyclic peptides attractive models to study the structural and functional aspects of proteins and enzymes.[5–8] In such a model, by varying the number and the nature of the amino acid units, one can obtain cyclic peptides with different cavity sizes, different coordination environments, and different amino acid side chains that are important for the functional sites of proteins and enzymes. Among the amino acid residues, the histidyl side chain is probably the most frequently encountered metal-binding site in biological systems. The histidyl residue plays an important role in the active sites of many enzymes[9–13] and the metal-transport sites of several transport proteins.[14–20] Thus, information obtained from metal complexes with synthetic model compounds containing histidyl residues are very useful.

Our investigations, thus far, have been devoted to the interactions of diagmagnetic metals with the side-chain residues of model cyclic peptides.[5,6] In the present investigation, we chose to synthesize *cyclo*-(Gly-L-His-Gly-L-His-Gly-L-His-Gly) (herinafter denoted as G4H3) and study its complexation properties with the paramagnetic metal Cu(II) in aqueous solution over a wide pH range using electronic absorption, EPR, and ^{1}H and ^{13}C NMR spectroscopy. From analyses of the spectral data, it is concluded that Cu(II) binds to G4H3 in two modes: one involving three imidazole residues at neutral pH and the other involving four deprotonated peptide amide nitrogens at basic pH. An unusual NMR observation of a paramagnetic Cu(II) compound is reported.

Table I. Spectroscopic Data for G4H3–Copper(II) Complexes[a]

pH	λ_{max}, nm (ϵ)	$A_{\parallel}$, G	$g_{\parallel}$	$g_{\perp}$
5.3	620 (9)	176	2.254	2.058
6.3	590 (19)	175	2.252	2.059
7.0	560 (27)	174	2.250	2.060
8.5	610 sh	174	2.252	2.059
	540 (33)	188	2.204	2.052
10.0	540 (35)	190	2.202	2.052
11.2	540 (43)	190	2.200	2.050

[a] sh = shoulder.

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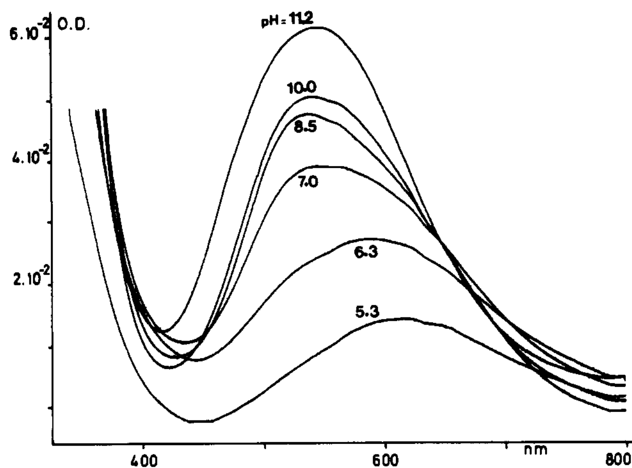

Figure 1. Visible absorption spectra as a function of pH for the G4H3–Cu(II) system at room temperature (ligand:metal ratio 1:1, [G4H3] = 7.14×10^{-3} M).

Experimental Section

Materials. The synthesis and characterization of the cyclic peptide was described in an earlier report.[6] $CuCl_2 \cdot 2H_2O$ was purchased from Fisher Scientific Co. (Ottawa, Canada), and sodium 4,4-dimethyl-4-silapentane-1-sulfonate was from Aldrich Chemical Co. (Milwaukee, WI). D_2O (99.93% D) from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France) was used as a solvent.

Instrumentation. All pH values were measured on a Knick digital pH meter calibrated with standard buffers (Merck Titrisol) and are quoted without correction for the D isotope effect. Solutions with variable Cu(II) concentrations were made up by micropipetting from a concentrated D_2O solution of the anhydrous $CuCl_2$. Care was taken about exposure of our samples to air in order to minimize the uptake of H_2O and hence the amplitude of the H_2O peak.

^1H NMR spectra at 250 MHz and ^{13}C NMR spectra at 62.8 MHz were recorded on a Bruker WM 250 spectrometer equipped with an Aspect 2000 computer system. Field stabilization was provided by an internal deuterium lock signal. Samples were examined at 23 ± 1 °C. The usual ^1H spectrometer conditions were 2200-Hz sweep width, 1-s cycle time, 16K data points, and 250 scans. The HOD signal was considerably reduced by saturation between scans. ^{13}C NMR spectra were recorded with quadrature detection and broad-band proton decoupling. Sample volumes were generally 1 mL, contained in a spinning 10 mm o.d. Wilmad NMR tube with Teflon plug for vortex suppression. Spectra were obtained by accumulating 40 000–70 000 transients for a 5×10^{-2} M solution of G4H3 with 16 K data points. A radio-frequency pulse of 90° was used, with a spectral width of 15 000 Hz and a repetition time of 0.5 s. To improve the peak definition, the sine bell routine was employed.

Visible absorption measurements over the range 340–800 nm were made in 2-mm cells at room temperature with a Cary 2300 spectrophotometer. Ligand–Cu(II) solutions of 1:1 ratio (7.14×10^{-3} M) were prepared and adjusted at different pH values.

X-Band spectra were obtained on a Bruker ER200D spectrometer at 110 and 293 K. Samples (10^{-2} M) were frozen in liquid N_2 in quartz tubes. The variation of the spectrum of the G4H3–Cu(II) complex as a function of pH was carried out by titrating the complex at room temperature.

Results

Visible Absorption Spectra. Formation of different G4H3-Cu(II) complex species with increasing pH can be followed spectrophotometrically from 340 to 800 nm. Absorption spectra were obtained at various points during the titration of G4H3–

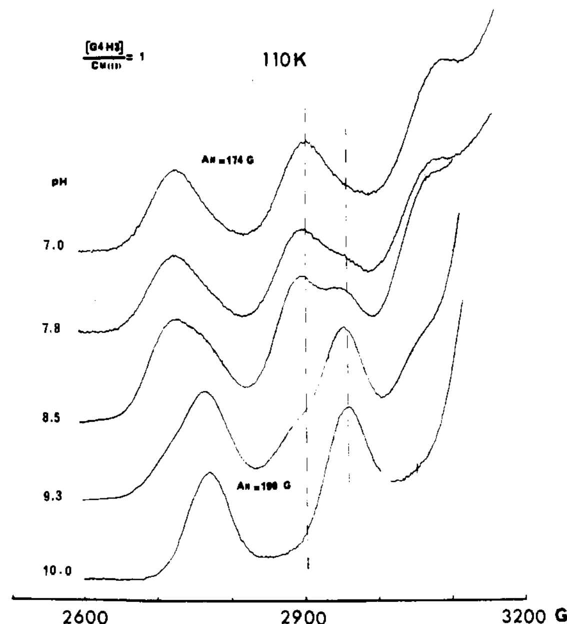

Figure 2. EPR spectra of solutions containing G4H3 and Cu(II) (ligand:metal ratio 1:1) at 110 K at different pH values (part corresponding to the $g_\parallel$ region).

Cu(II) from pH 4.4 to 11.2, and the corresponding data and spectra at different pH values are reported in Table I and Figure 1. At low pH (<5) the absorption spectrum of G4H3–Cu(II) presents a broad maximum near the infrared region with a very small molar absorbance. When the pH is raised, the absorption band in the visible region shifts to higher energy with a concomitant increase in extinction coefficient. Up to pH 7.0 the visible spectrum had an absorption maximum at 610 nm with a shoulder near 540 nm. As the pH was increased above 7.0, the absorbance at 610 nm decreased, while that at 540 nm increased. Finally up to pH 10.0 only the 540-nm absorbance is observed.

EPR Spectra. EPR spectra of G4H3–Cu(II) solutions show clear evidence of paramagnetic complex formation. Figure 2 shows the spectral features of G4H3–Cu(II) upon variation of pH at low temperature (110 K) corresponding to the $g_\parallel$ region. Three of the four well-resolved Cu(II) hyperfine splittings are observed, which result from the interaction between the electron spin and the nuclear spin of Cu(II) ($I = {}^3/_2$). As illustrated in this figure, two distinct types of Cu(II) signals are observed during its binding to G4H3 over pH 7.0–10.0. But we did not detect additional EPR absorptions at ~1500 G near $g = 4$ observed in a spin-coupled Cu(II) dimer. Hence, these Cu(II)–peptide complexes are considered to be mononuclear. In Figure 2, at pH ≤7, the observed spectrum is almost entirely due to a single species. Between pH 7.0 and pH 10.0, the EPR spectra are approximately made up of the superimposition of the two types while, at pH ≥10, only one form is present. These results are in accord with those obtained by visible spectroscopy. The low-pH form of the G4H3–Cu(II) complex exhibits smaller $A_\parallel$ and larger $g_\parallel$ values than the corresponding values obtained for its high-pH form. The EPR parameters for the first species are (the spectra are assumed to be axial; i.e., $g_x \simeq g_y$) $g_\parallel = 2.25$, $g_\perp = 2.06$, and $A_\parallel = 174$ G, and those for the second species at basic pH are $g_\parallel = 2.20$, $g_\perp = 2.05$, and $A_\parallel = 190$ G.

Extensive superhyperfine structure is seen at room temperature. These fine-structure transitions arise from the interaction of the nuclear spin of nitrogen ligands ($I = 1$) on G4H3 with the unpaired electron density on Cu(II). An examination of the EPR spectrum at pH 7.0 shows (Figure 3a) the presence of seven lines, while nine lines are observed under more basic conditions (pH 10.0, Figure 3b).

NMR Spectra. The presence of an unpaired electron in a molecule gives rise to large shifts and broadening effects of the NMR resonances of nearby nuclei. If the rates of dissociation (k_{diss}) of the transition-metal ion from the ligand are in the NMR

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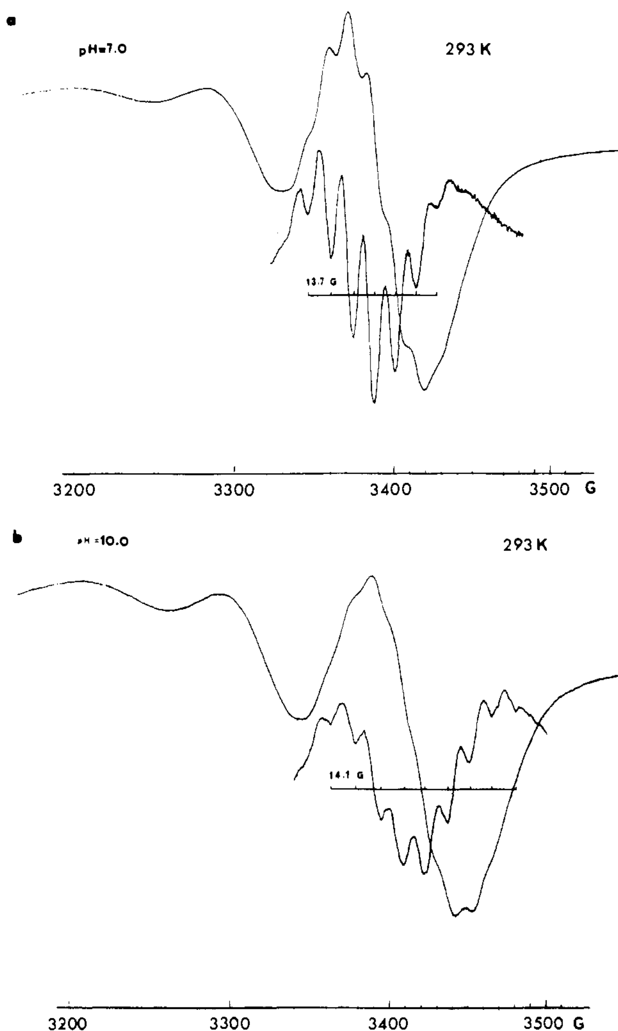

Figure 3. EPR spectra of 1:1 molar ratio solutions for G4H3:Cu(II) at pH 7.0 (a) and 10.0 (b) and at room temperature. The insert spectra represent the second derivative and show a nitrogen nuclear superhyperfine structure.

slow-exchange limit on the chemical shift time scale (which are of the order of 10–10^3 s^{-1}), the resultant shifted and broadened resonances will be observed separate from the resonances of the diagmagnetic ligand. As Cu(II) ions generally exhibit a relatively long relaxation time for the unpaired electron ($\tau_s^{-1} \approx 10^8$ s^{-1}),[21] its dominant effect is line broadening; the relaxation in the bound sphere is so efficient that $1/T_{2M^2} \gg \Delta\omega_{M^2}$, and one can assume that $\Delta\omega_M$, the shift between the resonances of free and bound ligand, is negligible. This is consistent with different observations on Cu(II)-containing systems.[22-24] Nevertheless, Bertini et al.[25] have reported ^1H shifts of greater than 20 ppm for Cu(II) complexes of a series of bis(*N*-alkylsalicylaldiminato) derivatives, ligands which are very different from peptides. The theory of the paramagnetic line broadening of NMR spectra undergoing chemical exchange has been treated elegantly by Swift and Connick[21] and by Luz and Meiboom.[26] The readers are directed to these papers for more information.

As has been generally encountered, the large paramagnetic broadening observed in the presence of small amounts of Cu(II) indicates that the metal ions are effectively exchanging between the various donor sites. Under these conditions, each resonance

Table II. ^{13}C Chemical Shifts and Line Widths of G4H3 and G4H3–Cu(II) in D_2O at 62.8 MHz (pH 10.8)

resonances	free ligand δ	1:1 complex δ	1:1 complex $\Delta\nu_{1/2}$, Hz
His CβH_2	30.70		
	30.84	30.14	20
	31.14		
Gly CαH_2	44.96		
	45.34	44.32	250
	45.69		
His CαH_2	56.19		
	56.40	58.38	20
	56.46		
His C(2)H	119.74		
	119.83	119.76	100
His C(5)	135.45		
	135.56	135.50	100
His C(4)H	138.79	138.72	100
Gly CO	173.85	173.82	
	173.91		
	174.08	173.99	80
	174.64	174.52	
His CO	176.03		
	176.09	177.90	20
	176.35		

corresponds to the weighted average of unbound and bound ligand. The broadening studies rely on "fast exchange" conditions.[22,27]

NMR spectra obtained from the complexation studies of G4H3 with Cu(II) show a rather curious and unusual behavior of a Cu(II)-bound peptide. Indeed, unlike the case for the other peptide–Cu(II) complexes, where the ligand to metal ratio must be as high as 10^2–10^3, it is necessary to use a very low ligand: Cu(II) ratio to detect changes in the behavior of certain resonances. Thus, for a ligand to metal ratio of 30 as one goes from acidic to basic conditions, the signals of the imidazole protons His C(2)H and His C(4)H and, to a lesser extent, the His CβH_2 protons broaden with increasing pH until neutral pH and again sharpen when the pH rises above 8. The Gly CαH_2 protons seem not to be affected. Similar observations have been encountered by ^{13}C resonances under similar conditions.

However, the most surprising and interesting results come from the spectra under basic conditions. Indeed at pH 10.8 and at a G4H3 to Cu(II) ratio as low as 5, there is no evidence of a change in ^1H resonances compared to those of the free peptide (cf. Figure 4). But at high levels of Cu(II) (G4H3:Cu(II) = 2), we can see in the downfield part of the spectra the appearance of two new resonances corresponding to the C(2)H and C(4)H protons of the imidazole residues. These resonances are shifted downfield by less than 0.1 ppm. The increasing addition of Cu(II) to the ligand results in a progressive increase of intensity of both C(2)H and C(4)H corresponding to the complex while those corresponding to the free ligand decrease and disappear near the 1:1 stoichiometry. Similarly, in the region 3–4 ppm, the His CβH_2 resonances shift downfield by ~0.2 ppm upon complexation while the resonances corresponding to the four Gly CαH_2 protons broaden and disappear in the noise with increasing concentration of Cu(II).

Striking observations are also made in the ^{13}C NMR spectra. Addition of less than stoichiometric amounts of Cu(II) to a solution of G4H3 at pH 10.8 results in a decrease of the intensity of the resonances for free G4H3 with a proportional appearance of a new set of resonances. At a G4H3:Cu(II) ratio of 1:1, the ^{13}C NMR spectrum does not indicate any resonance due to metal-free G4H3. Due to its very low T_1 relaxation time (vide infra) the spectrum corresponding to the 1:1 complex shows a very poor signal to noise ratio. The chemical shifts of the metal complex and the free ligand, as well as the line width of the complex at

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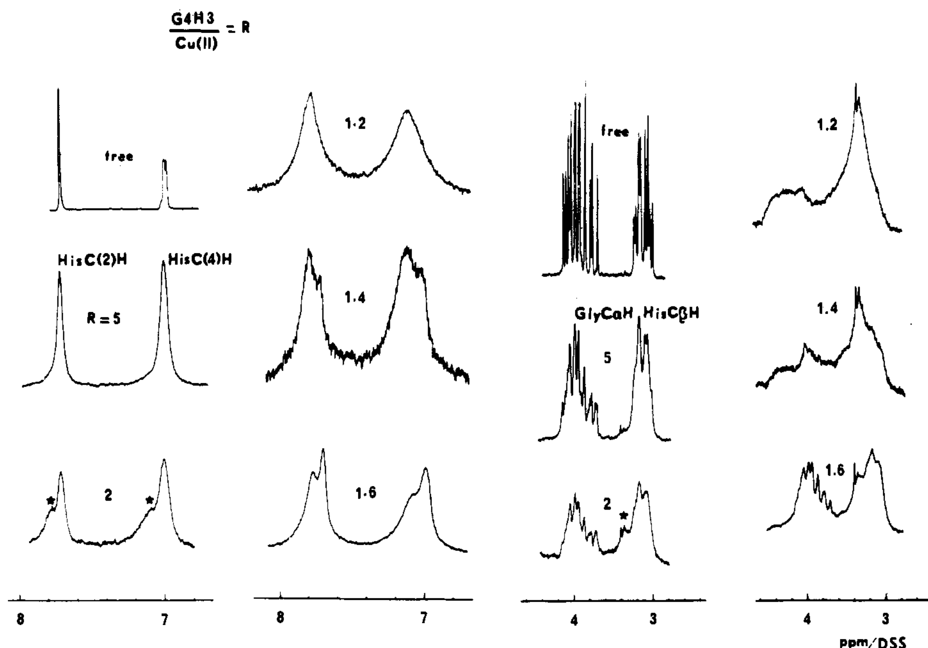

Figure 4. ^{1}H NMR spectra (7–8 and 3–4 ppm region) of G4H3 for different R ratios at pH 10.8. Asterisks denote formation of the 1:1 complex.

half-height, are presented in Table II. Both downfield and upfield shifts are observed upon complexation. The largest changes occur for the carbon resonances of the following substituents: His CαH (Δ = 2.03 ppm), His CO (1.80 ppm), and, to a lesser extent, Gly $C\alpha H_2$ (−1.00 ppm) and His $C\beta H_2$ (−0.70 ppm). The resonances corresponding to His C(2)H, C(4)H, and C(5) and Gly CO are not shifted. In addition, we observed the largest broadening occurring for the $C\alpha H_2$ protons of the glycine residues ($\Delta\nu_{1/2} \approx$ 250 Hz), while the three resonances of the imidazole moiety show an average broadening of ~100 Hz. All the other resonances are unaffected, having $\Delta\nu$ values less than 20 Hz.

Discussion

The electronic spectra of the G4H3–Cu(II) system recorded at various pH values exhibit broad absorption bands in the visible region. As shown in Figure 1, an isosbestic point at 580 nm indicates that two compounds are present in the solution. According to the classical ligand field theory, as stronger ligands enter the Cu(II) coordination sphere and replace water molecules, the crystal field strength increases and the absorption shifts to higher energies. Hence, the red shifts of the bands indicate that the donor atoms of the species predominant in the low-pH region exert a smaller ligand field strength as compared to the high-pH ones. Thus, λ_{max} > 700 nm corresponds to a Cu(II) complex bound to oxygen atoms whereas the blue color of peptide complexes with $\lambda_{max} \approx$ 610 nm is typical of square-planar coordination involving a 3 N, O configuration.[28] The color change to violet under alkaline conditions and the blue shift absorption (λ_{max} = 500 nm) are consistent with the involvement of a 4 N configuration around Cu(II).

The EPR spectra also show the existence of two paramagnetic species in solution where relative abundance depends on the pH of the solution. Under acidic conditions, a broad absorption results from the rapid tumbling of the relatively small hydrated Cu(II) ions. Above pH 4, when a stronger chelatelike complex begins to form, additional absorptions appear at higher fields and we observe the partly resolved four-line pattern of a typical peptide–Cu(II) complex. Random motion of solute molecules at 293 K yields an isotropic spectrum from which g_0 and the nuclear hyperfine structure constant A_0 can be measured. Thus, at pH 6.5, the observed spectrum is almost entirely due to a single species with g_0 = 2.123 and an isotropic hyperfine coupling constant of about 70 G, while under basic conditions, the hyperfine coupling constant is larger, A_0 = 92 G, and g value smaller, g_0 = 2100. Furthermore, Figure 3 clearly indicates that the EPR splitting corresponds to the superhyperfine interaction with neighboring nuclei, namely, nitrogen (^{14}N, I = 1) atoms. Seven and nine superhyperfine lines, at pH 7.0 and 10.0, respectively, support strongly the coordinations of three and four nitrogen atoms with Cu(II).

Frozen solutions (110 K) of the G4H3–Cu(II) complexes give resolved anisotropic EPR spectra. In liquid solution, the complexes are randomly oriented and rotating freely, whereas in the frozen state they are fixed. From this, the values of $g_\parallel$ and $A_\parallel$ can be measured accurately. The data observed show the pattern typical for nearly tetragonal symmetry ($g_\parallel > g_\perp$), having well-resolved parallel hyperfine structure and large $A_\parallel$ values. These $g_\parallel$, $g_\perp$, and $A_\parallel$ values are in the range expected for N- or O-bonded Cu(II). Furthermore, Peisach and Blumberg[29] have pointed out that $A_\parallel$ and $g_\parallel$ vary with the composition of the ligand atoms bound to Cu(II). Thus a structural assignment of a given Cu(II) complex can be made on the basis of its position in the plot of $A_\parallel$ vs. $g_\parallel$; e.g., the effect of increased electron-donating ability on the EPR parameters is to decrease $g_\parallel$ and increase $A_\parallel$. Our results confirm the presence of two complexes having an axial symmetry and can be assigned to that of a mononuclear Cu(II) complex with $d_{x^2-y^2}$ ground state. At neutral pH, these results are compatible with three-nitrogen and one-oxygen (3 N, 1 0) ligation around Cu(II), while at basic pH four nitrogens (4 N) are involved in the coordination.

Nevertheless, our NMR observations are certainly the most significant, as they have never been observed in peptide–Cu(II) complexes. Here also, two independent complexes are clearly evident in pH-variation experiments. From the pH dependence of ^{1}H line width it is clear that no complexation occurs at pH <4. In contrast, at higher pH, the large and specific broadening of the $C\beta H_2$ histidine and C(2)H, C(4)H imidazole protons together with the negligible effects observed on the glycine protons clearly indicate, around neutral pH, the binding to the three imidazole functions. As was always observed with peptide ligands,[22-24] no chemical shift changes are detected for a peptide to metal ratio as low as 30. Consequently, the paramagnetic line-broadening study can be treated by the Swift and Connick relations.[21] The study of the broadening provides information about the dynamics of the exchange of G4H3 between its free form and the G4H3–

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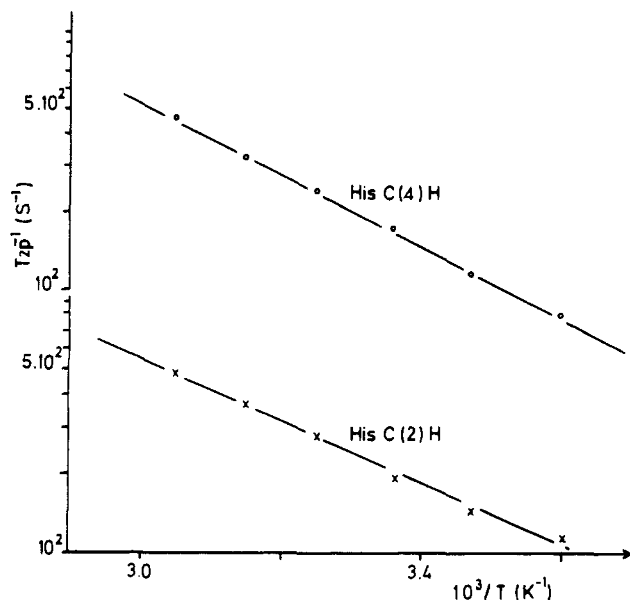

Figure 5. Temperature variation of experimental relaxation time (T_{2p}) for G4H3–Cu(II) at pH 6.40 (T_2 values measured by the line width at half-height).

Cu(II) complex. In order to elucidate the mechanism of the relaxation time, T_{2p}, we studied the temperature dependence of line broadening. Indeed, the effect of temperature on the observed values of T_{2p}^{-1} can be used to determine the dominant (slowest) process.[26] The logarithm of the broadening of the C(2)H and C(4)H resonances by Cu(II) as a function of the inverse of the temperature is shown in Figure 5 at pH 6.40. The line broadening was found to increase with increasing temperature in the range from 5 to 65 °C. In this temperature region, therefore, the system is in slow exchange on the ^{1}H NMR time scale between free and bound environments, and the line-broadening effects are governed by the exchange rate $1/\tau_M$,[21,30] which is equal to the dissociation rate constant of the G4H3–Cu(II) complex. Since the temperature dependence of the line broadening indicates that our system is in the regime of slow exchange, the residence time τ_M of the ^{1}H nucleus in the first coordination sphere of the complex can therefore be obtained from the ^{1}H line widths of the cyclic peptide in the presence of Cu(II). A plot of the total G4H3 concentration vs. the reciprocal line broadening, $1/\Delta\nu_{1/2}$, yields a straight line with a slope corresponding to $1/\tau_M$. From our experimental plots we obtain a value of ca. 8×10^{-4} s for the residence time and a value of 1.25×10^3 s^{-1} for the dissociation constant. An identical value has recently been obtained by Kodaka et al.[31] with a cyclic hexapeptide containing histidyl residues, where it was suggested that the imidazole groups are directly coordinated to Cu(II). These values correspond to the lower limit of the slow exchange;[24] hence, our system is in "quasi" slow exchange. The assumption was made that no chemical shifts occurred between free and complexed ligand (vide supra). But, as will be discussed below regarding the phenomenon observed at basic pH, chemical shifts can take place between the bound and unbound states. Furthermore, since τ_M corresponds to the lower limit of the slow process, an exchange-broadening effect may also be operative. Under these circumstances, our residence time value should be taken with caution.

Results obtained at basic pH are unambiguous and more informative. As was observed until now in peptide–Cu(II) complexes, no shifting of resonances is expected to result from the presence of unpaired electrons on the metal ion; however, broadening occurs very strongly.[22,23,27,32] Our results seem to be very different from these observations. We report in Figure 4 and Table II the data obtained by ^{1}H and ^{13}C resonances at pH 10.8. From these data, the following points have emerged.

(i) Unlike the case for the other peptide–Cu(II) complexes, the very high concentration of Cu(II) used does not affect the good quality of the spectra.

(ii) When Cu(II) is added to G4H3 solution, there is a significant perturbation in the frequencies arising from several groups in this peptide (both shifting and broadening).

(iii) From Figure 4, it is clear that the histidine $C\beta H_2$ and aromatic resonances show approximately the same broadening ($\Delta\nu_{1/2} \approx$ 30–40 Hz) and are all shifted downfield by ~0.1–0.2 ppm in the presence of Cu(II), while the glycine $C\alpha H_2$ resonances are broadened beyond detection by increasing the concentration of Cu(II). Similarly, the ^{13}C results show that the Gly $C\alpha H_2$ resonances are the most broadened (Table II). Both negative and positive shifts are observed upon complexation. Interestingly, the resonances of the CO groups of the histidine moiety are all shifted downfield by Cu(II), whereas the Gly CO peaks seem to be slightly affected.

(iv) The fact that a new set of resonances is observed and that increasing additions of Cu(II) increase the intensity of these resonances with a proportional disappearance of some resonances corresponding to the free ligand indicate that the G4H3 is in slow exchange on the ^{1}H and ^{13}C NMR chemical shift time scale between its free and complexed states.

(v) At a G4H3:Cu(II) ratio of 1:1, both ^{1}H and ^{13}C spectra do not indicate any resonances due to metal-free G4H3. These results strongly suggest that we have a 1:1 complex.

No attempts were made to explain the direction (both upfield and downfield) or the magnitude of the chemical shifts that occur upon binding of the peptide to the Cu(II) ion. However, in relation to different observations, the delocalization in the electron density about Cu(II) has been shown to affect strongly the broadening of protons and carbons in the vicinity of the metal ion, i.e., the Gly $C\alpha H_2$ nuclei in our case. On the basis of these observations, we suggest, in agreement with the visible and EPR spectra, that the four deprotonated peptide Gly NH nitrogens are involved in the site of metal ion complexation.

Since the resonances corresponding to the bound and unbound ligand are clearly observed, the Swift and Connick[21] relations are not valid. However, in these circumstances, the upper limit on the exchange rate in the "well-observed" case can be mathematically described by the condition

$$2\pi(\Delta\nu_M)\tau_M \gg 1 \quad (1)$$

where $\Delta\nu_M$ is the chemical shift difference (in Hz) between bound and free ligand and τ_M is the lifetime of the ligand bound to the metal ion.[33] This condition refers to all individual resonances, and it is applied to the different $\Delta\nu_M$ values. The smallest chemical shift perturbation induced by Cu(II) was found to be ca. 20 Hz at 250 MHz for the imidazole resonances. From this ^{1}H chemical shift separation, we estimate that the upper limit of the lifetime of the G4H3–Cu(II) complex is $\tau_M \gg 10^{-2}$ s and, consequently, the exchange rate or the dissociation rate (k_{diss}) of the metal between these species is estimated to be $k_{diss} \ll 100$ s^{-1}.

The present data revealed some significant and heretofore unknown properties that may have relevance in studying the paramagnetic behavior of Cu(II) by NMR spectroscopy. One of the interesting questions arising from these results is how to explain this rather unusual and unexpected NMR observation. We suggest, on the basis of our study, that the metal is firmly trapped inside the cycle, similarly to what occurs with cryptates. The Cu(II) ions are tightly bound to the macrocyclic ligand and form a kinetically stable complex.

In the past few years, Solomon's equation,[34] giving the transverse relaxation time T_{2M}, has been the basis of many NMR line-broadening studies in which $1/r^6$ dependence has been used to delineate the nuclei closest to the metal center and subsequently

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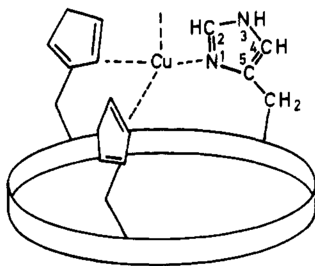

Figure 6. Proposed structure of the Cu(II)–G4H3 complex at neutral pH.

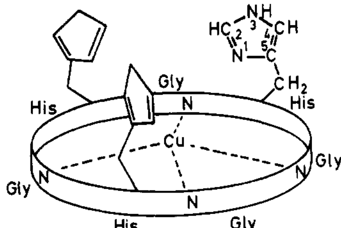

Figure 7. Proposed structure of the Cu(II)–G4H3 complex at basic pH.

determine the geometry of Cu(II) complexes in solution.[35] Unfortunately, as pointed out by Espersen and Martin,[27] a large difference between the spin–lattice relaxation time (T_{1M}) and the transverse relaxation time (T_{2M}) is not unexpected for copper complexes ($T_{1M} \gg T_{2M}$), indicating in the T_{2M} relation an important contribution from the scalar term, which is not distance-dependent. Our results[23,32] and those of many others[22,36] agree well with this assumption. Consequently, selective broadening experiments may not reflect the explicit r^{-6} distance dependence of the dipolar term, even though they may still be used to determine the sites of Cu(II) binding.

On the basis of these observations, it will be interesting to know what the predominant factor is that controls the line broadening in our 1:1 complex. Our first results obtained by measuring the relaxation parameters show that the values of T_{1M}/T_{2M} ratios are less than 2. Consequently, the measured T_{2M} values are largely determined by the dipolar term, implying therefore the possibility of the determination of the copper–nuclei distances.[37] If such major findings are confirmed, they should provide an important contribution to the knowledge of NMR spectroscopy of paramagnetic Cu(II) complexes of biological molecules.

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Concluding Remarks

The following are the main conclusions drawn from the present studies:

(1) The NMR, EPR, and visible spectral data presented herein give direct evidence that, in aqueous solution, the Cu(II) ion interacts with G4H3 to give two kinds of complexes depending on the pH. Around neutral pH, binding occurs through the three N(1) imidazole residues (Figure 6), whereas at basic pH Cu(II) binds via four Gly NH deprotonated peptide nitrogens (Figure 7).

(2) The NMR investigation gives evidence that both complexes are in "slow exchange" conditions, contrary to the general assumption of "fast exchange" in such systems.

(3) A major finding concerns the unusual behavior of G4H3-bound Cu(II) at basic pH, which reveals for the first time the observation of the NMR spectra for a 1:1 Cu(II) complex with this kind of ligand. This phenomenon suggests that Cu(II) is very tightly bound in the cyclic cavity and forms an anomalous long-lived complex.

(4) Unlike the general features underlying the paramagnetic line-broadening properties of Cu(II), our relaxation parameters seem to indicate the predominance of the dipolar term in the T_{2M} relation. Consequently, and for the first time to date, it appears that the ^{1}H and ^{13}C NMR technique will be very useful for obtaining structural information about such complexes in solution. These results raise some important questions in relation to the interpretation of the NMR data of paramagnetic Cu(II) complexes in solution made by several authors.

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Oxygen Atom Transfer in the Oxidation of Triphenylphosphine by Molecular Oxygen Catalyzed by an Ru(III)–EDTA–PPh_3 Complex

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The oxidation of PPh_3 to $OPPh_3$ in the presence of Ru(III)–EDTA, PPh_3 (1:10), and molecular oxygen in 50% dioxane–water proceeds through the formation of intermediate complexes $[Ru^{III}(EDTA)(H_2O)]$ (**1**), mixed-ligand $[Ru^{III}(EDTA)(PPh_3)]$ (**2**), and the (μ-peroxo)ruthenium(IV) complex $[Ru^{IV}(EDTA)(PPh_3)]_2O_2$ (**3**). The formation of complexes **1–3** has been supported by potentiometric, spectrophotometric, electrochemical, and kinetic measurements. The rate of oxidation of PPh_3 is first order with respect to Ru(III)–EDTA complex, first order with respect to PPh_3 and one-half order with respect to molecular oxygen, thus supporting the formation of **3** as an intermediate in the oxygenation reaction of PPh_3. The proposed rate-determining step in the oxidation of PPh_3 is the formation of an intermediate ruthenyl complex species $[Ru^{V}{=}O(EDTA)(PPh_3)]$ (**4**) followed by a fast inner-sphere transfer of oxygen atom from **4** to PPh_3 to form $OPPh_3$ and the regeneration of $[Ru^{III}(EDTA)(H_2O)]$ (**1**). The system represents an example of a truly catalytic Ru(III) oxygen atom transfer system with a simple non-porphyrinic ligand and molecular oxygen as the oxidant.

Introduction

The oxidation of triphenylphosphine to triphenylphosphine oxide has attracted much interest in recent years due to its relevance to oxygen atom transfer reactions catalyzed by cytochrome P-450 oxidase.[1-3] A number of oxygen atom transfer reactions to various substrates were reported with Fe(III) ion[4] and Fe(III) porphyrins.[5-12] In these reactions the catalytically active $Fe^{IV}{=}O$ species

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