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Spectroscopic and potentiometric study of copper(II) complexes with L-histidyl-glycyl-L-histidyl-glycine in aqueous solution

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Abstract

The complex formation between copper(II) and the tetrapeptide L-histidyl-glycyl-L-histidyl-glycine (HL) has been studied in aqueous solution in the pH range 2–10.5, by potentiometric and spectroscopic methods (visible, CD, EPR, ¹H NMR and Raman scattering). Between pH 3 and 6 the species [CuHL]²⁺ and [CuH₁L] have been detected. The former complex co-ordinates through two nitrogen and two oxygen atoms in the equatorial plane while, for the latter, the spectroscopic data (particularly Raman spectra) suggests an unusual structure with the two imidazole rings and two peptide NH in the plane. The species at pH 7, [CuH₂L][−], is involved in the co-ordination by the amino group, the His¹ imidazole and two peptide nitrogens, while, for the complex [CuH₃L]^{2−}, which seems to be predominant in alkaline medium, a third deprotonated backbone NH, replaces the imidazole nucleus. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Copper complexes; His containing peptides; Tetrapeptides; NMR, EPR, IR, Raman, UV and CD spectra

1. Introduction

Histidyl residues play an important role in peptide and protein co-ordination with biological metal ions. Enzymes like superoxide dismutase,

carbonic anhydrase and nitrogenase as well as transport proteins like albumin offer one or more imidazole rings on histidine side chains into the co-ordination sites [1–5]. On the other hand, Cu^{II} is one of the more involved ions in protein-mediated biological processes. For this reason a wide range of oligopeptide–copper complexes mimicking the functionality of the enzyme active sites,

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have been studied and among them, histidine containing sequences have been subjected to special attention. In particular, copper complexes of linear di-, tri-, tetra-, and penta-peptides with one or two histidine residues were extensively investigated [6–17]. The anchoring sites for the copper ion in these peptides is a competition between the NH_2 and the COOH terminal groups, the deprotonated peptide NH , in addition to the N^π of the imidazole ring. It was claimed that the latter is the first anchor site of the metal to the peptide, even in the presence of the free terminal amino group [6–13]. The researches have pointed out that the position of the histidine in the peptide sequence has a great influence on the structure and stability of the Cu^{II} complexes. The presence of a histidine moiety in the second or third position exerts a co-operative effect on the deprotonation of the adjacent peptide NH [7,8]. As a consequence of the different coordination sites of the Cu^{II} ion dramatic changes in the biological properties of these complexes may also arise, i.e. in the mechanism of the O_2^- dismutation [17].

In the course of our researches on the synthesis and properties of highly flexible linear and cyclic histidyl-glycine peptides we prepared the tetrapeptide $\text{H}-(\text{L-His-Gly})_2\text{-OMe}$ (L_{Me}) [18]. We also reported a preliminary EPR study on the interactions of this peptide with the paramagnetic ions Cu^{II} , Mn^{II} and $\text{V}^{\text{IV}}\text{O}$ at different pH and metal-to-ligand molecular ratios [19]. Then we undertook to investigate the properties of the copper complexes of the free carboxylic acid $\text{H}-(\text{L-His-Gly})_2\text{-OH}$ (HL). This substance has been previously considered by Ueda et al. [20] only at pH 8, suggesting a different role of the two histidyl residues but without giving information on the stoichiometry of the involved species. We also carried out in vitro and in vivo researches on the activity of HL complexes with copper ions which will be published elsewhere. All these considerations prompted us to extend the physico-chemical study of this system over a wider pH range (2–11) and at different ligand-to-metal ratios. To distinguish and characterise the equilibrium species in aqueous solution, a multitask approach is suitable [21]. The study herein reported was performed by integrating several spectroscopic tech-

niques (UV/Vis, CD, EPR, ^1H NMR and Raman), combined with potentiometric determinations and computer modelling optimisation [22].

2. Experimental

The hydrolysis of the trifluoroacetate salt of the pure L_{Me} [18] to the free HL was performed in NaOH solution at pH 10.5–11.0, leaving the solution to stand for 3 h at room temperature (RT). A sample gave only one peak by analytical RP-HPLC, carried out on a Beckman System Gold apparatus using a Vydac ODS 218TP54 column (250×4 mm) with a linear gradient of CH_3CN -0.1% TFA in H_2O -0.1% TFA from 40 to 80% in 15 min. FAB MS, $[\text{M} + \text{H}]^+$ 429 (calculated for the sodium salt, $[\text{M}]^+$ 428.3). Preparative RP-HPLC gave a solution that, evaporated and treated with ether, afforded a colourless product (found: C, 34.2; H, 4.0; N, 14.1. $\text{C}_{16}\text{H}_{22}\text{N}_8\text{O}_5 \cdot 3\text{TFA} \cdot 1.5 \text{H}_2\text{O}$, requires: C, 34.1; H, 3.6; N, 14.4%). FAB-MS spectra were obtained on a VG 7070EQ spectrometer and elemental analysis with a Perkin–Elmer 240C apparatus.

2.1. Potentiometric measurements

$\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (Aldrich) was used in order to minimise competing anionic complexation. Potentiometric titrations were carried out in 0.1 M NaCl at a constant temperature of 25°C , following a procedure described elsewhere [23]. A digital Radiometer PHM-84 potentiometer and a Multidosimat piston burette were connected to an Olivetti M20 computer to control titrations. A Ross glass electrode (Orion, mod. 81-01) and a Ross reference electrode (Orion, mod. 80-05), immersed in the aqueous solution contained in a magnetically stirred and thermostated glass cell, were used for each experiment. A nitrogen stream over the surface of the solution avoided carbon dioxide contamination.

The protonation equilibria of L_{Me} were studied by dissolving a weighed amount of the solid compound in ca. 100 ml of 0.1 M NaCl containing a known quantity of HCl in a large excess. Titra-

Table 1

Experimental data of the potentiometric measurements for the Cu^{II}-complex formation and/or protonation of the ligands L_{Me} and HL in 0.1 M NaCl at 25°C

Ligand × 10 ³ (mol) ^a	Copper(II) × 10 ³ (mol) ^b	H ⁺ × 10 ³ (mol) ^c	Titrant (mol dm ⁻³) ^d	pH-range	Number of points ^e
H-(L-His-Gly) ₂ -OMe					
0.0474		0.2659	-0.1130	4.73–8.92	31
0.0484		0.3924	-0.1139	4.31–8.13	31
0.2032		0.8024	-0.0974	5.20–7.85	62
H-(L-His-Gly) ₂ -OH					
0.0474		-0.0495	0.1237	8.86–3.34	42
0.0484		-0.2363	0.1237	7.93–2.83	66
0.0484		0.6296	-0.1139	3.30–8.08	48
0.0350		-0.0323	0.1242	9.00–3.27	73
0.2032		-0.3190	0.0964	8.08–2.42	118
0.0484	0.0262	-0.0729	0.1236	7.40–3.31	54
0.0461	0.0453	0.5017	-0.1139	3.29–7.17	64
0.2032	0.0541	1.2234	-0.0974	3.25–9.15	93

^a Initial amount of ligand.

^b Initial amount of copper(II) ions.

^c Initial amount of hydrogen ions.

^d HCl titrant concentration (negative values refer to NaOH).

^e Number of points from titration curve.

tions were made by stepwise addition of 0.1 M NaOH. Voltage (mV) and volume (ml) of added titrant were paper-printed and automatically recorded. Backward titrations with 0.1 M HCl revealed a lower amount of hydroxide ions in excess, the difference being attributed to the alkaline hydrolysis reaction that quantitatively occurred within 3 h. These potentiometric titration data were used to evaluate the protonation constants of HL. Stability constants of the system HL/Cu^{II} were studied using 1:1, 2:1 and 4:1 ligand-to-copper(II) molar ratios.

Experimental details for both protonation and complex-formation studies are summarised in Table 1. In all cases, the thermodynamic equilibrium constants were evaluated with the Superquad program [24], running on an Apple (Macintosh LC II) computer.

2.2. Spectroscopic measurements

¹H and ¹³C NMR spectra were acquired on a Varian Gemini-200, VXR-300 and, for large spectral width acquisitions (350 ppm), on a Bruker Advance 500 apparatus, in D₂O solutions, using

standard pulse programs. Chemical shifts are reported in ppm (δ) from TSP as internal standard. The relevant amount of Cu^{II} ion was taken from a 0.190 M solution (determined by atomic absorption on a Perkin–Elmer 5000 spectrometer) of anhydrous CuSO₄ (Merck) in D₂O. All ‘pH’ val-

Table 2

Basicity constants of the ligands L_{Me} and HL and stability constants of HL complexes with copper(II) in 0.1 M NaCl at 25°C^a

	log <i>K</i>	log β
L _{Me} + H ⁺ ⇌ HL _{Me} ⁺	7.338 (6)	
HL _{Me} ⁺ + H ⁺ ⇌ H ₂ L _{Me} ²⁺	6.370 (6)	
H ₂ L _{Me} ²⁺ + H ⁺ ⇌ H ₃ L _{Me} ³⁺	5.333 (7)	
L ⁻ + H ⁺ ⇌ HL	7.332 (11)	
HL + H ⁺ ⇌ H ₂ L ⁺	6.545 (10)	
H ₂ L ⁺ + H ⁺ ⇌ H ₃ L ²⁺	5.327 (12)	
H ₃ L ²⁺ + H ⁺ ⇌ H ₄ L ³⁺	3.082 (17)	
Cu ²⁺ + L ⁻ + H ⁺ ⇌ [CuHL] ²⁺		15.09 (2)
Cu ²⁺ + L ⁻ ⇌ [CuH ₋₁ L] + H ⁺		5.70 (2)
Cu ²⁺ + L ⁻ ⇌ [CuH ₋₂ L] ⁻ + 2 H ⁺		-0.54 (2)
Cu ²⁺ + L ⁻ ⇌ [CuH ₋₃ L] ²⁻ + 3 H ⁺		-8.52 (3)

^a Values in parentheses are standard deviations.

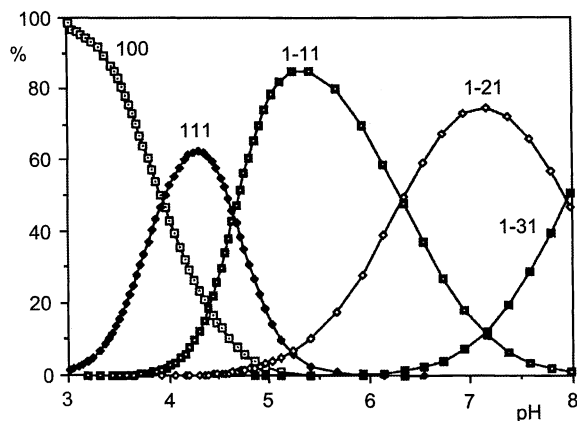


Fig. 1. Computed species distribution curves for the HL/Cu^{II} system (ligand-to-metal ratio 1:1), at 25°C in 0.1 M NaCl. Each species $M_pH_qL_r$ is indicated by subscripts p, q and r.

ues (uncorrected pH-meter readings) were determined, at RT, using a 302E Orma Electronic Digital pH meter equipped with a Wilmad glass electrode, into the NMR tubes, adjusting the pH with NaOD or DCl solutions (Fluka).

X-band EPR spectra were recorded at RT with a Bruker ER 200DD-SRC spectrometer. The operational frequency (9.44 GHz) was tested with a Hewlett–Packard X5-32 B wavemeter and the magnetic field was calibrated by DPPH as field marker. X-band EPR spectra at low temperature (LN) were recorded on a Varian E-9 spectrometer

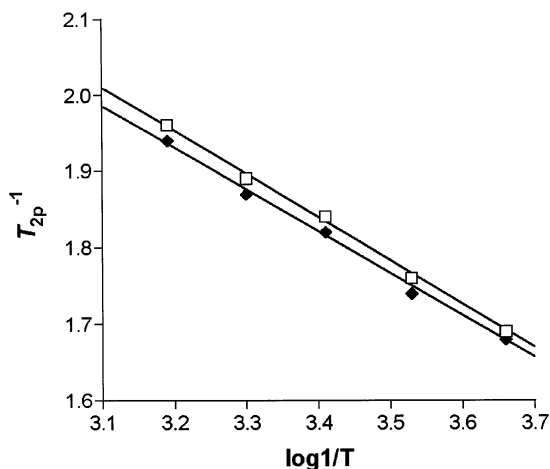


Fig. 2. Temperature variation of the experimental relaxation time (T_{2p}) at pH 6.15, ◆, His¹ 2-H, □, His³ 2-H.

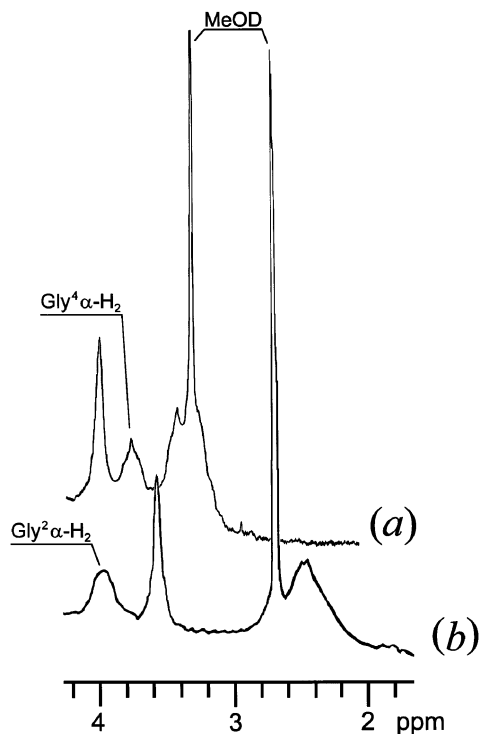


Fig. 3. ¹H NMR spectra of HL/Cu^{II} system in D₂O solution, (a) pH 2.9 with ligand-to-metal ratio 3; (b) pH 6.8 with ligand-to-metal ratio 20:1.

equipped with an on-line computer for data treatment. The g values were obtained by reference to the signal at $g = 2.0008$ [25].

Visible absorption data were recorded at RT on a Shimadzu 1601PC spectrophotometer and CD spectra on a Jasco J-500C spectropolarimeter. Raman spectra were registered using the 406.7, 457.9, 488.0, 514.5 and 568.2 nm exciting lines of an Argon or a Krypton ion laser, a Jobin–Yvon HG-25 monochromator, an RCA-C31034A cooled photomultiplier and a data processing system.

3. Results

3.1. Potentiometric titration analysis

3.1.1. Basicity constants

The basicity constants of HL were studied in 0.1 M NaCl at 25°C and the results are reported in Table 2.

From the potentiometric titration data only three $\log K$ values were evaluated for L_{Me} . They were consistent with the basicity of one amino and two imidazole nitrogens present in the molecule. The hydrolysis process occurred in the alkaline solution, due to the excess of hydroxide ions added during the forward titration. The back-titration with HCl solution displayed an early end-point in the potentiometric curve. This was attributed to the hydrolysis reaction for the consumption of hydroxide ions, the difference being equivalent to the esterified compound. In this condition, both forward and backward titrations confirmed four basic sites to be protonated. Each protonation step was easily recognised by the comparison of the $\log K$ values. For the esterified tetrapeptide the first and third protonation constants were closer to the corresponding $\log K$ of the hydrolysed compound. They were attributed to the amino, and imidazole nitrogens, respectively, belonging to the first histidyl residue. The second protonation step involves the imidazole nitrogen of the second histidyl residue that is close to the carboxyl group. The latter, when in the free form, shows more electronic withdrawing properties that leads to an increased value of $\log K_2$.

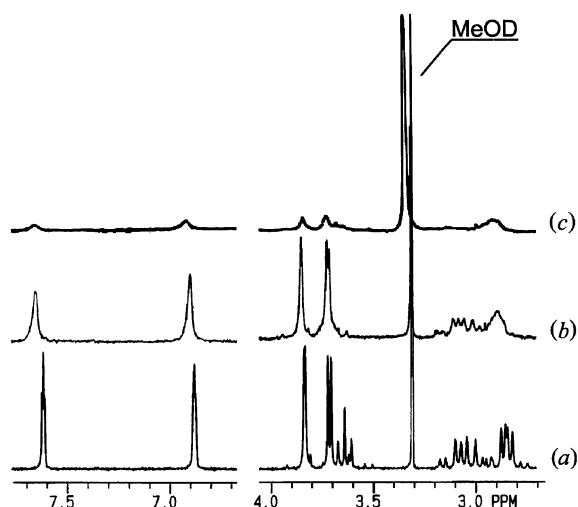


Fig. 4. ^1H NMR spectra of HL/ Cu^{II} system in D_2O solution, (a) pH 11, ligand alone; (b) pH 10.3 with ligand-to-metal ratio 5:1; (c) pH 10 with ligand-to-metal ratio 1.1:1.

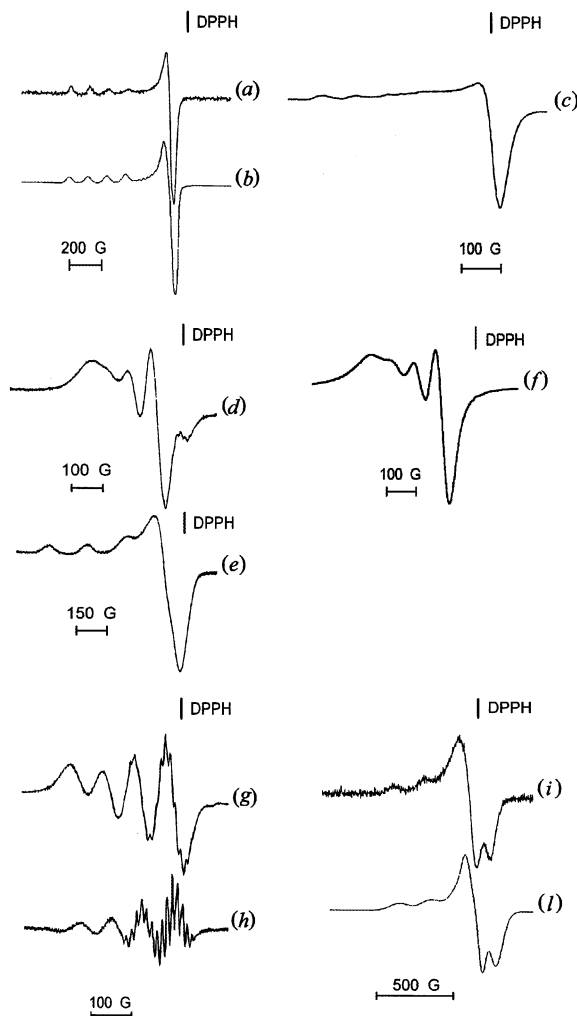


Fig. 5. Temperature dependent X-band EPR spectra of ligand/ Cu^{II} (5:1) in D_2O at different pH ($[\text{Cu}^{2+}] = 1 \times 10^{-3} \text{ mol dm}^{-3}$); (a) Cu^{II} - D_2O experimental (exp) spectrum at 100 K, (b) relevant computed (comp) spectrum; (c) ligand- Cu^{II} exp spectrum at 100 K, pH 2.7; (d) and (e) ligand- Cu^{II} exp spectra at 298, and 100 K, respectively, pH 5.5, (f) relevant comp spectrum at 298 K; (g) and (h) exp spectra (first, and second derivative, respectively) at 298 K, pH 7.0; (i) exp spectrum at 100 K, pH 7.0; (l) relevant comp spectrum at 100 K.

3.1.2. Stability constants

The behaviour of the ligand HL towards its complexing ability with copper(II) ion was studied in 0.1 M NaCl at 25°C. The potentiometric titration curve revealed end-points consistent with the displacement of protons equivalent to the amount

Table 3

Temperature and pH dependent X-band EPR parameters of the ligand–Cu^{II} system at ligand-to-metal ratio 5:1

pH range	g_{iso}	$g_{\parallel}$	$g_{\perp}$	$\langle g \rangle$	A_{iso}	$A_{\parallel}$	$A_{\perp}$ ^a	$\langle A \rangle$	$A_{\text{iso}}(\text{N})$
2.3 ^b	2.197	2.424	2.090	2.201	4.6	12.9	1.1	4.6	–
1.7–3.2	2.185	2.390	2.078	2.182	≤ 5.1	14.4	≤ 1.1	≤ 5.5	≤ 1.3
4.0–5.0	2.146	2.278	2.063	2.135	6.5	18.8	≤ 0.9	≤ 7.0	≤ 1.3
5.0–6.5	2.125	2.255	2.049	2.118	6.9	19.2	1.3	6.9	≤ 1.3
7.0–9.5	2.090	2.176	2.043	2.087	8.4	20.7	2.1	8.6	1.3

^a Computed value. $[\text{Cu}^{2+}] = 1 \times 10^{-3} \text{ mol dm}^{-3}$; $A_{\text{i}} \equiv 10^{-3} \text{ cm}^{-1} (\pm 0.6 \times 10^{-3})$; $g_{\text{i}} (\pm 0.006)$; $\langle g \rangle = 1/3(g_{\parallel} + 2g_{\perp})$; $\langle A \rangle = 1/3(A + 2A_{\perp})$.

^b Cu–H₂O binary system.

of Cu^{II} in solution. Several complex species were considered to be present in solution (pH range 3–9) and the best model, fitting potentiometric data, account for the following four species $[\text{CuHL}]^{2+}$, $[\text{CuH}_1\text{L}]$, $[\text{CuH}_2\text{L}]^{-}$ and $[\text{CuH}_3\text{L}]^{2-}$. For the ligand-to-metal ratios used, all other species introduced were discarded during the evaluation of $\log \beta$ by the Superquad program, as being negative or excessive in their standard deviations. This set of complexes shows some similarities with other findings in closely related systems, without being identical to any one of those previously reported [9,10,26]. In Fig. 1 are reported the significant complex species in the pH range of 3–8, as computed by the $\log \beta$ values.

3.2. Visible spectra

Visible optical spectra were obtained for solutions containing L/Cu^{II} = 4:1 and 1:1, for concentrations varying from 5×10^{-3} to $2 \times 10^{-2} \text{ M}$. In the experimental conditions used and depending on the pH values, three maxima were detectable in the range 500–600, 600–700, and above 700 nm, respectively.

3.3. NMR spectra

The ¹H signals of the ligand HL were assigned by H–H correlated (COSY) spectra and by comparing the chemical shifts taken for several pH values. This latter method was useful especially for the 2- and 5-H resonances of the two imidazole nuclei which were subjected to large and

different upfield shifts following the increasing pH. The α -H triplet of His¹ lies at higher field with respect to the corresponding His³ resonance [27,28], and it is markedly shielded by enhancing the alkalinity. The α -H₂ signals of Gly² and Gly⁴ were assigned observing that one of the two signals (namely the Gly⁴) was shifted upfield by raising the pH in the range 2.5–9.0. The same signal arose from the unique Gly α -H₂ resonance of L_{Me} during the alkaline hydrolysis. To detect broadening in the ¹H resonances we could use low HL/Cu^{II} ratios (from 2 to 30) instead of the usual range of 10^2 – 10^3 of non-peptide Cu^{II} complexes. The measured T_{2p}^{-1} was independent on the observation frequency (200 or 500 MHz). Further, no shift of the resonances of the free ligand in the bulk solution was observed even for low HL/Cu^{II} ratios. This fact suggests that $\Delta\omega_{\text{M}}$ (i.e. the shift of the resonances of free and bound ligand) is negligible, as found for several Cu^{II} complexes of amino acids and peptides [16,29]. Freeman [29], and Martin [30], demonstrated that, in the fast exchange conditions between the free and bound ligand, the distances between the Cu^{II} ion and the binding sites can be calculated by selective broadening studies for amino acid and peptide complexes with Cu^{II} only if $\Delta\omega_{\text{M}}$ is negligible and the scalar term is not predominant in the transverse relaxation mechanism. In our case, the temperature variation of the line broadening for both imidazole rings, tested at pH 6.15 (HL/Cu^{II} ratio 30) (Fig. 2), revealed that the system was not in fast exchange at intermediate pH and, consequently, T_{2M} could not be determined only on the basis of the line-broadening experiments.

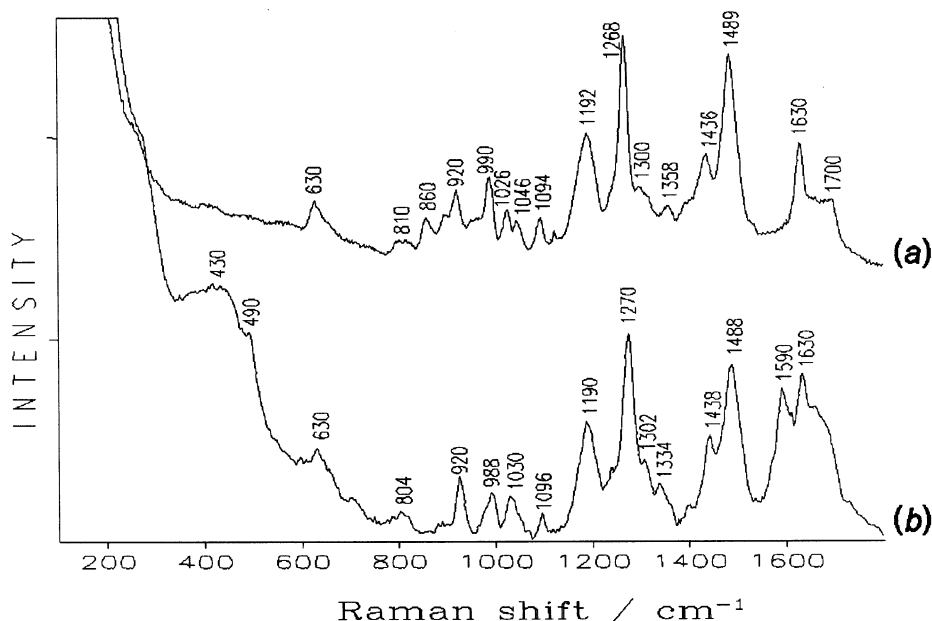


Fig. 6. Raman spectra of (a) the ligand HL and (b) HL/Cu^{II} system in 5×10^{-2} M aqueous solutions at pH 2.9 (457.9 nm exciting line).

Despite this fact, the works of Sarkar and Kodaka showed that, in peptide and protein/Cu^{II} complexes, selective broadening could be an efficient probe for testing which amino acid residues were involved in the co-ordination [16,31,32].

His¹ 2- and 5-H were first affected by the paramagnetic broadening with respect to the corresponding His³ lines. At pH values below 3 a significant line broadening for the His¹ signals appeared only when HL/Cu^{II} ratio was less than 5. Paramagnetic relaxation involved differently Gly² and Gly⁴ α -H₂ resonances, as a function of pH: at pH ca. 3 and for HL/Cu^{II} ratio 3, only the higher field signal corresponding to Gly⁴ lost the multiplet aspect and broadened (Fig. 3a). No distinction could be made between the His¹ and His³ β -H₂ resonances as both the multiplets broadened and overlapped. Raising the pH and for ligand-to-metal ratios in the range 3–5, all the signals progressively broadened and, at pH around 5, the peptide resonances were under the detection limit. At pH values above 6 and for a ligand-to-metal ratio as high as 20, the signals became detectable again. Now, the broadening of the Gly² and Gly⁴ CH₂ groups is reversed, with the Gly² α -H₂ much

more affected than the carboxy terminal amino acid (Fig. 3b). This behaviour indicated that, at these pH values, Gly² NH was involved in the Cu^{II} co-ordination also in the presence of a strong excess of ligand.

On going on from pH 8 to more alkaline solutions all the signals gradually sharpened and at pH above 10 the line width approximated that found in the free peptide, also for a ligand excess as low as 2 or 3 (Fig. 4a and 4b). Only for a very low ligand-to-metal ratio (1.1:1) broadening was still detectable (Fig. 4c, taken at pH 10). This behaviour recalls that which was reported by Sarkar et al. for cyclo(Gly-His-Gly-His-Gly-His-Gly) [16], and by Gajda and co-workers for glycylglycylhistamine [26]. The integrated intensities of the ligand signals were compared with the line of the CH₃OD formed from the in situ hydrolysis of L_{Me}. This line was unaffected by the presence of Cu^{II} in the whole pH range. The relative areas, measured for different ligand-to-metal ratios, were in good agreement with the corresponding percentage of the unbound peptide. Studies on the linewidth/temperature dependence, carried out in the range 0–80°C, showed no significant line broadening.

3.4. X-band EPR analysis

In Fig. 5 are collected the temperature dependent X-band EPR spectra [RT and LN temperature ($T=100$ K)] of the copper-tetrapeptide solution in D_2O (ligand-to-metal ratio = 5:1) as a function of pH. Computed spectra are shown in order to verify the accuracy of the best-fit parameters, which are reported in Table 3.

The EPR lineshape analysis of the cupric complexes is performed referring to the maxima in the plot of the complex species distribution (Fig. 1). The actual cupric $S=1/2$ Electron Spin Hamiltonian has the general form:

$$H = (\beta H g S + IAS) + \sum_i I_i(N)A_iS \quad (1)$$

with the unpaired electron mainly located in the cupric $3d_{x^2-y^2}$. In Fig. 5a is shown the axial LN spectrum of the $Cu^{II}-D_2O$ system ($g_{\parallel} > g_{\perp} > g_e = 2.0023$), with the corresponding computed spectrum in Fig. 5b [33]. The lineshape and the relevant parameters differ from those of the HL/ Cu^{II} system at pH 2.7 (ligand-to-metal ratio = 5:1), suggesting that at acidic pH the cupric ion interacts with the ligand. The corresponding LN spectrum is shown in Fig. 5c. The spectrum Fig. 5d refers to the $[CuH_{-1}L]$ species (pH 5.3, $T=298$ K), while Fig. 5e shows the relevant glassy spectrum. The RT computed spectrum is shown in Fig. 5f ($g_i > g_e$). At neutral pH, the new $[CuH_{-2}L]^-$ species becomes predominant. The relevant RT and glassy spectra are displayed in Fig. 5(g, i). The RT spectrum displays well resolved shpf(N) structures, particularly in the sec-

ond derivative mode (Fig. 5h), while the glassy corresponding one (Fig. 5l) exhibits only the cupric hpf resolution of the axial structure ($g_i > g_e$). The corresponding RT spectrum exhibits a noticeable $m_l(Cu)$ dependence of the overall ΔH lineshape, particularly on the fourth hpf absorption ($m_l = +3/2$) [34], due to incomplete averaging of the g_i and A_i anisotropies. Here, the resolution of the nitrogen shpf structure gives the direct evidence for the magnetic interaction of the unpaired electron with the equatorially bound nitrogen atoms. Higher pH than 7.0 does not induce significant changes in the temperature dependent cupric lineshape while, getting back to the previous acidic conditions, the reversal spectral sequence clearly indicates that the overall chemical equilibrium is totally reversible.

It must be pointed out that lower ligand-to-metal molar ratios (from 5:1 to 1:1) do not show significant variations both on the corresponding temperature dependent lineshape and on the relevant paramagnetic parameters. In particular, there is X-band EPR evidence for a better spectral resolution at the highest HL/ Cu^{II} molar ratios (5:1).

3.5. Raman spectra

Raman spectra of oligopeptides exhibit a common spectral feature, due to the presence of the amide bond. They consist of strong bands at ca. 1650 (amide I) and 1260 cm^{-1} (amide III), together with those, usually occurring in the 870–930 and 1000–1050 cm^{-1} regions, originated by

Table 4
Spectroscopic data (visible and CD) for copper(II)–peptide complexes^a

Species	λ (nm) d–d ^b absorbance maximum	CD ^c	
$[CuHL]^{2+}$	761 (20)	491 (0.92)	582 (–0.11)
	671 (42)		
$[CuH_{-1}L]$	530 (122)	492 (1.19)	593 (–0.135)
$[CuH_{-2}L]^-$	525 (153)	495 (1.29)	614 (–0.084)
$[CuH_{-3}L]^{2-}$	524 (172)	499 (1.40)	631 (–0.114)

^a L/ Cu^{II} = 1.1; $[Cu^{2+}] = 5 \times 10^{-3}$ mol dm^{–3}.

^b ϵ (dm^{–3} mol^{–1} cm^{–1}) in parentheses.

^c $\Delta\epsilon$ (dm³ mol^{–1} cm^{–1}) in parentheses.

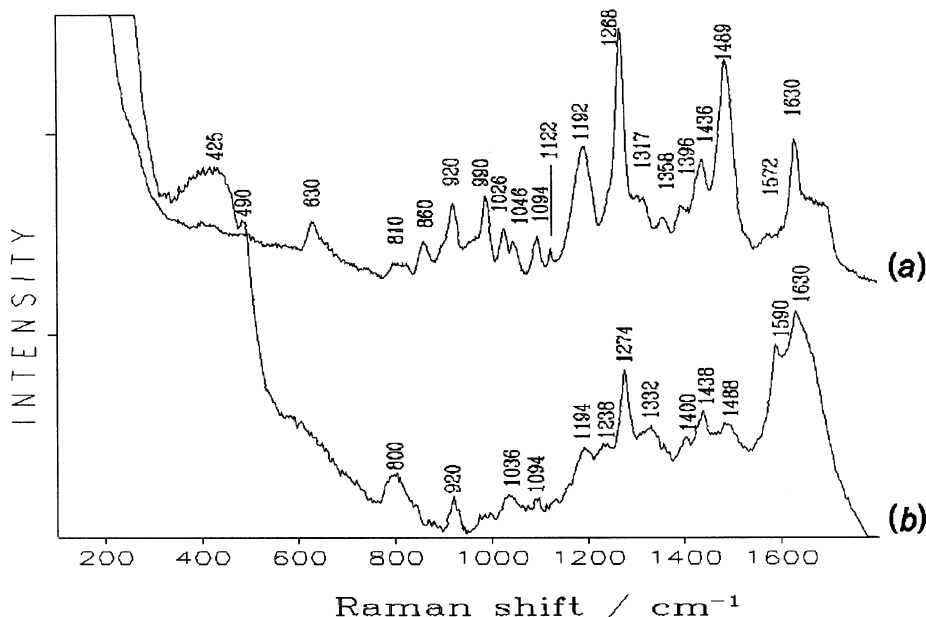


Fig. 7. Raman spectra of (a) the ligand HL and (b) HL/Cu^{II} system in 5×10^{-2} M aqueous solutions at pH 4.3 (457.9 nm exciting line).

C–C skeletal vibrations and C–N stretching modes [35–39]. These bands are easily identified in the Raman spectra of glycine, diglycine and triglycine molecules because their intensity depends from the increased number of peptide bonds. In the case of the peptide HL, most of the Raman bands observed at the different pH correspond to the strongest Raman bands of histidine (Figs. 6a, 7a and 8a). A suitable identification of the bands of the backbone and the histidine residue was a necessary prerequisite to detect the groups involved in the co-ordination with Cu^{II} and was performed by comparing the frequencies with the literature data [35–39]. A dramatic increase of the Raman signal is achieved through the Resonance Raman (RR) scattering, which occurs when the excitation light coincides with a UV-visible absorption band of the complex. Because only the vibrations associated with atoms of the chromophore are resonance-enhanced, the RR spectroscopy offers a selective probing of the metal-ligand centres of the oligopeptide, without interference from the peptide backbone. The visible absorption spectra of ligand-to-metal ratio 1:1 complex exhibit

maxima at ca. 530 nm in the pH range 4.6–10. Hence, the Raman signal obtained with the corresponding exciting frequency gains intensity from resonance contribution. Actually, more information arises from the pre-resonance spectra obtained with the 457.9 nm line (Figs. 6b, 7b, 8b and 9b), where both enhanced and non-enhanced bands can be detected, preventing photodegradation effects, which can occur under resonance conditions.

4. Discussion

At metal-to-ligand equimolecular or lower ratio (1:1, 1:2, 1:4) the pH-metric titration curves accounted for the same five species in the pH range 3–8 (Fig. 1). No dinuclear species nor bis-complexes were detected. Four peptide-to-copper bonded species, namely [CuHL]²⁺, [CuH₋₁L], [CuH₋₂L]⁻ and [CuH₋₃L]²⁻, are predominant at pH 4.3, 5.5, 7.0, and 8.5, respectively. At low pH values (ca. 3) the potentiometric analysis could not show species other than the Cu^{II} hexa-aquo ion. This could be due to the lower concen-

tration of ligand molecules with respect to the other techniques used in this paper. On the other hand, a higher degree of protonation of the carboxylate group ($\log K$ ca. 3) needs a greater concentration of hydrogen ions and this could affect the liquid junction potential. Nevertheless, the visible spectrum at pH 3.1 suggested the presence of planar donor groups different from H_2O (λ_{max} about 780 nm). This was confirmed by EPR data at $T = 100$ K, which showed significant differences between the actual spectrum and that of the 'pure' Cu^{II} aquo ion. In particular, the Raman spectrum (Fig. 6) supported the involvement of the carboxylic group in the Cu^{II} co-ordination because of the shift of the COOH band from 1700 to 1590 cm^{-1} (COO–Cu bond) [40]. The same spectrum excluded the imidazole N^π owing to the imidazolium band at 1489 cm^{-1} appeared to be unaffected. The broadening of Gly^4CH_2 group in the NMR spectrum at pH 3.9 (Fig. 3a) confirms this hypothesis, it being unlikely, at this pH, the deprotonation of the peptide NH [41].

The visible spectrum of the first cupric complex detected at pH 4.3 (1:1 molar ratio), corresponding to the species $[\text{CuHL}]^{2+}$, showed $\lambda_{\text{max}} = 671$

nm (Table 4). At the same pH and for $\text{HL}/\text{Cu}^{\text{II}}$ ratio as high as 5, the EPR parameters accounted for a histamine-like structure. However, the temperature dependent X-band EPR data did not show direct shpf interaction of the unpaired electron with the equatorial donor nitrogens, due to the experimental linewidth largely overlapping the relevant nitrogen couplings [$\Delta H_i > A_i(\text{N})$]. On the other hand, on the basis of the Blumberg–Peisach approach [42], the paramagnetic parameters fit the N_2O_2 equatorial atom donor set (Table 3), with an upper limit for this interaction [$A_{\parallel}(\text{N}) \leq \Delta H_{\parallel}/4$; $A_{\perp}(\text{N}) \leq \Delta H_{\perp}/4$]. Accordingly, in the ^1H NMR spectra, the broadening of the $\text{His}^1\alpha\text{-H}$, 2- and 5-H indicated the involvement of the amino and imidazole groups of the first amino acid residue in the copper co-ordination. At the same time, in the Raman spectrum (Fig. 7), the decreased intensity of the imidazolium cation bands indicated that at least one imidazole ring is co-ordinated. Again, the presence of the band at 1590 cm^{-1} supported some COO–Cu interactions. The formation of a co-operative binding between the terminal amino group and the imidazole N^π of the His^1 is possible, as it was reported for the $\text{L-His-Gly}/\text{Cu}^{\text{II}}$

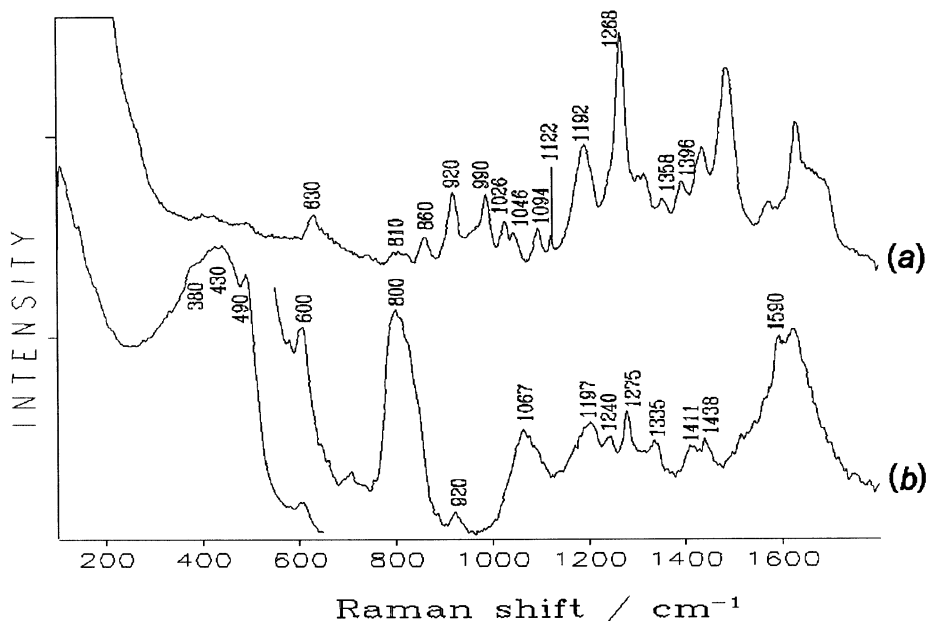


Fig. 8. Raman spectra of (a) the ligand HL and (b) $\text{HL}/\text{Cu}^{\text{II}}$ system in 5×10^{-2} M aqueous solutions at pH 5.1 (457.9 nm exciting line).

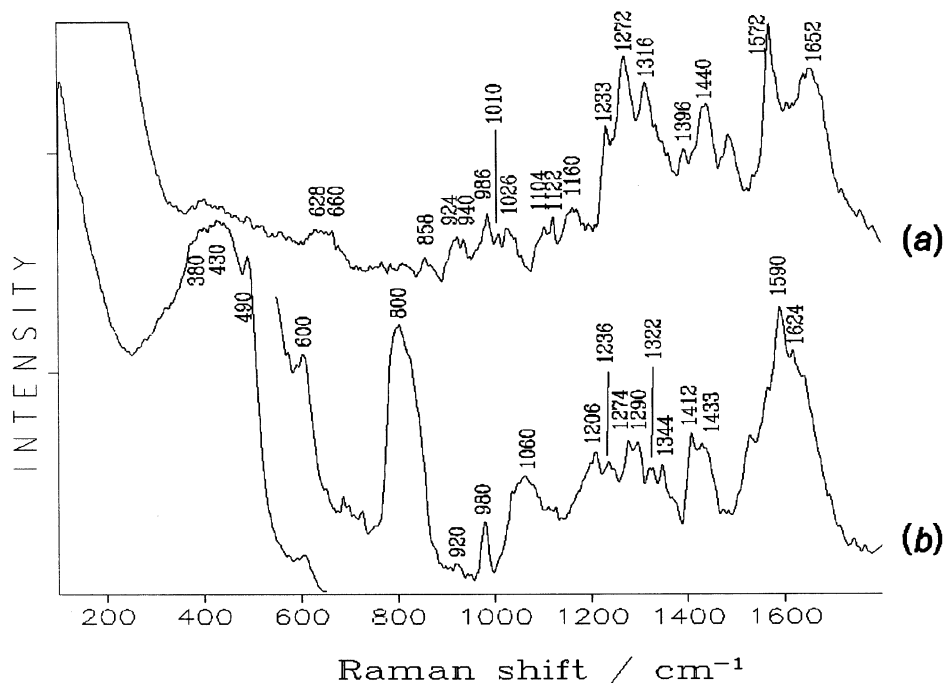


Fig. 9. Raman spectra of (a) the ligand HL and (b) HL/Cu^{II} system in 5×10^{-2} M aqueous solution at pH 8.7 (457.9 nm exciting line).

complex [15]. However, the concomitant presence of different cupric complexes at this pH did not allow us to clarify the nature of the equatorial oxygen donors (water molecule or carboxylate group). Following the equation of Billo [43], the calculated λ_{max} for NN'OO' equatorial co-ordination set is 655 nm while for the set NN'OO, i.e. with two water molecules in the equatorial plane, is 673 nm, close to our experimental result (671 nm, Table 4). This suggests that this is the more probable co-ordination mode, because the in plane co-ordination of the carboxy group should involve a 14-membered ring in the plane with a very low enthalpic contribution.

At pH 5.5, with the [CuH₋₁L] species being predominant, the visible spectrum showed $\lambda_{\text{max}} = 531$ nm, supporting the presence of four nitrogen atoms in the equatorial plane. The visible CD spectrum showed a strong positive contribution respect to the sample at pH 4.3, confirming the formation of a tetradentate species with 4N donors atoms (Table 4) [9]. The bands at 490–500 nm range can be assigned to d–d transitions [44].

Again, the EPR spectra (Fig. 5) did not show resolved nitrogen shpf splittings [$\Delta_i H > A_i(\text{N})$]. The relatively lower values of $g_i(\text{Cu})$ and the higher A_i (Table 3), with respect to those of the previous pH value, agree with a higher number of N atoms equatorially bound. On the other hand, in the presence of some structural hindrance of the actual cupric complex, a relatively long rotational correlation time, τ_r , may induce effective spectral line broadening, in turn reflecting the lacking of shpf resolution [10]. Moreover a significant molecular slow tumbling could be caused by a not co-ordinated, but solvated ligand moiety such as the $-\text{NH}_3^+$ group. Furthermore, the large broadening of 2- and 5-H resonances of His¹ and His³ in the ¹H NMR spectra suggests that the imidazole rings are involved in the complexation, even if, at the ligand-to-metal ratios as high as 20 or more, other species might be present [45].

From the inspection of the Raman spectra at pH 5.1 (Fig. 8) it was evident that the strong band of the imidazolium cations at 1489 cm⁻¹ in the free peptide disappeared in the complex. At the

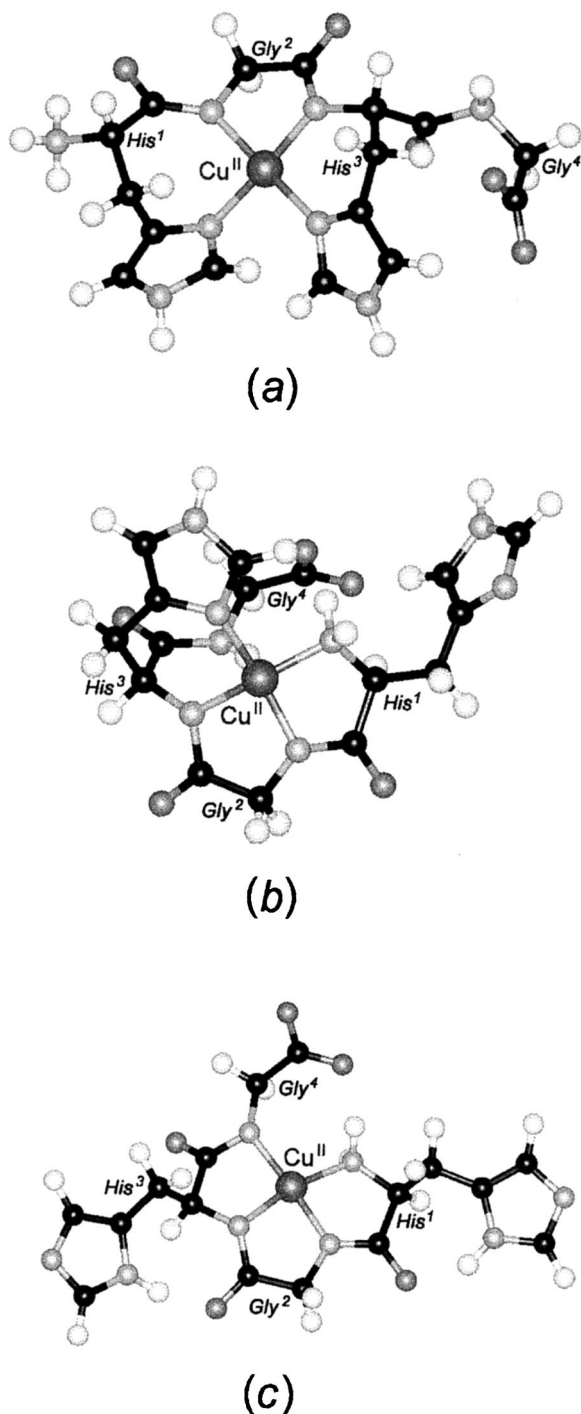


Fig. 10. Optimised structures of the main HL/Cu^{II} complexes at (a) pH 5.1, (b) pH 7.0 and (c) pH > 8, obtained from PM3(tm) semi-empirical calculations.

same time the broad band at 430 cm^{-1} , attributable to the Cu–imidazole bond [40,46] became very strong as well as the imidazole breathing mode at 800 cm^{-1} . These data strongly suggest the involvement of both histidine side chains in the metal ion co-ordination. The bathochromic shift of the amide I band also indicated the presence of Cu–N[−] bonds. From the experimental data we proposed the structure of Fig. 10a where the copper co-ordination took place by two imidazole side chains and two NH of the backbone. Here, the deprotonation of the second peptide NH should be favoured by the presence of the histidine residue in third position [7]. If we consider this set of donors strictly in the equatorial plane, for calculating the λ_{max} of the complex we can use the formula of Kurganov and Davankov [47], which takes into account the contribution of both the equatorial and axial substituents. For this structure, with two apical water molecules, the calculated λ_{max} is 532 nm (if Δ , the steric interaction correction factor, has the value of 0.7).

Another structure could be proposed, involving the terminal NH₂ group, two imidazole rings and the His³ peptide NH. However, in this case, one of the co-ordinated nitrogen atoms lies far from the equatorial plane (60°), as shown by the computed structure, making this arrangement unlikely.

As the pH raised, the NMR signals pattern did not change; at pH 7.0, when the species $[\text{CuH}_{-2}\text{L}]^-$ is predominant, the His¹, His³ and Gly² residues appeared to be interested in the co-ordination. While the NMR data could not clarify if His³ NH was also deprotonated, the $\lambda_{\text{max}} = 525\text{ nm}$ confirmed the presence of four nitrogen atoms in the equatorial co-ordination sphere. The corresponding EPR spectra recorded at RT showed resolved nine signals shpf structure in accordance with four nearly magnetically equivalent nitrogen atoms equatorially bound (N_4 equatorial donor set). The relevant spectra are shown in Fig. 5 (g, h). Accordingly, the unresolved glassy spectrum displays, in the $g_{\parallel}$ region, hpf parameters fitting those resulting from the RT lineshape (Blumberg–Peisach approach). The CD difference spectrum (Fig. 11b) shows a significant

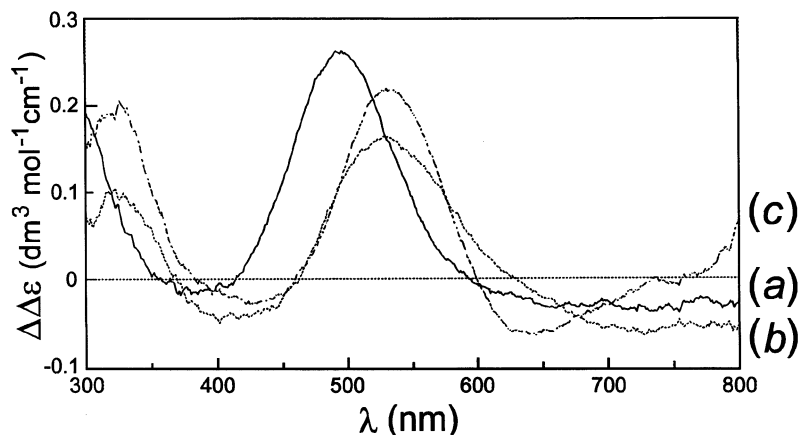


Fig. 11. Difference between the CD spectra of the HL/Cu^{II} system registered at (a) pH 5.15 and 4.0, (b) 6.95 and 5.15 and (c) 9.6 and 6.95.

modification of the ellipticity in the d–d range, suggesting a change around the chiral centre [9], while, in the charge transfer (ct) region, a minor contribution of the negative Im–Cu (π_1) transition seems probable [14].

From the collected data, we propose the structure Fig. 10b with one imidazole ring (His³), the NH₂ group and two peptide NH (Gly² and His³) in the co-ordination plane. The relevant calculated λ_{max} (528 nm, $\Delta = +0.8$, with two apical water molecules) [47], agrees well with the experimental data, provided the planarity of the equatorial donor system is retained. This is the same structure hypothesised by Ueda at pH 8.0 [20]. The potentiometric data state that, already at the neutral pH, the [CuH₂L][−] species is nearly equimolecular with the daughter [CuH₃L]^{2−} species (Fig. 1). This latter is predominant at higher pH values and showed the same visible maximum of the preceding structure of Fig. 10b, indicating that the predominant square planar arrangement is in favour of an N₄ macrochelate coordination. This allowed us to exclude the simple entry of an OH[−] group in the apical position by enhancing the pH, which, on the contrary, would afford a consistent bathochromic shift [41].

No appreciable differences have been found between the EPR spectra of the system at pH 7.0 and 9.0. It suggested that the variation in the actual N atom composition of the equatorial co-ordination sphere for [CuH₃L]^{2−} did not affect

the temperature dependent paramagnetic parameters, as it is likely to occur when the nitrogen atom hybridisation is similar (sp^2). This behaviour is analogous to that reported for the glycylglycyl-histamine/Cu^{II} system (L'/Cu^{II}) at pH 8 and 12.5, where the EPR spectra of the two species, [CuH₂L'][−] and [CuH₃L']^{2−}, containing an equatorially bound imidazole ring and an imidazolate ion, respectively, displayed identical paramagnetic features [26]. Nevertheless, the deprotonation of the NH pyrrole group must be discarded for our ligand, because the relevant $pK^H = 7.98$, calculated from the data of Table 2 for the reaction [CuH₂L][−] = [CuH₃L]^{2−}, is by far lower than the reported values (9.6–11.4) [9,26,41]. The Raman band at 990 cm^{−1}, belonging to the ring deformation of the histidine residue, absent in the spectrum at pH 5.1, is still present at this pH so are the weak bands of the free imidazole ring at 1230, 1270, 1320 and 1440 cm^{−1} (Fig. 9). This observation accounts for the presence of only one imidazole involved in complexation. No information could be gained about the charge or the position (axial or equatorial) of the heterocycle ring. The presence of co-ordination bonds between the N atoms of the backbone and the metal ion is confirmed by the downshift of the amide band from 1650 to 1620 cm^{−1}. The line sharpening occurring in the ¹H NMR spectra at this pH could be explained by the mechanism suggested by Gajda et al. for their system. These

authors proposed the formation of labile bis-complexes in rapid exchange with the free ligand in the bulk solution, whose amount rapidly decreased in alkaline medium [26]. However, the lack of broadening of the ^1H NMR lines by increasing the temperature suggests, in our case, the share of a new chelate compound, i.e. the species $[\text{CuH}_3\text{L}]^{2-}$, which accounts for the probable deprotonation of the third peptide NH (Fig. 10c). The visible data (Fig. 4) are in accordance with the presence of the NH_2 , N^- , N^- , N^- equatorial donor set. Because of the presence of two histidine side chains on the same side of the structure Fig. 4c, one imidazole ring and a water molecule may be the axial groups of a octahedrally distorted geometry. In this case, the calculated λ_{max} is 526 nm ($\Delta = +0.8$) [47]. The CD difference spectrum (Fig. 11c) seems also to support this structure, owing to the higher contribution, for this species, of the $\text{Cu}-\text{N}^-$ transitions in the ct range (300–350 nm) [8,14].

5. Conclusions

The ligand $\text{H}-(\text{L-His-Gly})_2\text{-OH}$ demonstrates peculiar behaviour towards the copper(II) complexation. The potentiometric data, taken also for ligand-to-metal ratios as high as 4, do not show the presence of dimer structures. The peptide acts through a 4N donor set since pH 5, where the Raman spectra support the involvement of the two imidazole rings in the co-ordination plane. This behaviour is quite different from the isomer $\text{H}-(\text{Gly-L-His})_2\text{-OH}$ that showed no more than 3 N atoms in the equatorial plane for the whole pH range [10]. At neutral pH the system $\text{HL}/\text{Cu}^{\text{II}}$ shows an albumin-like coordination [32], whereas, in strong alkaline medium, a stable chelate is formed involving the deprotonation of all the amide bonds.

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