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# Equilibrium and structural studies on copper(II) complexes of tetra-, penta- and hexa-peptides containing histidyl residues at the C-termini

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The stoichiometry, stability constants and solution structure of the complexes formed in the reaction of copper(II) with oligopeptides containing histidyl residues at the C-termini ($Gly_3His$, $Gly_4His$ and $Gly_5His$) have been determined by potentiometric, UV–VIS and EPR spectroscopic methods. The formation of the species $[CuHL]^{2+}$, $[CuL]^{+}$, $[CuH_{-1}L]$, $[CuH_{-2}L]^{-}$ and $[CuH_{-3}L]^{2-}$ was detected in all cases. Binding modes of the species $[CuL]^{+}$, $[CuH_{-1}L]$ and $[CuH_{-2}L]^{-}$ were characterized by the metal ion co-ordination of the terminal amino group, carbonyl oxygen or one or two deprotonated amide nitrogens in joined five-membered chelates from the N-termini, while the fourth co-ordination site of the metal ion was occupied by nitrogen donors of imidazole in the form of a macrochelate. The stability of the macrochelate was decreased upon increasing the length of the peptide molecule. For the penta- and hexa-peptides the species $[CuH_{-3}L]^{2-}$ was characterized as a 4N-complex with equatorial co-ordination of the terminal amino group and subsequent three deprotonated amide nitrogens, with unco-ordinated imidazolyl residues, while a 5N-species was suggested to form for $Gly_3His$ with axial interaction of the imidazole-N donor atom. Copper(II) complexes of $Gly_2His$ and pentaglycine were also investigated for reliable comparison.

## Introduction

It is well known that histidyl residues of proteins are the most common binding sites for copper(II) ions in a number of metalloenzymes. The biological relevance and abundance of copper(II)–N(imidazole) interactions explain the huge number of studies on the co-ordination chemistry of histidine containing peptides.[1–5] It is clear from these studies that complex formation processes of histidine containing peptides largely depend on several crucial factors including the nature of metal ions, the metal ion to ligand ratio and especially the location of histidyl residues in the amino acid sequence.

It is widely accepted that the interaction of copper(II) with N-terminal histidyl peptides is characterized by the formation of a dimeric species $[Cu_2H_{-2}L_2]$ with GlyGly-like co-ordination and imidazole bridging in equimolar solutions, while stable bis complexes with 4N-co-ordination without amide deprotonation are formed in the presence of excess of dipeptides.[6] The insertion of histidine in the second position of a peptide chain results in enhanced metal binding capability of the ligands, which is explained by the ($NH_2$, $N^-$, N(Im)) co-ordination of the first two amino acid residues.[6–11] For GlyHis and related ligands it is especially important to note that deprotonation and co-ordination of the amide nitrogen are also promoted by zinc(II) and cobalt(II) in the physiological pH range.[12,13]

The tripeptides containing C-terminal histidyl residues have been widely studied and used as the simplest models for human serum albumin.[14–18] The interaction of copper(II) with GlyGly-His is characterized by the co-operative deprotonation of two subsequent amide nitrogens and by the formation of $[CuH_{-2}L]^{-}$ which is a very stable 4N-complex. The binding sites of GlyGlyHis–*N*-methylamide[16] and GlyGly–histamine[17] have been described in a similar way supporting the outstanding metal binding capability of peptides containing histidine as the third amino acid residue.

Copper(II) and nickel(II) complexes of a few peptide molecules containing histidyl residues in the fourth or fifth positions have already been studied, but the results cannot be generalized, because the interactions are much more complicated. The anchoring effect of histidyl residues for amide binding has been established and subsequent co-ordination of deprotonated amide nitrogens preceding the C-terminal histidyl residue was concluded. For the copper(II)–Ac-GlyGlyGlyHis system (where Ac represents the acetyl group, $CH_3CO$) this resulted in the formation of a 4N-complex in slightly alkaline media.[19,20] Transition metal complexes of a number of tetra- to hexa-peptides representing the oligopeptide segment of angiotensin II and containing C-terminal histidyl residues were studied by Kozlowski and co-workers.[21–25] The anchoring capacity of histidyl residues was observed in all cases and the formation of various macrochelates was proposed in the species [CuL], without amide binding. The existence of similar macrochelates was proposed in palladium(II) complexes of ProGlyAlaHis at low pH values.[26] However, in the copper(II)–GlyGlyGlyHis and copper(II)–AlaGlyGlyHis systems the binding modes of the final 4N-species, $[CuH_{-3}L]^{2-}$, were explained by a structural rearrangement of the macrochelates with an unco-ordinated amino group,[1,23] and co-ordination of N(imidazole) and preceding three amide nitrogens was suggested. The co-ordination properties of the penta- and hexa-peptides, ArgValTyrIleHis and AspArgValTyrIleHis were interpreted in a similar way *via* the formation of a macro-

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**Table 1** Stability constants (log $\beta_{pqr}$) of the proton and copper(II) complexes of oligopeptides: $T = 298$ K, $I = 0.2$ mol dm$^{-3}$ KCl (standard deviations are in parentheses)

| Species | $Gly_2His$ [18] | $Gly_3His$ | $Gly_4His$ | $Gly_5His$ | Triglycine [31] | Tetraglycine [31] | Pentaglycine |
|---|---|---|---|---|---|---|---|
| HL | 8.06(2) | 8.03(1) | 8.05(2) | 8.00(1) | 7.93 | 7.94 | 7.95(1) |
| $H_2L$ | 14.88(3) | 14.89(2) | 14.98(2) | 14.87(2) | 11.25 | 11.18 | 11.31(1) |
| $H_3L$ | 17.68(3) | 17.55(3) | 17.88(3) | 17.73(2) | — | — | — |
| $[CuHL]^{2+}$ | 12.40(15) | 12.24(15) | 12.16(23) | 12.29(5) | 9.51 | 9.09 | — |
| $[CuL]^{+}$ | 7.60(20) | 8.47(1) | 8.50(2) | 8.11(2) | 5.25 | 5.06 | 5.31(5) |
| $[CuH_{-1}L]$ | 2.50(20) | 1.63(3) | 2.41(3) | 2.63(2) | −0.16 | −0.50 | −0.29(3) |
| $[CuH_{-2}L]^{-}$ | −1.55(2) | −5.79(3) | −5.39(4) | −5.06(3) | −7.02 | −7.41 | −7.10(3) |
| $[CuH_{-3}L]^{2-}$ | — | −16.67(4) | −15.71(4) | −15.67(3) | −18.30 | −16.59 | −14.99(3) |
| p$K$(His) | — | 3.77 | 3.66 | 4.18 | — | — | — |
| p$K_1$(amide) | — | 6.84 | 6.09 | 5.48 | 5.41 | 5.56 | 5.60 |
| p$K_2$(amide) | — | 7.42 | 7.80 | 7.69 | 6.86 | 6.91 | 6.81 |
| p$K_3$(amide) | — | 10.88 | 10.32 | 10.61 | — | 9.18 | 7.89 |

chelate, but the binding sites of the 4N-species were described in the "classical" co-ordination of peptides, namely with the involvement of terminal amino and subsequent three amide nitrogens in metal binding.[25] The striking differences in the co-ordination chemistry of the tetra-, penta- and hexa-peptides were explained by steric effects caused by the non-co-ordinating (*e.g.* Tyr) side chain residues.

Taking into account the great variety of the possible co-ordination sites of the above mentioned peptides it is worthwhile to study copper(II) complexes of oligopeptides containing only glycyl and C-terminal histidyl residues. Here, we report the results of combined potentiometric, UV–VIS and EPR spectroscopic studies on the copper(II) complexes of GlyGlyGlyHis ($Gly_3His$), GlyGlyGlyGlyHis ($Gly_4His$) and GlyGlyGlyGlyGlyHis ($Gly_5His$). Copper(II) complexes of the tripeptide GlyGlyHis ($Gly_2His$) and pentaglycine were also studied for comparison.

## Experimental

### Materials

The tripeptide $Gly_2His$ was purchased from Serva, pentaglycine was purchased from Bachem and their concentrations were checked by potentiometric titrations. The metal ion stock solutions were prepared from analytical grade copper(II) chloride and the concentration was checked gravimetrically *via* oxinates.

All solvents and chemicals for commercial sources used for synthesis were of the highest available purity and used without further purification. The protected L-amino acids (Fmoc-His-($N^{im}$-Mtt)–OH, Fmoc-Gly–OH (Fmoc = fluoren-9-ylmethoxycarbonyl, Mtt = 4-methyltrityl)) and the resin 2-chlorotrityl chloride were from CBL Chemicals Ltd., Patras, Greece.

### Peptide synthesis

The peptides $Gly_3His$, $Gly_4His$ and $Gly_5His$ were synthesized in the solid state, using the 2-chlorotrityl chloride resin [27] (substitution 1.1–1.6 mequivalents Cl g$^{-1}$) as the solid support. The Fmoc group was used for temporal protection of the α-amine group and dicyclohexylcarbodiimide (dcc)–1-hydroxybenzotriazole were used as coupling reagents. The procedure followed is described in detail in the literature.[28] Peptide cleavage from the resin was performed using a $CH_3CO_2H$–$CF_3CH_2OH$–$CH_2Cl_2$ (1 : 2 : 7 v/v) solution. The imidazole protecting group (Mtt) was removed using 50% trifluoroacetic acid in $CH_2Cl_2$–$CF_3CH_2OH$ (6 : 1 v/v). Peptide purity was controlled by thin layer chromatography using the solvent mixtures BuOH–pyridine–$CH_3CO_2H$–water (4 : 1 : 1 : 2 v/v) and verified by proton NMR (Bruker AMX 400 spectrometer).

### Potentiometric measurements

The pH-potentiometric titrations in the pH range 2.5–11.0 were performed in 4 cm$^3$ samples in the concentration range of 2–4 × 10$^{-3}$ mol dm$^{-3}$ at metal ion to ligand ratios of between 1 : 1 and 1 : 3. The number of experimental points was 50–70 (cm$^3$–pH) for each titration curve. Argon was bubbled through the samples to ensure the absence of oxygen and for stirring the solutions. All pH-metric measurements were carried out at 298 K, at a constant ionic strength of 0.2 mol dm$^{-3}$ KCl. The measurements were made with a Radiometer PHM 84 pH-meter equipped with a 6.0234.100 combined electrode (Metrohm) and a Dosimat 715 automatic burette (Metrohm) containing carbonate-free potassium hydroxide at known concentration. The pH-readings were converted to hydrogen ion concentration [29] and the overall stability constants (log $\beta_{pqr}$) of the various species ($[M_pH_qL_r]$) were calculated by means of a general computational program (PSEQUAD).[30]

$$pM + qH + rL \rightleftharpoons M_pH_qL_r$$

$$\beta_{pqr} = \frac{[M_pH_qL_r]}{[M]^p[H]^q[L]^r}$$

### Spectroscopic measurements

Visible spectra of the copper(II) complexes were recorded on a JASCO UVIDEC 610 double beam spectrometer or on a HP-8453 diode array spectrophotometer in the same concentration range used for potentiometric studies. Anisotropic X-band EPR spectra (9.15 GHz) of frozen solutions were recorded at 120 K using a Varian E-9 spectrometer after addition of ethylene glycol to ensure good glass formation in frozen solutions. Copper(II) stock solutions for EPR measurements were prepared from $CuSO_4 \cdot 5H_2O$ enriched with $^{63}Cu$ to obtain better resolution of EPR spectra. For this purpose metallic copper (99.3% $^{63}Cu$ and 0.7% $^{65}Cu$) was purchased from JV Isoflex, Moscow, Russia and converted to the sulfate.

## Results and discussion

Stability constants of the proton and copper(II) complexes of $Gly_3His$, $Gly_4His$, $Gly_5His$ and pentaglycine were determined by potentiometric measurements and the data are collected in Table 1. Equilibrium parameters of the copper(II) complexes of $Gly_2His$,[18] tri- and tetra-glycine are also listed for comparison.[31]

It can be seen from Table 1 that the stoichiometry of the complexes formed in the copper(II)–oligopeptide systems are similar to each other. For histidine containing peptides the titration curves of all metal ion to ligand ratios can be fitted with the assumption of 1 : 1 complexes differing only in the number of protonated binding sites. Inclusion of bis- or poly-nuclear complexes into the computational models did not provide better fitting while spectral parameters are also in agreement with the exclusive formation of 1 : 1 species. It is also important to note that for $Gly_3His$, $Gly_4His$ and $Gly_5His$ the values of stability constants are quite similar to each other, but significantly differ from those of $Gly_2His$. The copper(II)–

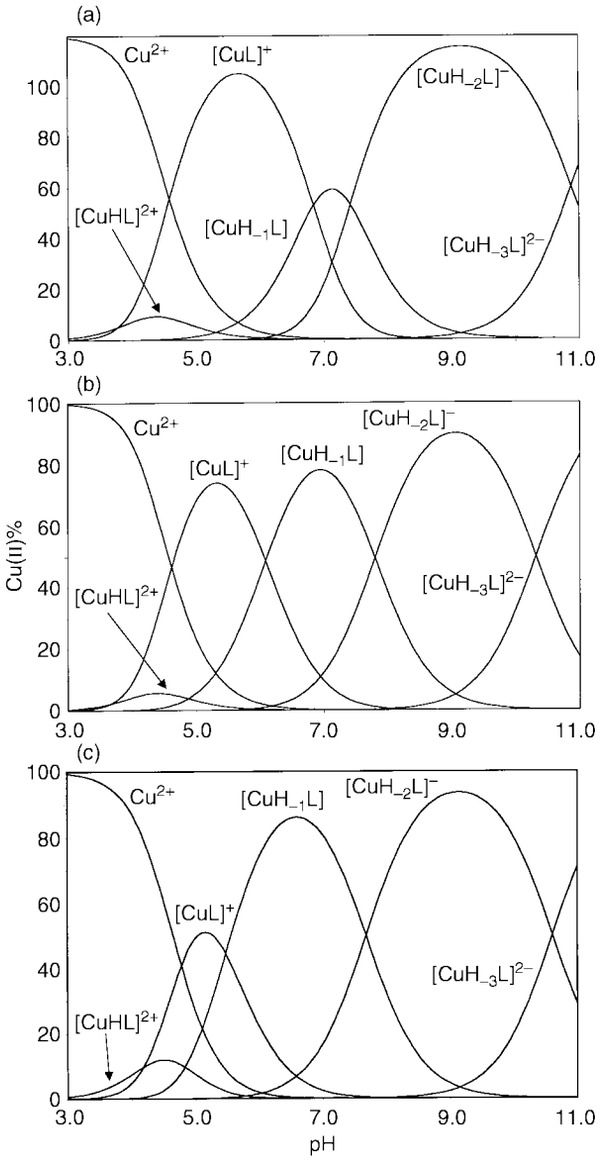



**Fig. 1** Species distribution of the complexes formed in the copper(II)–$Gly_nHis$ ($n = 3$–5) systems at equimolar concentration ($4 \times 10^{-3}$ mol $dm^{-3}$): (a) L = $Gly_3His$, (b) L = $Gly_4His$, (c) L = $Gly_5His$.

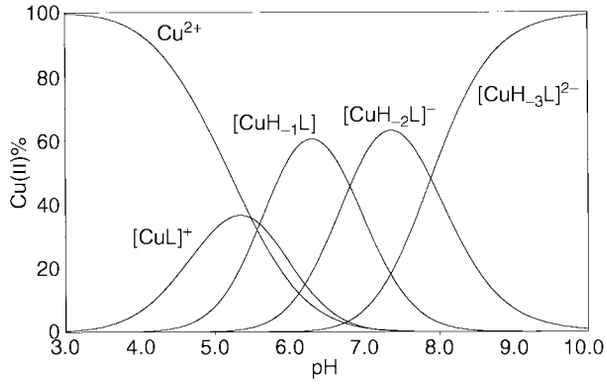



**Fig. 2** Species distribution of the complexes formed in the copper(II)–pentaglycine system at equimolar concentration ($4 \times 10^{-3}$ mol $dm^{-3}$).

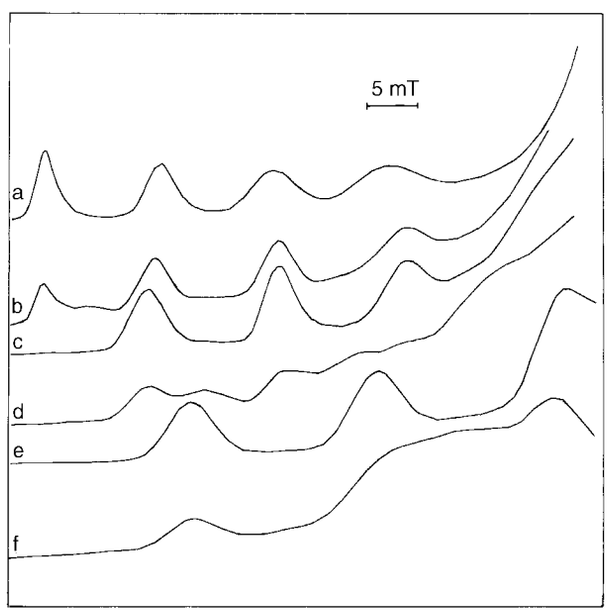



**Fig. 3** Parallel region of EPR spectra recorded at 120 K in aqueous solutions of $^{63}Cu$(II) and $Gly_3His$ in equimolar concentration as a function of pH: (a) pH = 2.95, $[Cu(H_2O)_6]^{2+}$; (b) pH = 4.35, $[Cu(H_2O)_6]^{2+}$ + $[CuHL]^{2+}$; (c) pH = 5.48, $[CuL]^+$; (d) pH = 6.81, $[CuL]^+$ + $[CuH_{-1}L]$ + $[CuH_{-2}L]^-$; (e) pH = 9.02, $[CuH_{-2}L]$; (f) pH = 12.10, $[CuH_{-3}L]^{2-}$.

$Gly_2His$ system can be characterized by the predominant formation of the species $[CuH_{-2}L]^-$ with 4N-co-ordination. The other monomeric species, $[CuHL]^{2+}$, $[CuL]^+$ and $[CuH_{-1}L]$ are present in very low concentration, because of the co-operative deprotonation and co-ordination of two amide nitrogens.[1,14,15,18] This co-operativity, however, does not hold for the other peptides containing histidine, for which successive deprotonation and co-ordination of amide nitrogens is observed. This differing behaviour is best represented by the corresponding species distribution curves depicted in Fig. 1(a)–(c) for $Gly_3His$, $Gly_4His$ and $Gly_5His$, respectively, and in Fig. 2 for pentaglycine.

It is obvious from Fig. 1 that the relative concentration of the various complexes is different in all cases and is reflected in the different p$K$ values of amide deprotonation. The interpretation of these differences, however, requires careful examination of the thermodynamic and spectral parameters of the various species. The parameters of the EPR spectra are given in Table 2, while selected examples of EPR spectra are shown in Figs. 3 and 4.

It is clear from the comparison of Table 1 and Fig. 1(a)–(c) that the species $[CuHL]^{2+}$ is always present in very low concentration. Its binding mode can be interpreted either by the co-ordination of the terminal amino and neighbouring carbonyl oxygen donors with the protonated imidazole side chain or by the monodentate co-ordination of an imidazole nitrogen with a protonated amino group. Earlier studies led to the conclusions that $N^3$ of imidazole is the primary binding site of these peptides,[1,19,23,24] but careful consideration of the spectroscopic parameters obtained for the species formed at higher pH strongly supports the existence of amine binding for all possible stoichiometries. The p$K$ values for the deprotonation of $[CuHL]^{2+}$ are around 4 for all His containing peptides, which can correspond to both possible binding modes. Unfortunately, the low concentration of this species does not make it possible to calculate the EPR parameters of the species, but its presence can be verified in all cases (see Fig. 3(b)) and its signal overlaps well with those of pentaglycine with ($NH_2$, CO)-co-ordination. On the other hand, the low concentration of $[CuHL]^{2+}$ of $Gly_nHis$ peptides as compared to that of $[CuL]^+$ of pentaglycine can be explained by the subsequent co-ordination of imidazole nitrogen donors, which results in enhanced stability of the species $[CuL]^+$. The enhancement in the stability of the species $[CuL]^+$ slightly suppresses amide deprotonation which is reflected in the p$K$ values for the first amide nitrogens (p$K_1$(amide)). The p$K$ values for $Gly_nHis$ are 6.84, 6.09 and 5.48 for $n = 3$–5, respectively, the latter value being very close to that of pentaglycine and supports the formation of macrochelates,

**Table 2** Parameters of the parallel region of EPR spectra of frozen solutions containing $^{63}$Cu(II) complexes of oligopeptides

| Species | Donor set | $Gly_3His$ | | $Gly_4His$ | | $Gly_5His$ | | Pentaglycine | |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^4 A_\parallel$/cm$^{-1}$ | $g_\parallel$ | $10^4 A_\parallel$/cm$^{-1}$ | $g_\parallel$ | $10^4 A_\parallel$/cm$^{-1}$ | $g_\parallel$ | $10^4 A_\parallel$/cm$^{-1}$ | $g_\parallel$ |
| $[CuHL]^{2+}$ | ($NH_2$, CO) | +[a] | + | + | + | + | + | — | — |
| $[CuL]^+$ | ($NH_2$, CO) + N(Im) | 139 | 2.299 | 137 | 2.298 | + | + | — | — |
| | ($NH_2$, CO) | — | — | — | — | — | — | 151 | 2.331 |
| $[CuH_{-1}L]$ | ($NH_2$, $N^-$) + N(Im) | + | + | 156 | 2.230 | 156 | 2.227 | — | — |
| | ($NH_2$, $N^-$) | — | — | — | — | — | — | 175 | 2.251 |
| $[CuH_{-2}L]^-$ | ($NH_2$, $N^-$, $N^-$) + N(Im) | 200 | 2.194 | 200 | 2.199 | 199 | 2.196 | — | — |
| | ($NH_2$, $N^-$, $N^-$) | — | — | — | — | — | — | 191 | 2.219 |
| $[CuH_{-3}L]^{2-}$ | five-co-ordinated | + | + | — | — | — | — | — | — |
| | ($NH_2$, $N^-$, $N^-$, $N^-$) | — | — | 206 | 2.171 | 206 | 2.172 | 206 | 2.171 |

[a] Detected but parameters cannot be estimated because of low concentration or overlap of several species.

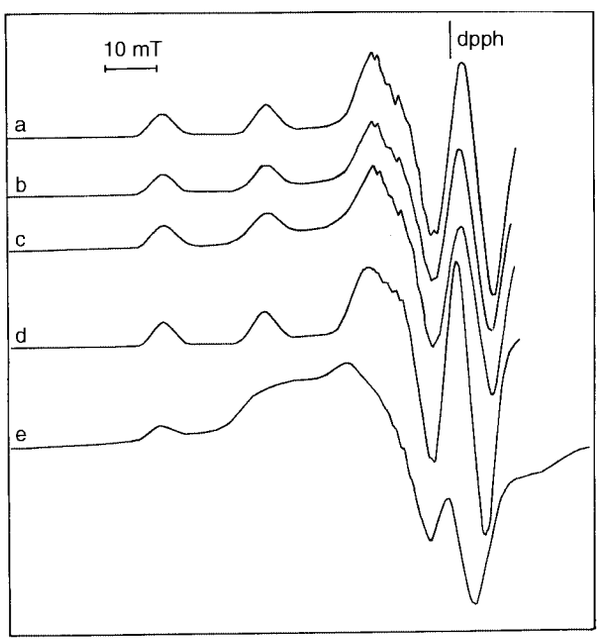
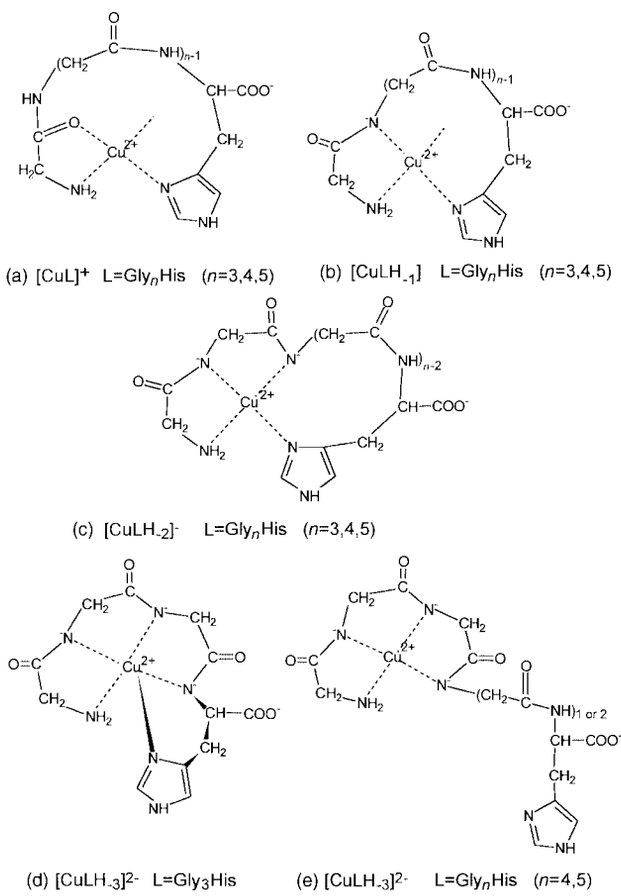



**Fig. 4** Low temperature (120 K) EPR spectra of the species formed in equimolar solutions of $^{63}$Cu(II) and (a) pentaglycine, (b) $Gly_5His$, (c) $Gly_4His$, (d) GlyGlyHis and (e) $Gly_3His$. Spectra (a), (b) and (c) belong to $[CuH_{-3}L]^{2-}$, (d) to $[CuH_{-2}L]^-$ and (e) to a mixture of $[CuH_{-3}L]^{2-}$ and $[CuH_{-2}L]^-$.




**Scheme 1**

which is most favoured for $Gly_3His$, while it does not have significant effect on the co-ordination properties of the hexapeptide. The EPR parameters of this species (Fig. 3(c)), namely the low $A_\parallel$ and high $g_\parallel$ values, strongly indicate a significant distortion in the coordination sphere of copper(II) and the existence of a macrochelate in the molecule (Scheme 1(a)). The macrochelates (or loops) correspond to the co-ordination of 2N donors from both the N- and C-termini and contain 14, 17 or 20 atoms for $Gly_3His$, $Gly_4His$ and $Gly_5His$, respectively.

The thermodynamic stabilities and concentrations of the species $[CuH_{-1}L]$ change in the opposite manner relative to $[CuL]^+$. The base consuming process can only be explained by the deprotonation and co-ordination of the first amide nitrogen of the peptide molecules, but it requires careful examination of all experimental data to deduce the location of this amide residue. In previous studies on copper(II) complexes of $Gly_3His$ and segments of angiotensin II, the species $[CuH_{-1}L]$ was assigned as a 3N-complex co-ordinated *via* the amide groups preceding the histidyl residue resulting in the formation of six- and five-membered joined chelates.[21–25] In other words, a rearrangement of the co-ordination sphere of the central metal ion and the reprotonation of the unco-ordinated terminal amino group is suggested. However, careful comparison of potentiometric and spectroscopic data obtained in this study on the copper(II) complexes of tetra- to hexa-peptides led to different conclusions. Namely, in agreement with previous findings, $[CuH_{-1}L]$ is a 3N-complex, but with the involvement of terminal amino and subsequent deprotonated amide nitrogens in metal binding (five-membered chelate, as usual for common peptides). The third nitrogen donor atom comes from the imidazole side chain at the C-terminus leading to a macrochelate in this species (Scheme 1(b)). There are several arguments for this conclusion:

(i) The species $[CuH_{-1}L]$ has the same absorption and EPR spectra for all ligands supporting the same binding mode in the corresponding copper(II) complexes. The concentration of the species $[CuH_{-1}L]$ is rather low in the case of $Gly_3His$, which results in poor resolution of EPR spectra (see Fig. 3(d)), but the spectral parameters can be readily obtained for $Gly_4His$ and $Gly_5His$. The relatively low hyperfine coupling constants ($A_\parallel \approx 156 \times 10^{-4}$ cm$^{-1}$, $g_\parallel \approx 2.23$) corresponds again a highly distorted geometry, since 3N species with joined chelate rings generally possess much higher coupling constants.[28] Furthermore, the N-protected peptide Z-GlyHis forms a species $[CuH_{-2}L]^-$

by pH 8, for which $A_{\parallel} = 199 \times 10^{-4}$ cm$^{-1}$ and $g_{\parallel} = 2.220$ which corresponds to equatorial co-ordination of two amide and one imidazole nitrogen donor atoms (five + six membered chelates).[32]

(ii) The absorption maxima of $[CuH_{-1}L]$ is at 620 ± 5 nm in all cases ($\varepsilon \approx 80$ dm$^3$ mol$^{-1}$ cm$^{-1}$). Such values are too high for the equatorial coordination of three chelating nitrogen donors, but can be interpreted by the assumption of a highly distorted macrochelate around the central metal ion.

(iii) Finally the most important and convincing proof for the existence of a macrochelate in the species $[CuH_{-1}L]$ (and $[CuH_{-2}L]^-$) comes from comparison of the p$K$ values and spectral parameters of the species $[CuH_{-1}L]$, $[CuH_{-2}L]^-$ and $[CuH_{-3}L]^{2-}$. Namely, if $[CuH_{-1}L]$ were a chelate from imidazole and preceding amide nitrogens then the N-protected peptides of histidine and its derivatives should form similar species with copper(II) as characteristic for the peptides $(Gly)_n$-His. This, however, is not the case, and N-protected derivatives of histidine are not able to prevent metal ion hydrolysis in equimolar solutions.[33] On the other hand, copper(II) complexes of Ac-$Gly_2$His and Ac-$Gly_3$His have already been studied and the anchoring ability of imidazole for the co-ordination of the preceding amide groups was definitely established. For example, for Ac-$Gly_3$His, values of p$K_1 = 6.50$, p$K_2 = 7.35$ and p$K_3 = 8.80$ were reported for the ionization of the first, second and third amide groups, respectively.[20] These values are not very far from those of Table 1, especially for the first two p$K$(amide) values of $Gly_3$His and $Gly_4$His and thus it can be concluded that the binding sites are similar. However, the first p$K$ for the amide ionization in the copper(II)–$Gly_5$His system is the same as that of pentaglycine and would also be similar for oligoglycine-like binding sites. As a consequence, the protonation constants of the amide groups in metal complexes cannot be used to distinguish between the various binding sites, especially in the pH range below the p$K$ values of the free peptides. The various parameters of the species $[CuH_{-2}L]^-$ and $[CuH_{-3}L]^{2-}$ and the spectral changes accompanying the formation of these species, however, provide unambiguous proof for the co-ordination of the N-termini in all possible stoichiometries. The species $[CuH_{-2}L]^-$ is a predominating complex in all systems and the p$K$ values for its formation could be interpreted either by deprotonation of the free ammonium group or by the deprotonation and co-ordination of the second amide nitrogen. Taking into account that the formation of $[CuH_{-2}L]^-$ is accompanied by a significant blue shift of absorption spectra and the characteristic changes of EPR parameters in the pH range 7–8, the co-ordination of one more nitrogen donor cannot be questioned (p$K_2$(amide)). As a consequence, $[CuH_{-2}L]^-$ is definitely a 4N-complex, but the fourth co-ordination site is still occupied by imidazole-N in the form of a macrochelate. The basicity of the amino groups of peptides definitely support that the last deprotonation of the complexes (p$K_3$(amide) > 10) can arise only from amide co-ordination, therefore the terminal amino group should be involved in metal binding in this and in all previous species. Of course, the deprotonation of third amide group is hindered as compared to that of Ac-$Gly_3$His (p$K_3 = 8.80$),[20] since for $Gly_n$His peptides, the binding of third amide nitrogen is replacing the macrochelate in the fourth co-ordination site of the metal ion.

As stated above, the species $[CuH_{-2}L]^-$ is a 4N-complex in all cases as corroborated by spectral parameters. The absorption maxima appear at 560 ± 2 nm with molar absorptivity $\varepsilon \approx 125 \pm 5$ dm$^3$ mol$^{-1}$ cm$^{-1}$. The EPR parameters, $A_{\parallel} = (200 \pm 1) \times 10^{-4}$ cm$^{-1}$, $g_{\parallel} = 2.197 \pm 0.002$, correspond to a 4N-complex with slightly distorted tetragonal structure (see Fig. 3(e)). The structures are best described if the co-ordination of one more amide nitrogen from the N-terminal side is suggested and the fourth co-ordination site is occupied by the imidazole group (Scheme 1(c)). The extent of distortion of the geometry is significantly decreased going from $[CuL]^+$ to $[CuH_{-2}L]^-$, since in the latter species three equatorial sites are already occupied by chelating donor groups.

Deprotonation and co-ordination of the third amide nitrogen take place above pH 10 and result in the formation of the species $[CuH_{-3}L]^{2-}$. The base consuming process is accompanied with a significant blue shift of the absorption spectra and characteristic changes of EPR parameters suggesting that a mixed hydroxo complex is not formed. The replacement of imidazole binding by the co-ordination of an amide nitrogen was also suggested by Gyurcsik *et al.*[34] in the copper(II)–LysLeuAlaHisPheGly system on the basis of UV–VIS and CD measurements. At the same time it is important to note that the spectral parameters of the $[CuH_{-3}L]^{2-}$ complex of $Gly_3$His significantly differ from those of the penta- and hexa-peptides. This is clearly seen in Fig. 4, which shows the complete EPR spectra of frozen solutions containing the species $[CuH_{-3}L]^{2-}$ of all peptides studied. Fig. 4 and Table 2 indicate that pentaglycine, $Gly_4$His and $Gly_5$His have very similar EPR and absorption spectra ($\lambda_{max} = 512 \pm 2$ nm, $\varepsilon_{max} = 140 \pm 5$ dm$^3$ mol$^{-1}$ cm$^{-1}$). However, the other two 4N-species have completely different EPR spectra with $[CuH_{-2}L]^-$ of $Gly_2$His being co-ordinated *via* the terminal amino, two deprotonated amide and the imidazole N$^3$ nitrogen donor atoms for which $A_{\parallel} = 209 \times 10^{-4}$ cm$^{-1}$, $g_{\parallel} = 2.178$ and $\lambda_{max} = 525$ nm. These values are in good agreement with the equatorial co-ordination of 4N donors in joined five,five,six-membered chelates. Both UV–VIS and EPR characteristics of the species $[CuH_{-3}L]^{2-}$ formed with $Gly_3$His are, however, significantly different from the above example. Although detected in a mixture with $[CuH_{-2}L]^-$ (see Fig. 4(e)) $[CuH_{-3}L]^{2-}$ exhibits EPR features (*e.g.* strong rhombic distortion with one $g$ value near 2.0) supportive of a penta-co-ordinated species in a rather distorted environment.[35] The $[CuH_{-2}L]^- \longrightarrow [CuH_{-3}L]^{2-} + [H^+]$ reaction is accompanied with a small blue shift of the absorption spectra suggesting that the base consuming process is amide deprotonation. On the other hand, the absorption maxima appears at $\lambda_{max} = 550$ nm, $\varepsilon_{max} = 140$ dm$^3$ mol$^{-1}$ cm$^{-1}$, which is significantly red shifted as compared to those of the penta- and hexa-peptides and can be explained by the axial interaction of the side chain imidazole nitrogen atoms (Scheme 1(d)). In principle, the axial co-ordination of C-terminal His residues would be possible in the $[CuH_{-3}L]^{2-}$ complexes of both $Gly_4$His and $Gly_5$His. The much longer distance between the last co-ordinated amide nitrogen and the free imidazole in the complexes of $Gly_4$His and $Gly_5$His as compared to that of $Gly_3$His (six-membered chelate), however, will reduce the bonding strength of axial interaction and it cannot compete with the free rotation of subsequent non-co-ordinating amino acid residues. As a consequence, the penta-, hexa- or longer peptides with C-terminal His residues are best characterized by oligoglycine-like co-ordination with free imidazole side chains (Scheme 1(e)).

## Conclusions

Stability constants and spectral parameters of the copper(II) complexes of peptides clearly demonstrate that imidazole side chains have a very significant impact on the co-ordination properties of these ligands. For the widely studied GlyHis and GlyGlyHis this results in the enhanced metal binding ability of the molecules due to the change of the characteristic binding sites of common peptides. For tetrapeptides or higher oligomers containing histidine the number of all nitrogen donors is too high to co-ordinate in the equatorial plane of copper(II), but the Cu(II)–N(im) interaction remains a governing factor during complex formation.

The species $[CuL]^+$ is formed with all peptides containing C-terminal His residues, $Gly_3$His, $Gly_4$His and $Gly_5$His. It is a 2N-complex formed *via* the co-ordination of terminal amino-N and carbonyl-O donors in a five-membered chelate completed by the binding of imidazole in the form of a macrochelate

(Scheme 1(a)). The unusually low hyperfine coupling constants of the species indicate a large extent of distortion in the co-ordination geometry. The stability of this macrochelate is decreased with an increase of the number of amino acid residues in the peptide chain. An increase of the pH of the solutions results in the deprotonation and co-ordination of amide nitrogens subsequent to the terminal amino group. There is a dichotomy in the literature as to the location of the deprotonated amide groups in such copper(II) complexes. In the previous studies it was concluded that imidazole-N and preceding nitrogen donors are the binding sites in the species $[CuH_{-1}L]$ and $[CuH_{-2}L]^{-}$. Comparison of the thermodynamic and spectral parameters of the copper(II) complexes of $Gly_3His$, $Gly_4His$ and $Gly_5His$ and consideration of the metal binding capability of N-protected peptides, however, led to the differing conclusions. The co-ordination geometry of $[CuH_{-1}L]$ is described as 3N-complex containing a five-membered chelate ($NH_2$, $N^-$) at the N-termini completed with the binding of imidazole side chain in the form of a macrochelate (Scheme 1(b)). The existence of a macrochelate results in some distortion of geometry as reflected in the spectral parameters. The binding sites of the species $[CuH_{-2}L]^{-}$ are similar to those of $[CuH_{-1}L]$ except for the presence one more amide nitrogen saturating the co-ordination sphere of copper(II), ($NH_2$, $N^-$, $N^-$) + N(Im) in a macrochelate (Scheme 1(c)). In other words, below pH 10 all three peptides, $Gly_3His$, $Gly_4His$ and $Gly_5His$, have very similar metal ion speciation and the same binding modes, which is best characterized in terms of the existence of a macrochelate around the central metal ion. As one of the consequences of these macrochelates, the deprotonation and co-ordination of the third amide nitrogens are significantly suppressed ($pK_3$(amide) > 10) as compared to that of pentaglycine ($pK_3$-(amide) = 7.89). In connection with the results obtained for pentaglycine it is important to note that this p$K$ value is significantly lower than that of tetraglycine ($pK_3$(amide) = 9.18). This seems to be in contradiction with the expectations, but it is in good agreeement with earlier literature studies.[36] Taking into account the fact that $[CuH_{-3}L]^{2-}$ of tetraglycine and pentaglycine have the same binding patterns in the crystal structure of solid samples[37,38] the difference in p$K$ values can originate only from the slightly suppressed deprotonation of the third amide nitrogen of tetraglycine. This probably can be explained by a weak interaction between the metal ion and the carboxylate residue, which are much closer to each other in tetraglycine.

The species $[CuH_{-3}L]^{2-}$ of $Gly_nHis$ peptides ($n$ = 3–5) show two different binding modes in the copper(II) complexes. The penta- and hexa-peptides are characterized by a pentaglycine-like co-ordination with unco-ordinated imidazole residues (Scheme 1(e)). This assumption is in good agreement with the spectroscopic parameters and can be explained by the large separation between the N-terminal binding sites and histidyl side chains. For the tetrapeptide, $Gly_3His$, the equatorial sites are occupied by the same donor atoms, but the imidazole residue is close enough to the metal ion to be involved in axial co-ordination, which results in the formation of a penta-co-ordinated complex (Scheme 1(d)).

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