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Is the monomeric prion octapeptide repeat PHGGGWGQ a specific ligand for Cu^{2+} ions?

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Ac-PHGGGWGQ-NH_2, an octarepeat peptide fragment of prion, is a relatively effective ligand for Cu^{2+} ions. At a pH of about 7.4 the major binding sites involve the imidazole nitrogen and two amide nitrogens of ^{3}Gly and ^{4}Gly giving a $CuH_{-2}L$ species. The stability of the complex formed is similar to other peptides having a similar type of coordination. The NMR spectra indicate that in $CuH_{-2}L$ the complex side chain of the Trp residue is located very close to the metal ion. The geometry around the Cu^{2+} ion seems to be slightly distorted from the tetragonal one. In strongly basic solution the coordination involves an additional amide nitrogen. In $CuH_{-2}L$, $CuH_{-3}L$ and $CuH_{-4}L$ complexes the amide nitrogens involved in the metal ion binding are those placed towards the C-terminal from the His residue. The N-terminal of the unprotected octapeptide is very effective in binding the Cu^{2+} ion although at high pH the imidazole nitrogen may not be involved in metal ion binding.

There are strong indications that prion protein (PrP) may play a role in copper metabolism.[1–6] The N-terminal part of the apoprotein consisting of residues 29–124 is unstructured.[7,8] Residues 51–91 contain an unusual glycine-rich repeat every eight residues. Residues 60–91 consist of four octarepeat sequences (PHGGGWGQ), while the 51–59 homologous fragment lacks the histidine residue (PQGGGWGQ). PrP was shown to selectively bind copper within the octarepeats region.[9] Copper complexes of PrP and PrP-derived synthetic peptides have been extensively investigated by UV-vis, CD, Raman, EPR and ENDOR spectroscopy and cyclic voltamperometry.[10–12] The imidazole side chain of the His residue was proposed to be a major binding site for Cu^{2+} ions[2,12] although the consecutive binding sites may depend on the peptide fragment used. In the case of two-octarepeat sequences a single Cu^{2+} ion is bound, while a four-octarepeat peptide binds four metal ions in a cooperative manner.[2] In all the species formed the imidazole nitrogen is a major binding site and an amide nitrogen between Pro and His completes the peptide coordination to the Cu^{2+} ion. Studies on Cu^{2+}ion binding to a monomeric octarepeat fragment[12] also suggest an imidazole nitrogen as an anchoring site. However, in the major species formed between pH 6 and 8, besides the imidazole site, coordination to three amide nitrogens is suggested. This type of coordination at a pH below 7 suggests an unusually high ability of the octarepeat sequence to bind Cu^{2+} ions, similar to that of albumin-like peptides.[13–15] In such peptides the four nitrogen coordination occurs at physiological pH but the binding mode involves N-terminal amino and imidazole nitrogens together with two amide nitrogens. The similar binding ability of the octarepeat peptide to that of albumin-like peptides could suggest a specific role of Gly residues in the very effective stabilization of metal ion complexes with the PrP octapeptide. The binding of the Cu(II) ion to the imidazole of His and the amide nitrogens of the Gly residues located on the C-terminal side of His is a rather unusual coordination mode as for the planar complex the formation of a less favorable 7-membered $\{N_{imid},N^{-}\}$ chelate ring is necessary. It is likely, however, that the imidazole can coordinate to Cu^{2+} ions apically. In this work we have used potentiometric titrations to establish the stoichiometry of the complexes formed between Cu^{2+} ions and the octarepeat peptide and the spectroscopic techniques (UV-VIS, CD, EPR, NMR) to evaluate their structures in solutions. Although the use of the spectroscopic methods is very efficient in the structural studies, the potentiometry seems to be more reliable for evaluating the complex stoichiometry.

Experimental

Synthesis of the peptides

Peptides were synthesized by the solid-phase method using Fmoc chemistry. The TentaGel S RAM (solutions of Fmoc groups 0.25 meq g^{-1}) (RAPP Polymere, Germany) was used as a support. The syntheses were carried out manually. During the syntheses the following amino acid derivatives were used: Fmoc–Gly, Fmoc–Pro, Fmoc–His(Trt), Fmoc–Trp(Boc), Fmoc–Gln(Trt). Deprotections were performed with 50% morpholine in DMF–NMP, (1 : 1, v/v) with addition of 1% Triton X-100. Couplings were achieved using 1 mM HOBt–1 mM DIPCDI (1 : 1, v/v) in the mixture DNF-NMP (1 : 1, v/v) with addition of 1% Triton X-100 for 60 min. After the syntheses had been completed the N-terminal amino group was acetylated with 0.2 M 1-acetylimidazole in DMF–DCM (1 : 1, v/v) for 40 min.[16] The peptides were removed from the resin together with the side chain protections in a one step procedure using Reagent-B – TFA–phenol–triisopropylsilane–H_2O (88 : 5 : 2 : 5, v/v) for 90 min.[17] The crude peptides were purified on a semipreparative C8 HPLC column (Kromasil-100 10*250 mm, 15 μM, Knauer) in the linear gradient 20–80% of B (A: 0.1% TFA; B: 80% acetonitrile in A) in 30 min. Elution profiles were monitored at 278 nm. The purity of the peptides

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was checked on an analytical C8 HPLC column (Kromasil-100 4.6*250 mm, Knauer) in the linear gradient 20–80 % of B (A: 0.1% TFA; B: 80% acetonitrile in A) in 30 min monitored at 276 nm, and for both peptides was greater than 96%. The synthesized peptides have shown the correct molecular mass, measured by mass spectroscopy using the ESI-MS technique.

Potentiometric measurements

Stability constants for protons and Cu(II) complexes were calculated from titration curves carried out at 25 °C using a total volume of 1.5 cm^3. NaOH was added from a 0.250 cm^3 micrometer syringe which was calibrated by both weight titration and the titration of standard materials. The metal ion concentration was 1×10^{-3} mol dm^{-3} and the metal to ligand ratio was 1 : 1.2. The pH-metric titrations were performed at 25 °C in 0.1 mol dm^{-3} KNO_3 on a MOLSPIN pH-meter system using a Russel CMAW 711 semi-combined electrode calibrated in hydrogen concentrations using HNO_3.[18] The SUPERQUAD program was used for stability constant calculations.[19] Standard deviations were computed by SUPERQUAD and refer to random errors only. They are, however, a good indication of the importance of a particular species in the equilibrium.

Spectroscopic measurements

Solutions were of similar concentrations to those used in the potentiometric studies. Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ESP 300E spectrometer at X-band frequency (9.3 GHz) at 120 K. The EPR parameters were calculated for the spectra obtained at the maximum concentration of the particular species for which well-resolved separations were observed. The absorption spectra were recorded on a Beckman DU 650 spectrophotometer. Circular dichroism (CD) spectra were recorded on a Jasco J 715 spectropolarimeter in the 800–230 nm range. The values of $\Delta\varepsilon$ (*i.e.* ε_l–ε_r) and ε were calculated at the maximum concentration of particular species obtained from the potentiometric data.

All the NMR experiments were carried out on a Bruker Avance DRX 600 spectrometer at controlled temperature (±0.2 K). Solutions were prepared in deuterium oxide or in de-ionized water containing 10% deuterium oxide, and were carefully deoxygenated with a few freezing–vacuum pumping–thawing cycles. Chemical shifts were referenced to internal [$^2H_4$]-trimethylsilylpropanesulfonate (TSP-d_4). The pH was adjusted to the desired value with either DCl or NaOD. A stock solution of copper nitrate either in D_2O or in H_2O–D_2O was used to obtain the desired copper concentration in the peptide solution. Proton spin lattice relaxation rates were measured with the inversion recovery pulse sequence and calculated by exponential regression analysis of recovery curves of longitudinal magnetization components. The proton signals were assigned using standard COSY, TOCSY, and NOESY experiments. TOCSY and NOESY spectra were performed in the phase sensitive mode using the TPPI method for quadrature detection. TOCSY experiments were run using a total spin-locking time of 75 ms given by the MLEV-17 sequence. NOESY spectra were obtained at different values of the mixing time in order to optimize cross peak intensities. For the assignment of carbon resonances, HMQC and HMBC experiments were carried out with standard pulse sequences. Spectral processing was performed on a Silicon Graphics O_2 workstation using the XWINNMR 2.5 software.

Molecular models were generated using the HYPERCHEM software package (HYPERCHEM, Hypercube release 5.0, Hypercube Inc., Waterloo, Canada, 1997) as implemented on a PENTIUM 120 MHz PC, by means of the Zindo-1-semi-empirical method (for charge calculations) and the MM^+ force field (for molecular mechanics and dynamics calculations).

Results and discussion

The N-terminal unprotected octapeptide (H_2L, NH_2^+-PHGGGWGQ-NH_2) exhibits two protonation constants with pK 8.37 and 6.16, which can be assigned to amino and imidazole nitrogens, respectively. Both values are typical for oligopeptides having a His residue close to the N-terminal amino group.[14] The calculations based on the potentiometric data indicate the formation of several complex species (Table 1, Fig. 1a). The minor CuHL complex usually is not observed in

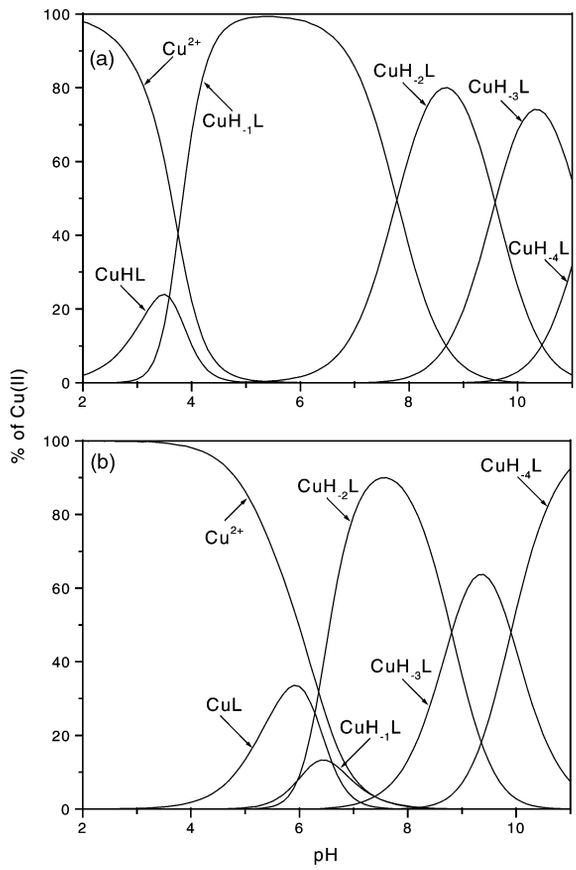

Fig. 1 Species distribution profile for Cu^{2+} complexes of PHGGGWGQ-NH_2 (a) and Ac-PHGGGWGQ-NH_2 (b) Cu^{2+} to peptide molar ratio 1 : 1, $[Cu^{2+}] = 0.001$ M.

systems containing the ^{2}His residue due to formation of {NH_2,N_{imid}} macrochelate binding.[14,20] The presence of a bulky ^{1}Pro residue seems to prevent the formation of CuL species with a macrochelate binding and the $CuH_{-1}L$ with the {NH,N^-, N_{imid}} binding mode is observed in pH range 3–9. Although the stability of the latter complex is relatively high[21] above pH 6 new deprotonation processes are observed and three species, $CuH_{-2}L$, $CuH_{-3}L$ and $CuH_{-4}L$ are formed. The spectroscopic data with d–d transition at 597 nm, g_{II} = 2.230 and A_{II} = 187 Gauss (Table 1) obtained for the $CuH_{-1}L$ complex are typical for the 3N species with a {NH,N^-,N_{imid}} binding mode.[14,22] The involvement of the imino, amide and imidazole nitrogens is clearly seen in the CD spectra, which exhibit the respective charge transfer bands (Table 1).[14,22,23] The formation of the $CuH_{-2}L$ species does not result in considerable changes in the absorption, CD or EPR spectra (Table 1). The d–d band moves to 588 nm and other parameters also change slightly. The major change is observed when the $CuH_{-3}L$ complex is formed. The d–d bands in absorption and CD spectra move distinctly to higher energy (Table 1) and the $N^- \longrightarrow Cu^{2+}$ CT band around 300 nm decreases its intensity considerably due to a high increase of the band at 266 nm, which could be assigned to Trp intra-ring transition. The CT band at 332 nm, which may contain the $N_{imid} \longrightarrow Cu^{2+}$

Table 1 Potentiometric and spectroscopic data for copper(II) complexes of Ac-PHGGGWGQ-NH_2 and PHGGGWGQ-NH_2

Species	log β	pK	UV-Vis		CD		EPR	
			λ/nm	ε/M^{-1} cm^{-1}	λ/nm	$\Delta\varepsilon$/M^{-1} cm^{-1}	$A_{\parallel}$/G	$g_{\parallel}$
PHGGGWGQ-NH_2								
HL	8.37(1)	pK_{NH} = 8.37						
H_2L	14.53(1)	pK_{im} = 6.16						
CuHL	13.85(7)		596	18	603	0.104		
					334	0.035		
$CuH_{-1}L$	6.67(3)		597	75	613	0.257	187	2.23
					515	−0.154		
					340	0.111		
					301	−0.359		
					238	−2.193		
$CuH_{-2}L$	−1.1(6)	7.78	588	98	604	0.360	187	2.23
					509	−0.146		
					338	0.125		
					303	−0.321		
					245	−1.197		
$CuH_{-3}L$	−10.69(8)	9.58	541	114	566	0.329	187	2.22
					483	−0.202		
					367	0.133		
					332	0.238		
					304	−0.052		
					266	−1.329		
$CuH_{-4}L$	−21.79(11)	11.1	505	211	583	0.161	212	2.17
					503	−0.14		
					302	0.491		
					268	−2.005		
Ac-PHGGGWGQ-NH_2 [a]								
HL	6.42(2) [6.41(1)]							
CuL	3.70(4) [3.69(3)]		742 [679]	22 [33]	734	−0.129	130 [142]	2.36 [2.36]
					602 [598]	0.275 [0.073]		
					338 [337]	0.196 [0.047]		
					303 [293]	−0.055 [0.093]		
					225	−0.378		
$CuH_{-1}L$	−2.90(4) [−2.80(2)]	6.6 [6.48]						
$CuH_{-2}L$	−8.87(2) [−8.99(3)]	6.0 [6.19]	621[624]	97 [90]	727 [722]	−0.429 [−0.314]	160 [160]	2.32 [2.32]
					601 [596]	0.594 [0.792]		
					338 [337]	0.781 [0.541]		
					261 [292]	−1.378 [−0.332]		
$CuH_{-3}L$	−17.68(3) [−17.97(5)]	8.81 [8.98]	589 [577]	99 [113]	730 [709]	−0.198 [−0.148]	164 [164]	2.24 [2.23]
					604 [604] [489]	0.348 [0.169] [0.098]		
					335 [334]	0.599 [0.414]		
					256 [300]	−2.925 [−0.545]		
$CuH_{-4}L$	−27.58(4) [−28.92(7)]	9.90 [10.94]	531 [540]	183 [139]	576 [591]	0.854 [0.385]	205 [204]	2.18 [2.19]
					495 [518] [466]	−0.89 [−0.121] [0.042]		
					325 [330]	0.184 [0.151]		
					256 [302]	−3.461 [−0.496]		

[a] Values for protected heptapeptide are given in brackets.

transition, is relatively strong suggesting that in the $CuH_{-3}L$ species the His residue may be still involved in the interaction with the metal ion. However, the distinct change in the d–d energy may suggest a change in the number of nitrogens bound to the Cu^{2+} ion. The most likely explanation seems to be the involvement of third amide nitrogen located on the C-terminal site of the ^{2}His residue. Thus, the binding {NH,3 × N^-} mode with possible involvement of the imidazole nitrogen in an apical position is likely. Above pH 10 the $CuH_{-4}L$ complex is formed with further very distinct changes in all spectral parameters studied (Table 1). The imidazole CT band around 340 nm disappears and the amide CT band changes its sign to positive. The d–d transition moves to 505 nm and the A_{II} increases to 212 Gauss. These changes may suggest the formation also of a 4N type of complex with even stronger four nitrogens bound to the metal ion. The most likely binding mode seems to be the {4 × N^-}donor set. The spectroscopic parameters are typical of a tetragonal Cu^{2+} complex with four strong peptide nitrogens involved in the metal ion coordination.[13–15,20,22] The disappearance of the $N_{imid} \longrightarrow Cu^{2+}$ CT, transition when the $CuH_{-4}L$ species is formed, suggests the removal of the imidazole nitrogen from the coordination to the metal ion. The deprotonation process observed may indicate the involvement of a fourth amide nitrogen derived from the ^{2}His or ^{6}Trp amide bond in metal ion coordination. As discussed above, the bulky Pro residue may disfavor to some extent the involvement of Pro NH in the metal ion coordination which results in its replacement at very basic pH by a fourth amide donor (*vide infra*).

The protected octapeptide and heptapeptide (Ac-PHGGGWGQ-NH_2 and Ac-PHGGGWG-NH_2, respectively) both behave as a HL ligand with protonation constant ≈6.42 assigned to the imidazole side chain (Table 1). Both peptides

behave identically in the metal ion coordination. It means that the C-terminal Gln residue does not affect the binding ability of the peptides at all (Table 1).

The protection of the N-terminal nitrogen of ^{1}Pro dramatically changes the coordination, which begins at a pH two units higher than that discussed above (Table 1, Fig. 1b). The anchoring site at the imidazole nitrogen is metal-bound in the CuL species. This complex undergoes a stepwise deprotonation processes giving $CuH_{-1}L$, $CuH_{-2}L$, $CuH_{-3}L$ and $CuH_{-4}L$ complexes. The major species in the "physiological" pH, 6–8 is the $CuH_{-2}L$ complex, *i.e.* the complex having two amide nitrogens bound to the Cu^{2+} ion. The d–d transitions are observed at 621 nm in the absorption spectrum and at 727 and 601 nm in the CD spectrum. These energies are considerably lower than those obtained for the $CuH_{-2}L$ complex of the N-terminal unprotected complex (*vide supra*, Table 1). The presence of the CD band at 338 nm corresponding to the $N_{imid} \longrightarrow Cu^{2+}$ CT transition indicates the involvement of the imidazole nitrogen in the metal ion binding.[23] The planar $\{N_{imid},2 \times N^{-}\}$ coordination results usually at d–d energies well below 600 nm.[24] The lower energy of the d–d transitions could suggest that the Cu^{2+} ion coordinates to three nitrogens with N_{imid} bound in an apical position or that the tetragonal geometry around the metal ion is strongly distorted. This coordination mode could be sterically forced when two amide nitrogens bound to the metal ion derive from ^{3}Gly and ^{4}Gly residues. The 3N coordination of the Cu^{2+} ion with His containing peptides is a typical one for neutral pH ranges when the N-terminal amino group is not available for binding.[14,24,25] This result differs from that reported earlier by Bonomo *et al.*[12] who proposed the binding of imidazole and three amide nitrogens in the major species present at neutral pH. The $\{N_{imid},2N^{-}\}$ coordination mode agrees to some extent with that proposed by Millhauser *et al.*[26]

Above pH 8 the third amide nitrogen deprotonates with pK 8.81 and the $CuH_{-3}L$ species is formed. This complex predominates at pH 10 and it corresponds most likely to the complex proposed in ref.12 as a major species at neutral pH. The coordination of the successive amide nitrogen is seen in the spectroscopic parameters, including shift of the d–d band from 621 to 589 nm and changes in the EPR parameters. The next proton dissociation resulting in the formation of $CuH_{-4}L$ complex considerably changes all spectroscopic data. The d–d band moves to 531 nm in the absorption spectra and A_{II} varies from 189 to 205 G. The changes in the CD spectra are even more pronounced. Two bands at 730 and 604 nm change their $\Delta\varepsilon$ signs and shift to 576 and 495 nm, respectively. These variations strongly suggest a distinct change in the coordination mode during the $CuH_{-3}L \longrightarrow CuH_{-4}L$ process (pK = 9.90). The pK value of the latter reaction is too low to correspond to the unbound imidazole nitrogen deprotonation (p$K \approx 11$[15]). The spectroscopic features of the $CuH_{-4}L$ species of both unprotected and protected peptides are very close to each other suggesting the same or a very similar binding mode in both types of complexes. These data strongly suggest the binding of the fourth amide nitrogen to the Cu^{2+} ion and removal of the His side chain from the coordination sphere in $CuH_{-4}L$ species. Equatorial coordination of four strong amide nitrogens makes apical binding of imidazole less likely. Thus, the data obtained for the protected peptide support the earlier suggestion that the NH Pro nitrogen coordination is not favored in very basic solutions and it is substituted by a fourth amide nitrogen in $CuH_{-4}L$ species.

As both protected peptides behave identically we have performed the NMR study only for the shorter one to avoid the complications derived from Gln protons. *Trans* and *cis* isomers of the Pro-amide bond were detected, as expected, in a ratio of 77 : 23, respectively, as determined by the relative intensities of NMR signals.

The assignments of ^{1}H- and ^{13}C-NMR spectra are reported in Tables 2 and 3. At pH = 7 all amide protons disappear

Table 2 ^{1}H chemical shift Ac-PHGGGWG-NH_2, 5.2 mM, pH = 7, T = 298 K, D_2O

^{1}H δ/ppm				
	Hα	Hβ	Hγ	Others
^{1}Pro	4.30	2.16–1.77	1.91–1.83	Hδ 3.56
^{2}His	4.63	3.14–3.07		H_2 7.82
				H_4 6.99
^{3}Gly	3.97			
^{4}Gly	3.92			
^{5}Gly	3.92			
^{6}Trp	4.65	3.33–3.27		H_2 7.26
				H_4 7.65
				H_5 7.17
				H_6 7.25
				H_7 7.50
^{7}Gly	3.77			

Table 3 ^{13}C chemical shift Ac-PHGGGWG-NH_2, 5.2 mM, pH = 7, T = 298 K, D_2O

^{13}C δ/ppm					
	C=O	Cα	Cβ	Cγ	Others
^{1}Pro	177.47	63.04	32.53	27.03	Cδ 51.54
^{2}His	176.36	56.22	30.62		C_2 138.42
^{3}Gly	174.82	45.42			
^{4}Gly	174.89	45.37			
^{5}Gly	174.22	45.18			
^{6}Trp	176.96	57.69	29.63		C_1 111.63
					C_2 127.39
					C_3 126.91
					C_4 121.14
					C_5 122.26
					C_6 124.86
					C_7 114.75
					C_8 138.97
^{7}Gly	176.83	44.97			

because of the exchange with deuterium atoms; for this reason we have chosen to also dissolve the prion peptide in deuterium oxide solution.

Copper addition was observed to selectively broaden some proton resonances (Fig. 2, the aromatic protons of the histidine

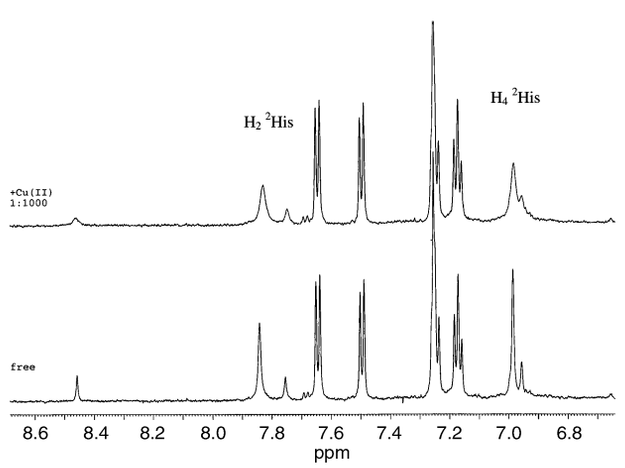

Fig. 2 Superimposed ^{1}H NMR spectra of Ac-PHGGGWG-NH_2, 5.2 mM (bottom) and Ac-PHGGGWG-NH_2, 5.2 mM, in the presence of Cu^{2+} ions, 5.24 μM, in D_2O (top) (pH = 7, T = 298 K).

residue being the most affected signals. Involvement of the His imidazole in copper binding was also shown by the calculated spin-lattice relaxation rate enhancements (Fig. 3 and 4) where binding to the metal is demonstrated either at pH 7 or pH 10.4.

The large R_{1p} value of the amide proton of ^{6}Trp suggests that this residue is close to the copper coordination sphere. This result might be important for the role of the tryptophan

residue, which has been previously shown to be a critical amino-acid involved in the oxidation–reduction couple with the metal ion.[27]

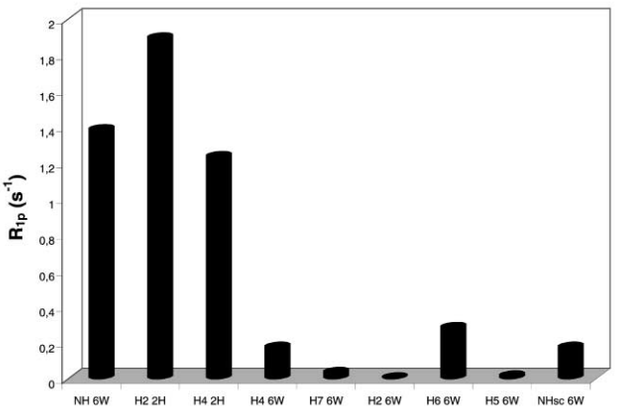

Fig. 3 Paramagnetic contributions, R_{1p} to spin-lattice relaxation rates of selected protons of Ac-PHGGGWG-NH_2, 5.2 mM, in H_2O $[Cu^{2+}]$ = 0.0052 mM pH = 7, T = 298 K.

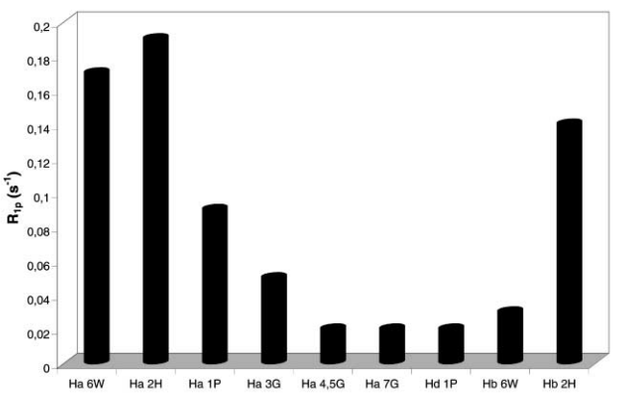

Fig. 4 Paramagnetic contributions, R_{1p} to spin-lattice relaxation rates of selected protons of Ac-PHGGGWG-NH_2, 5.2 mM, in D_2O $[Cu^{2+}]$ = 0.0104 mM pH = 10.4, T = 298 K.

The ^{13}C NMR analysis at pH = 7 reveals the most affected carbon resonances after copper addition (Fig. 5). In fact owing to the paramagnetic ion the ^{2}His, ^{3}Gly carbonic groups show either a broadening or a shift. These data strongly suggest that at pH ≈ 7 Cu^{2+} binds the amide nitrogens of the glycines following the histidine residue. All the NMR results completely support the coordination modes proposed by potentiometric and spectroscopic analysis.

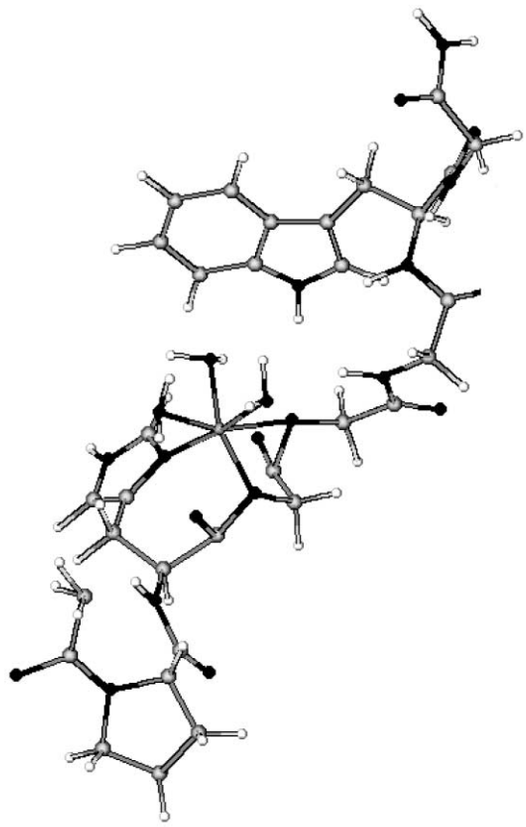

Fig. 6 Molecular model for the $CuH_{-2}L$ complex for the Ac-PHGGGWG-NH_2 peptide.

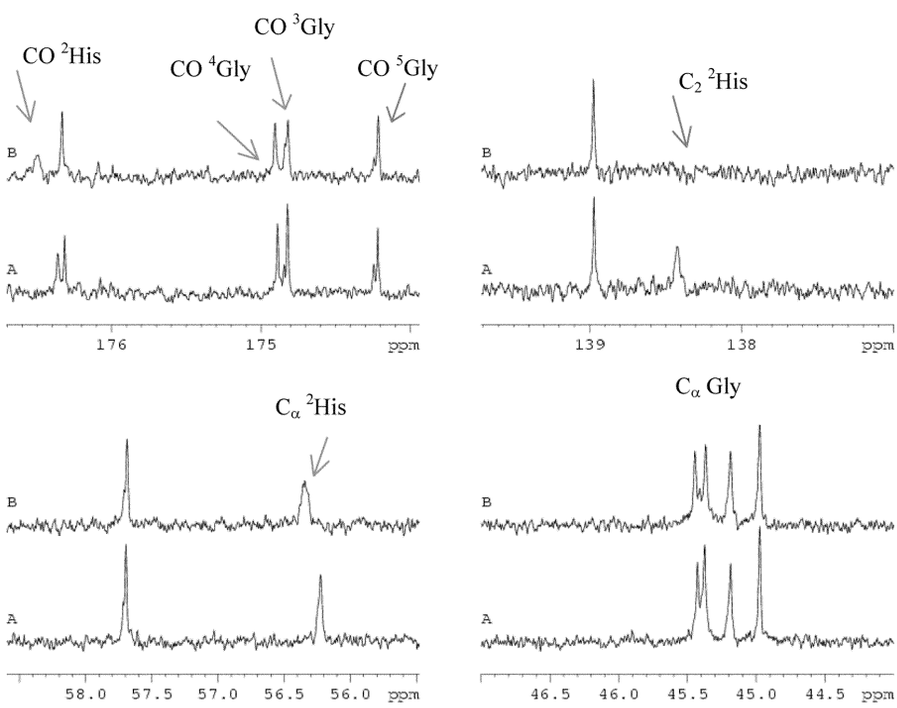

Fig. 5 Superimposed ^{13}C NMR spectra of Ac-PHGGGWG-NH_2, 5.2 mM, (bottom) and Ac-PHGGGWG-NH_2, 5.2 mM, in presence of Cu^{2+} ions, 0.026 mM, in D_2O (top) (pH = 7, T = 298 K).

In accordance with the obtained results we have calculated the model of the copper complex of prion peptide at pH ≈ 7 which is shown in Fig. 6.

The 3N complex with an $\{N_{imid}, N^-_{3Gly}, N^-_{4Gly}\}$ binding mode seems to have imidazole bound in an apical rather than an equatorial position and shows a distinctly distorted tetragonal geometry. As reported elsewhere[28] this type of geometry is an intermediate between the preferred geometry of the Cu^{2+} and Cu^+ pair and it could be suitable for the electron transfer proteins able to induce the very effective SOD-like activity of copper-bound prion protein.[29]

It is interesting to note also that the N-terminal Pro–His unit when unprotected is a quite specific sequence for interactions with Cu^{2+} ions. Its bulky ring prevents the formation of macrochelates involving the imidazole of the His residue and the N-terminal amino group. Protection of the N-terminal Pro nitrogen forces the cupric ion to coordinate with the amide nitrogens on the C-terminal side of the His residue.

The likely specificity of the –Gly–Gly–Gly sequence located on the C-terminal site of the His residue for cupric ion binding is under study.

Acknowledgements

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