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The possible role of Gly residues in the prion octarepeat region in the coordination of Cu^{2+} ions

Marek Łuczkowski,[a] Henryk Kozłowski,*[a] Anna Łęgowska,[b] Krzysztof Rolka[b] and Maurizio Remelli[c]

[a] *Faculty of Chemistry, University of Wroclaw, F. Joliot-Curie 14, 50-383 Wroclaw, Poland. E-mail: henrykoz@wchuwr.chem.uni.wroc.pl*

[b] *Faculty of Chemistry, University of Gdansk, Gdansk, Poland*

[c] *Department of Chemistry, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy*

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Spectroscopic and potentiometric data have shown that insertion of tripeptides other than the Gly_3 peptide fragment, Ala_3 or Lys_3, into the prion octarepeat region destabilizes the biologically relevant Cu^{2+} complex with the metal ion bound equatorially through the $\{N_{imid},2N^-\}$ donor set. The other likely role of the high glycine content could be enforcement of the high flexibility of the N-terminal prion region resulting in the unstructured protein organization. However, the insertion of bulkier amino acid residues does not change the basic coordination mode at physiological pH which involves imidazole nitrogen and two amide nitrogen donors from the third and fourth residues.

Recent work suggests that prion protein (PrP) may play a role in copper metabolism.[1–6] The N-terminal part of the apoprotein containing the fragment 29–124 is unstructured.[7,8] Residues 51–91 contain an unusual glycine-rich repeat every eight residues. PrP was shown to selectively bind copper within the octarepeats (PHGGGWGQ) region.[9] The solution and solid-state studies have shown that at around pH 7.4 the Cu^{2+} complex with metal ion coordinated to imidazole and two Gly amide nitrogen atoms dominates, both in solution[10,11] and the solid state.[11] According to single crystal X-ray studies the Cu^{2+} ion binds three nitrogen donors equatorially and is in close proximity to the Trp indole ring due to bridging by an axially bound water molecule.[11] The proximity of the Trp side-chain was also observed in solution studies.[10]

The binding of Cu(II) ion to the imidazole of His and the amide nitrogens of Gly residues on the C-terminal side of His is a rather unusual coordination mode as for the planar complex the formation of a less favorable seven-membered $\{N_{imid},N^-_{gly3}\}$ chelate ring is necessary.[10–13] The presence of three Gly residues on the C-terminal side of His could be structurally favored due, among other reasons, to the lack of bulky side chains and their larger flexibility derives from the Gly_3 insert. The Cu^{2+} binding *via* $\{N_{imid},N^-_{gly3},N^-_{gly4}\}$ is very specific for pH close to 7.0 and it may play a critical role in the binding/release mechanism of metal ions by PrP during endocytosis.[11] The latter process could facilitate molecular recognition between prion proteins, which could be the basis for trans-membrane signaling and likely the conversion to the pathogenic form.[11]

To test this hypothesis we have studied Cu^{2+} ion binding by two octarepeat analogues having Ala_3 and Lys_3 instead of Gly_3 by means of potentiometric and calorimetric titrations and spectroscopic techniques (UV-VIS, CD, EPR).

Experimental

Synthesis of the peptides

Both peptides were synthesized by the solid-phase method using Fmoc chemistry. The TentaGel S RAM (substitution 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-Ala, Lys(Boc), Fmoc-Pro, Fmoc-His(Trt), Fmoc-Trp(Boc), Fmoc-Gln(Trt), Ac-Pro. Blocking groups were removed with 20% piperidine in DMF/NMP, (1 : 1, v/v) with addition of 1% Triton X-100. Couplings were achieved using 1 M solutions of HOBt/DIPCDI (1 : 1, v/v) in a mixture of DMF/DCM (1 : 1, v/v) with addition of 1% Tritron X-100 for 60 min. After the syntheses were complete, the peptides were removed from the resin together with the side chain protection groups in a one step procedure using Reagent-B—TFA/phenol/triisopropylsilane/H_2O (88 : 5 : 2 : 5, v/v) for 90 min.[14] The crude peptides were purified on a semipreparative C8 HPLC column (Kromasil-100, 10 × 250 mm, Knauer) using a linear gradient, 20–80%, of B (A: 0.1% TFA; B: 80% acetonitrile in A) over 30 min. Elution profiles were monitored at 226 nm. The purity of the peptides was checked on an analytical C8 HPLC column (Kromasil-100, 4.6 × 250 mm, Knauer) using the same linear gradient over 30 min and monitoring at 226 nm, and was found to be >96% for all peptides studied. The synthesized peptides gave the correct molecular mass when measured by mass spectroscopy (ESI-MS technique).

Potentiometric measurements

Stability constants both for protons and Cu^{2+} complexes were calculated from three titrations carried out over the range pH 3–11 at 25 °C using a total volume of 1.5 cm^3. The purities and the exact concentration of the solutions of the ligands were determined by the method of Gran.[15] 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.[16] The SUPERQUAD program was used for stability constant calculations.[17] 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.

Calorimetric measurements

$\Delta H°$ values were determined for the Ala_3 analogue by titration calorimetry with a Tronac model 450 isoperibol calorimeter equipped with a 3 cm^3 reaction vessel. The calorimetric measurements were carried out by titrating in double aliquots of 2.5–2.8 ml of solutions of the same composition as in potentiometry with a standard solution of HNO_3. For each system at

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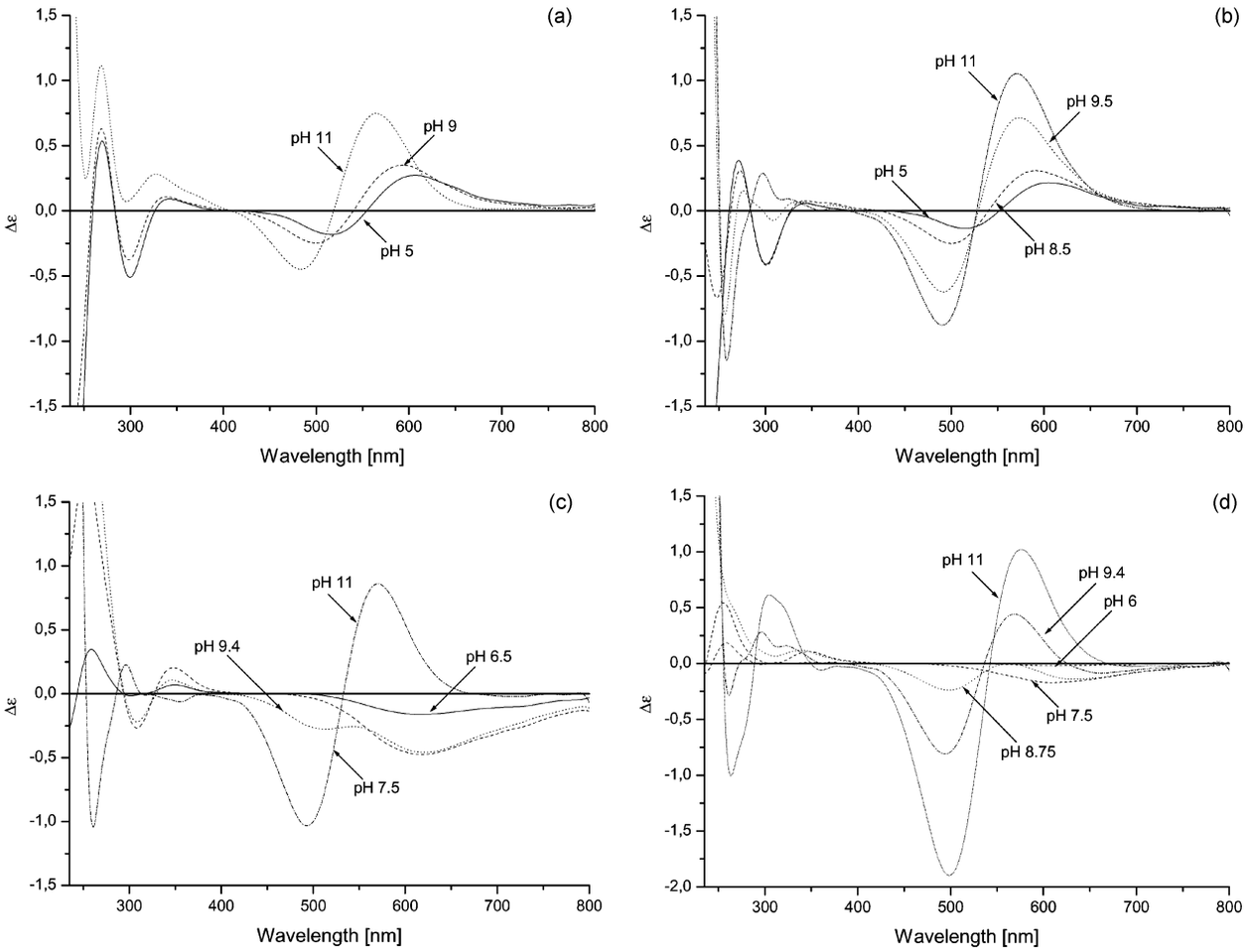


Fig. 1 CD spectra of Cu^{2+} complexes of PHAAAWGQ-NH_2 (a), PHKKKWGQ-NH_2 (b), Ac-PHAAAWGQ-NH_2 (c) and Ac-PHKKKWGQ-NH_2 (d). $[Cu^{2+}] = 1 \times 10^{-3}$ M, metal to ligand ratio 1 : 1.2.

least 250 experimental points were utilised to calculate the thermodynamic quantities. The reaction heats—corrected for non-chemical contributions which are particularly important when using small volume dewars,[18] and for the dilution heats computed from literature data[19]—were calculated by considering the calorie as equivalent to 4.187 J. Protonation and complex formation enthalpies and entropies were computed from experimental calorimetric titration by means of the computer program DOEC.[20] A pK_w value of 13.74 and a $\Delta H°_w$ value of 55.89 kJ mol^{-1}, determined by separate experiments, were employed in the calculations; the Cu(II) hydrolytic constants and the corresponding formation enthalpies have been taken from Arena *et al.*[21] The accuracy of thermodynamic parameters has been estimated as approximately three times the precision value given by DOEC, in the cumulative calculations, and it is expressed as a deviation over the last significant figure.

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 Jasco J 715 spectropolarimeter in the 750–230 nm range. The CD spectra at selected pH are shown in Fig. 1a–d. The values of $\Delta\varepsilon$ (*i.e.* $\varepsilon_l - \varepsilon_r$) and ε were calculated at the maximum concentration of the particular species obtained from the potentiometric data.

Results and discussion

The N-terminal unprotected octapeptides (NH_2^+-PHAAA-WGQ-NH_2, H_2L and NH_2^+-PHKKKWGQ-NH_2, H_4L) exhibit protonation constants for prolyl imino- and imidazole-nitrogens close to each other (Table 1). Both values are typical for oligopeptides having a His residue and they are close to parent octarepeat with Gly_3 insert.[10] The lysine analogue exhibits two additional protonation constants derived from Lys side chain amino groups (Table 1).

In the case of the unprotected Ala_3 oligopeptide (Table 3, later) the protonation enthalpy for the terminal amino group (−33 kJ mol^{-1}) is noticeably lower than reported for free Pro[22] (−44 kJ mol^{-1}). This result reflects the lower basicity of the oligopeptide (log K_1 = 8.40) with respect to the free amino acid (log K_1 = 10.5), in the same experimental conditions; the entropic contribution is instead almost identical in the two cases. No further comparison is possible with literature data since, to the best of the author's knowledge, this is the first protonation enthalpy reported for oligopeptides with a terminal Pro residue. The protonation enthalpy for the imidazole residue is in good agreement with the values reported earlier for His[23] (−29.3 kJ mol^{-1}) and for some oligopeptides containing two His residues[24] (−31 kJ mol^{-1}) for both the ligands.

For the Ala_3 analogue the calculations based on the potentiometric data indicate the formation of three complex species (Table 1). The coordination modes were established using the spectroscopic data (see Fig. 1a for CD spectra) as described earlier.[10] The minor CuHL and high pH $CuH_{-4}L$ species observed for Gly_3 peptide were not recorded for the alanine analogue. The complexes for Gly_3 octapeptide are dis-

Table 1 Potentiometric and spectroscopic data for proton Cu^{2+} complexes of PHAAAWGQ-NH_2, PHKKKWGQ-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})	$A_{\|}$/G	$g_{\|\|}$
PHAAAWGQ-NH_2						
HL	8.40(1)	pK_{NH} = 8.40				
H_2L	14.55(1)	pK_{im} = 6.15				
$CuH_{-1}L$ {NH, N_{im}, N^-}	6.53(2)		597 (74)	607 (0.273), 516 (−0.281), 342 (0.092), 299 (−0.510), 269 (0.535)	187	2.23
$CuH_{-2}L$ {NH, N_{im}, $2N^-$}	−1.6(4)	8.13	577 (99)	593 (0.531), 499 (−0.247), 339 (0.108), 298 (−0.367), 269 (0.631)	187	2.23
$CuH_{-3}L$ {NH, $3N^-$}	−11.55(5)	9.95	541 (114)	569 (0.621), 486 (−0.406), 328 (0.203), 269 (0.861), 251 (−0.095)	200	2.17
PHKKKWGQ-NH_2						
HL	10.21(2)	10.21				
H_2L	20.24(1)	10.03				
H_3L	28.55(1)	8.31				
H_4L	34.53(2)	5.98				
CuHL {NH, N_{im}, N^-}	26.80(1)		596 (51)	605 (0.216), 515 (−0.134), 343 (0.057), 301 (−0.409), 271 (0.387)	185	2.23
CuL {NH, N_{im}, $2N^-$}	19.04(4)	7.76	581 (98)	591 (0.309), 500 (−0.251), 372sh (0.057), 343 (0.076), 301 (−0.418), 273 (0.310), 248 (−0.665)	185	2.23
$CuH_{-1}L$ {NH, N_{im}, $2N^-$}–(H_2N_ε-Lys)	9.61(5)	9.43	549 (115)	574 (0.715), 491 (−0.621), 334 (0.068), 308 (−0.073), 276 (0.153), 257 (−0.792)	185	2.23
$CuH_{-2}L$ {NH, $2N^-$}	−0.12(5)	9.73	543 (128)	571 (0.873), 491 (−0.744), 293 (0.254), 257 (−0.926)	207	2.17
$CuH_{-3}L$ {NH, $3N^-$}–(N_2N_ε-Lys)	−10.74(7)	10.62	531 (147)	571 (1.055), 490 (−0.878), 297 (0.288), 258 (−1.150)	207	2.17
$CuH_{-4}L$ {$4N^-$}	−21.92(8)	11.18	525 (163)	575 (0.837), 497 (−0.841), 317 (0.145), 276 (−1.382)	207	2.17
PHGGGWGQ-NH_2 [10]						
HL	8.37(1)	pK_{NH} = 8.37				
H_2L	14.53(1)	pK_{im} = 6.16				
CuHL	13.58(7)		596 (18)	603 (0.104), 334 (0.035)		
$CuH_{-1}L$	6.67(3)		597 (75)	613 (0.257), 515 (−0.154), 340 (0.111), 301 (−0.359), 238 (−2.193)	187	2.23
$CuH_{-2}L$	−1.1(6)	7.78	588 (98)	604 (0.360), 509 (−0.146), 338 (0.125), 303 (−0.321), 245 (−1.197)	187	2.23
$CuH_{-3}L$	−10.69(8)	9.58	541 (114)	566 (0.329), 483 (−0.202), 367 (0.133), 332 (0.238), 304 (−0.052), 266 (−1.329)	187	2.22
$CuH_{-4}L$	−21.79(11)	11.1	505 (211)	583 (0.161), 503 (−0.14), 302 (0.491), 268 (−2.005)	212	2.17

tinctly more stable than the respective species obtained for the Ala_3 ligand (Table 1).

The $CuH_{-1}L$ complex with {NH_{pro},N^-_{his},N_{im}} binding mode has a very similar stability for both peptides, while for the $CuH_{-3}L$ complex with three amide nitrogens involved,[10] including two Ala (Gly) residues, the stability constant is almost one order of magnitude lower for the Ala_3 peptide. The steric hindrance and more rigid structure introduced by the Ala side-chain methyl groups could be the major reason for a lower stability as the pK values for the imidazole and imino group of Pro are very close to each other. The lower ability to deprotonate the consecutive amide protons by Cu^{2+} in the case of Ala_3 peptide excludes the formation of the $CuH_{-4}L$ complex in the pH range used in the potentiometric titrations.

The comparison of the stability constants for the complexes with Lys_3 peptide is rather complicated due to there being two Lys residues which deprotonate their side chains within the studied pH range (Table 1).

In the absence of thermo-chemical reference data for copper complexes with oligopeptides bearing Pro as N-terminal residue, we can compare the $\Delta H^\circ_{1\text{–}11}$ values found for the unprotected oligopeptide (Table 3, −26 kJ mol^{-1}) with the reported values for other ligands with His as second residue, like Gly-His-Lys (−42 kJ mol^{-1})[25] or Gly-His-Gly (−32 kJ mol^{-1}).[26] The difference between the literature and experimental values can be attributed to the steric hindrance caused by the Pro ring while it looks sufficiently low to suggest the same coordination mode: {$NH_{terminal}$,N^-_{His},N_{im}}. The subsequent deprotonation steps require approximately 24 kJ mol^{-1} each, a value slightly lower than that generally found for the amido nitrogen deprotonation/coordination to the copper ion (30–40 kJ mol^{-1}).[27]

The *protected* octarepeat analogues, Ac-PHAAAWGQ-NH_2 and Ac-PHKKKWGQ-NH_2 behave as HL and H_4L acids, respectively. While only one protonation site at His imidazole is seen for Ala_3 peptide, the lysine analogue shows three additional protonation constants for the Lys_3 fragment (Table 2).

The Ala_3 peptide forms the same set of complexes as Gly_3[10] except the minor $CuH_{-1}L$ species (Fig. 2, Table 2). The spectroscopic data collected in Table 2 (see Fig. 1c for CD spectra) show that the binding modes are the same as those found for the parent octarepeat peptide.[10,11] The speciation plots shows that there are two major differences between both peptides. The formation of $CuH_{-2}L$ and $CuH_{-3}L$ complexes is distinctly

Table 2 Potentiometric and spectroscopic data for proton and Cu^{2+} complexes of Ac-PHAAAWGQ-NH_2, Ac-PHKKKWGQ-NH_2 and Ac-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	$g_{\parallel}$
Ac-PHAAAWGQ-NH_2						
HL	6.32(1)	pK_{im} = 6.32				
CuL {N_{im}}	3.52(2)		654 (40)	615 (0.161), 347 (0.071), 311 (−0.085), 254 (0.918)	137	2.35
$CuH_{-2}L$ {N_{im}, $2N^-$}	−9.76(1)	13.28	628 (83)	618 (−0.474), 347 (0.204), 308 (−0.267), 252 (1.798)	160	2.30
$CuH_{-3}L$ {N_{im}, $3N^-$}	−19.12(2)	9.36	588 (99)	620 (−0.457), 513 (−0.277), 346 (0.107), 307 (−0.211), 251sh (2.697)	150	2.24
$CuH_{-4}L$ {$4N^{-4}$}	−28.84(2)	9.72	531 (180)	570 (0.856), 493 (−1.031), 296 (0.226), 355 (−0.062), 317 (−0.011), 260 (−1.041)	205	2.17
Ac-PHKKKWGQ-NH_2						
HL	11.02(4)	11.02				
H_2L	21.23(4)	10.23				
H_3L	30.98(5)	9.75				
H_4L	37.06(6)	6.08				
CuH_3L {N_{im}}	34.29(5)		769 (19)	609 (−0.020), 258 (0.183)	131	2.35
CuHL {N_{im}, $2N^-$}	21.00(3)	13.29	648 (54)	609 (−0.171), 347 (0.105), 255 (0.543)	160	2.28
CuL {N_{im}, $2N^-$}–(H_2N_ε-Lys)	12.07(6)	8.93	Minor			
$CuH_{-1}L$ {N_{im}, $3N^-$}	3.11(5)	8.96	556 (77)	633 (−0.141), 498 (−0.240), 339 (0.119), 262sh (0.554)	145	2.24
$CuH_{-2}L$ {$4N^-$}	−6.90(6)	10.01	537 (133)	570 (0.726), 495 (−1.212), 321sh (0.221), 298 (0.451), 280sh (−0.188), 261 (−0.781)	207	2.17
$CuH_{-3}L$ {$4N^-$}–(H_2N_ε-Lys)	−17.74(7)	10.84	531 (202)	578 (1.189), 500 (2.255), 310 (0.764), 280sh (−0.788), 263 (−1.184)	207	2.17
Ac-PHGGGWGQ-NH_2 [10]						
HL	6.42(2)					
CuL	3.70(4)		742 (22)	734 (−0.129), 602 (0.275), 338 (0.196), 303 (−0.055), 225 (−0.378)	130	2.36
$CuH_{-1}L$	−2.90(4)	6.6				
$CuH_{-2}L$	−8.87(2)	6.0	621 (97)	727 (−0.429), 601 (0.594), 338 (0.781), 261 (−1.378)	160	2.32
$CuH_{-3}L$	−17.68(3)	8.81	589 (99)	730 (−0.198), 604 (0.348), 335 (0.599), 256 (−2.925)	164	2.24
$CuH_{-4}L$	−27.58(4)	9.90	531 (183)	576 (0.854), 495 (−0.89), 325 (0.184), 256 (−3.461)	205	2.18

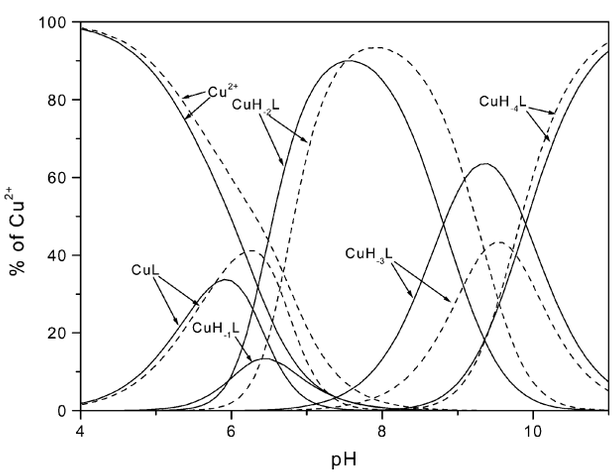


Fig. 2 Species distribution profile for Cu^{2+} complexes of Ac-PHGGGWGQ-NH_2 (solid line) and Ac-PHAAAWGQ-NH_2 (dashed line) at 25 °C and I = 0.1 M KNO_3. [Cu^{2+}] = 1 × 10^{-3} M, metal to ligand ratio 1 : 1.2.

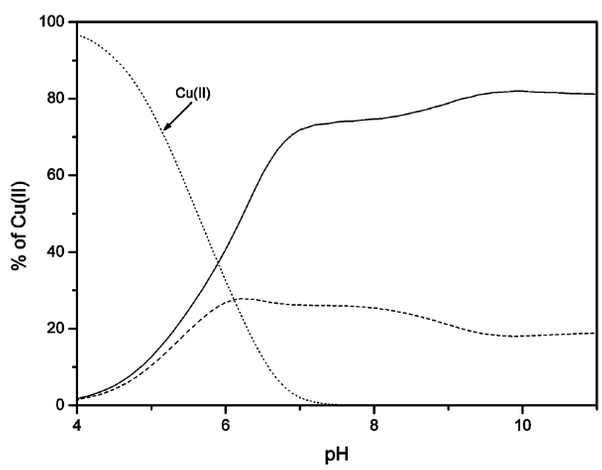


Fig. 3 Distribution profiles of free and complexed fractions of Cu^{2+} ions in the presence of both Ac-PHGGGWGQ-NH_2 (solid line) and Ac-PHAAAWGQ-NH_2 (dashed line) at 25 °C and I = 0.1 M KNO_3. [Cu^{2+}] = 1 × 10^{-3} M; ligand to metal to ligand ratio 1 : 1 : 1.

easier for the parent prion octarepeat when compared to the Ala_3 analogue. The competition plot showing the coordination ability of both ligands (Fig. 3) clearly shows that the prion octarepeat is a much more effective ligand for Cu^{2+} ions than the Ala_3 octapeptide.

The stability constants of both ligand complexes differ by

Table 3 Calorimetric data for Cu^{2+} complexes of PHAAAWGQ-NH_2, Ac-PHAAAWGQ-NH_2 and Ac-PHGGGWG-NH_2

Species	Stoichiometry	log β	$-\Delta G°$/kJ mol^{-1}	$-\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
PHAAAWGQ-NH_2					
HL	011	8.40(. .)	47.9(. .)	33(3)	49(12)
H_2L	021	14.55(. .)	83.0(. .)	58(4)	82(15)
$CuH_{-1}L$	1–11	6.53(2)	37.3(1)	26(3)	38(15)
$CuH_{-2}L$	1–21	−1.6(4)	−9.1(5)	2(5)	−39(18)
$CuH_{-3}L$	1–31	−11.55(5)	−65.9(1)	−22(6)	−146(20)
Ac-PHAAAWGQ-NH_2					
HL	011	6.32(2)	36.1(1)	29(2)	25(8)
CuL	101	3.52(2)	20.1(1)	25(3)	−16(12)
$CuH_{-2}L$	1–21	−9.76(1)	−55.7(1)	−46(3)	−32(10)
$CuH_{-3}L$	1–31	−19.12(2)	−109.1(1)	−86(4)	−77(15)
$CuH_{-4}L$	1–41	−28.84(2)	−164.5(1)	−110(4)	−182(15)
Ac-PHGGGWG-NH_2					
HL	011	6.41(1)	37(1)	28(2)	29(8)
CuL	101	3.69(3)	21.05(5)	37(6)	−46(21)
$CuH_{-1}L$	1–11	−2.80(2)	−15.97(4)	25(9)	−138(33)
$CuH_{-2}L$	1–21	−8.99(3)	−51.29(5)	−21(4)	−101(15)
$CuH_{-3}L$	1–31	−17.97(5)	−102.51(7)	−47(5)	−185(17)
$CuH_{-4}L$	1–41	−28.92(7)	−164.98(9)	−50(9)	−385(31)

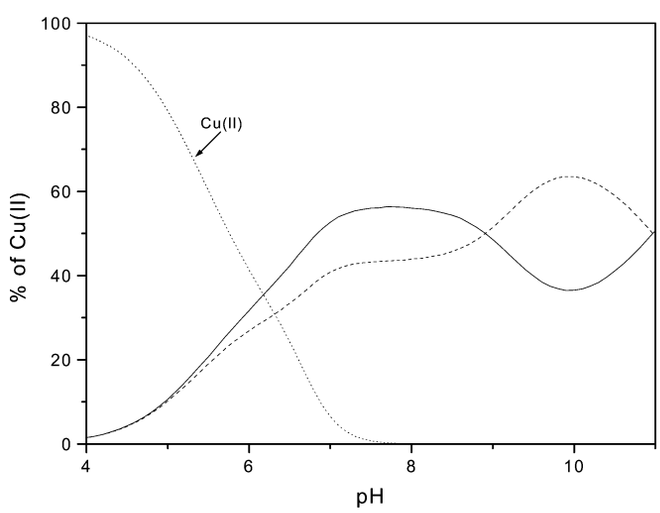
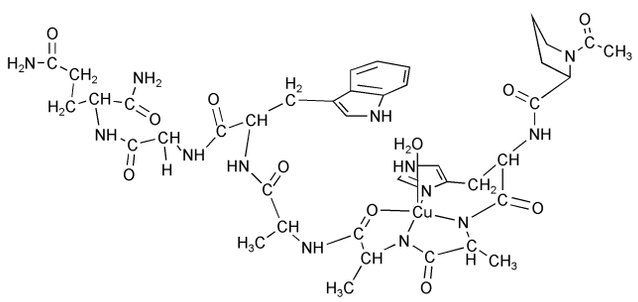


Fig. 4 Distribution profiles of free and complexed fractions of Cu^{2+} ions in the presence of both Ac-PHAAAWGQ-NH_2 (solid line) and Ac-PHKKKWGQ-NH_2 (dashed line) at 25 °C and $I = 0.1$ M KNO_3. $[Cu^{2+}] = 1 \times 10^{-3}$ M; ligand to metal to ligand ratio 1 : 1 : 1.

about one order of magnitude in favor of the parent peptide. The major differences between the most biologically relevant species, $CuH_{-2}L$ with $\{N_{imid},2N^-_{amid}\}$ binding mode (Scheme 1) for both peptides occurs around pH 6.5, which could be critical for the Cu^{2+} binding/release mechanism.[11] Comparison of the binding ability of prion peptide and its Lys analogue shows similar behavior. The $CuH_{-2}L$ (CuHL for Lys_3) species is again more stable for the parent prion octapeptide. The competition plot for Ala_3 and Lys_3 octapeptides shows that in the physiologically relevant pH range the alanine analogue is a slightly more efficient ligand than the lysine one (Fig. 4).

As far as the protected octapeptide is concerned (Table 3), its $\Delta H°_{101}$ value is sufficiently close to that reported[28] for the formation of the analogous species between copper and imidazole (−31.8 kJ mol^{-1} at $I = 0.16$ mol dm^{-3}) to support the hypothesis that the His residue is the first anchoring site for the metal ion. The $CuH_{-2}L$ complex formation is more endo-enthalpic by 71 kJ mol^{-1} with respect to that of the CuL species. This result is in excellent agreement with the hypothesis that two amido nitrogens subsequently bind the copper ion. The same is also true for the following two deprotonation steps. In particular, the formation of the $CuH_{-4}L$ species looks less endo-enthalpic and more endo-entropic than the previous ones, suggesting a more ordered structure.



Scheme 1

Conclusions

Comparison of the prion octarepeat peptide fragment with two analogues having Ala_3 or Lys_3 instead of Gly_3 shows that in all cases the major complex formed in the pH range 6–8 is the species with the $\{N_{imid},2N^-_{amide}\}$ binding mode as shown earlier by X-ray structure and solution studies.[10,11] The insertion of

bulkier Ala or Lys residues instead of a Gly_3 unit make this complex less stable. Although differences in stability are not very dramatic it could be critical in the pH range at which the Cu^{2+} binding/releasing mechanism occurs (6.5–7.0).[11] Thus, the glycine rich region in the octarepeat fragment of prion may play two basic roles (i) maintaining a high peptide flexibility for the unstructured prion N-terminal region and (ii) allowing very specific coordination of Cu^{2+} ions in the pH range important for binding and release of metal ion during the biological transportation process. The binding of Cu^{2+} results in partial protein organization which could be biologically relevant.[11]

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