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Coordination properties of Cu(II) ions towards the peptides based on the His-Xaa-His motif from *Fusobacterium nucleatum* P1 protein

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ABSTRACT

The coordination capacity of the copper(II) ions with peptides (fragments of the P1 protein - one of the outer membrane protein from *Fusobacterium nucleatum*) based on the His-Xaa-His motif was carried out using potentiometric measurements, mass spectrometry and spectroscopic techniques: UV–Vis, CD and EPR. The selected tetrapeptides (**Ac-HGHE-NH$_2$**, **Ac-GHEH-NH$_2$**, **Ac-HEHQ-NH$_2$** and **Ac-EHEH-NH$_2$**) form both mononuclear and bis-complexes with copper(II) ions. In the case of mononuclear complexes the CuL and CuLH$_{-2}$ species dominate in the solution, where the coordination sphere is create by $\{2 \times N_{Im}\}$ and $\{2 \times N_{Im}, 2 \times N^{-}_{amide}\}$, respectively. The **Ac-HGHE-NH$_2$** peptide form more stable the CuLH$_{-2}$ complex with the 4 N$\{2 \times N_{Im}, 2 \times N^{-}_{amide}\}$ binding site compared to the other ligands. The presence of glutamic acid residue in sequence **Ac-HEHQ-NH$_2$** produced the destabilization of the CuLH$_{-2}$ complex in comparison to that of the **Ac-HGHE-NH$_2$** sequence. For the CuLH$_{-3}$ complex the coordination process for complexes containing a histidyl residue in the first positions (H^1) proceed towards *C*-terminal sequence of the peptide. The bis-complexes are formed in the solution, where the metal ion is bounded by four imidazole nitrogen atoms $\{4 \times N_{Im}\}$.

1. Introduction

The interaction of transition metal ions with peptides is one of the main areas of bioinorganic chemistry due to the mimic properties of metallic centers of the relevant metalloproteins [1,2]. Proteins or peptides, which are composed of several amino acids are found to be highly specific and efficient ligands for many transition metals [3,4]. The presence of many different donor atoms allows the formation of complexes with various conformations [5]. Most importantly, the appropriate arrangement of amino acids in the peptide sequence has a significant effect on the stability of the formed complexes [6]. In addition, the stability of the formed complexes depends on the metal ion, the ability to chelating rings formation and the possibility to create the coordination bonds [7].

One of the strongest coordinators of transition metal ions is the histidyl residue [3,8]. Interestingly, this amino acid is the most common binding site in many metalloenzymes and metalloproteins [9]. The position of the histidyl residue in the amino acid sequence of the peptide and its number strongly affect the binding capacity of the metal ion by the peptide. Ligands containing at least two or more histidyl residues show large structural diversity of formed complexes. In addition, it is possible to create macrochelates and form not only mononuclear complexes [10].

The imidazole nitrogen atom is usually an anchoring site of metal ion irrespective of the position of the histidine residue in the amino acid sequence of the peptide [11]. The presence of the histidine residue in the first position of the peptide sequence with the blocked amino group results in the formation $\{N_{Im}, N^{-}_{amide}\}$ binding mode with an unstable seven-membered chelate ring. The metal ion (copper(II) or nickel(II)) is anchored by imidazole nitrogen atom and when the pH value of the solution is increased it is bound by subsequent amide nitrogen. Interestingly, the most thermodynamically stable complexes are created when the histidyl residue is in the third position with free amino group at *N*-terminal. This strong affinity towards copper(II) ions results from the ability to form three highly stable chelate rings (5-5-6), where the coordination process proceeds towards the *N*-terminal. At physiological pH, the formation of a complex with MLH$_{-2}$ stoichiometry is observed (where M is Cu(II) or Ni(II)) with the following set of donor atoms $\{NH_2, 2 \times N^{-}_{amide}, N_{Im}\}$. This coordination mode is often referred to as ATCUN motif (Amino Terminal CuNi binding site) and is responsible for the high binding affinity of copper(II) and nickel(II) ions of human serum albumin [4,12–17]. Importantly, the free amino group significantly influences on the binding process of the metal ion in the multi-histidine peptides. For these peptides usually the free amino group is primary binding site. In the case of peptides with a free amino group and containing histidine residues at position 4 and further,

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formation of macrochelates is observed [4,15,16]. This is due to the presence of two potential anchoring sites, whereby two metal ions can be bound. The coordination capability of the peptides relative to the transition metal ions is significantly reduced by blocking the *N*-terminal amino group [4,12–14].

As it was mentioned before, other factors affect on the stability of complexes, *e.g.* different donor atoms. It appears that an important binding site in metalloenzymes is the carboxyl group of glutamic or aspartic acid [4]. The number of these residues and their position in the peptide sequence, as in the case of the histidyl residue, determine the stability of the complexes. In the literature numerous examples show a significant effect of the aspartic acid on the coordination abilities [18]. The presence of the β-carboxyl and γ-carboxyl groups of Asp and Glu residues, respectively provide increase the negative charge of the complex. However, this effect is lesser in the case of glutamic acid residue due to the possibility of formation only seven-membered chelate ring. The position of glutamic acid in the peptide sequence can significantly affect the thermodynamic stability through the ability to hydrogen bonds formation. Furthermore, it is suggested that the carboxyl group of glutamic acid may stabilize the binding of the metal ion by the imidazole nitrogen atom [18,19].

Copper is a micronutrient necessary for the proper functioning of all organisms. It is the metal center of many enzymes involved in biological processes, *e.g.* electron transport, oxygen transport or cell signaling. Copper(II)-peptide complexes are especially interest because they serve as model systems of metalloproteins or naturally occurring enzymes. The complexity of the interaction between the metal ion and the peptide requires the use of small multihistidine peptides. This is very helpful in understanding and approximating the coordination process with the native molecules [2,8,20].

In this work we present the coordination process of copper(II) ions to tetrapeptides from the *Fusobacterium nucleatum* outer membrane P1 protein. The P1 protein consists of 483 amino acids and contains twelve histidine residues (Fig. 1). At *N*- and *C*-terminal of P1 protein single His residues are present while in the middle of the protein His-Gly/Glu-His sequences occur. This protein, which is the porine, is responsible for the transport of small molecules and long chain fatty acids inside the bacterial cell from the external environment. In addition, it is believed to play a role in pathogenicity due to interaction with the host immune system [21–25]. It is believed that this anaerobic, gram-negative bacterium is associated with the development of colorectal cancer. The exact mechanism of cancer with the participation of this bacterium is still unknown [26,27].

The presented studies were conducted for tetrapeptides based on histidine and glutamic acid residues in a different position in the amino acid sequence (**Ac-HGHE-NH$_2$**, **P^{I}**; **Ac-GHEH-NH$_2$**, **P^{II}**; **Ac-HEHQ-NH$_2$**, **P^{III}** and **Ac-EHEH-NH$_2$**, **P^{IV}**). Structural formulas of the corresponding ligands are shown in Fig. 2. It should be emphasized that the examined systems occur in the central part of the P1 protein and do not have a free amino group that can change the coordination process to copper(II) ions. We cannot refer the examined peptides to the whole P1 protein. It is known that the amino group is the primary group coordinating the copper(II) ions [4,28] but we would like to know how the sequences containing two histidine residues may coordinate the copper(II) ions. It should be also mentioned that the binding of copper(II) ions depend also on the availability of binding sites (protein structure).

The physicochemical properties of the obtained complexes with copper(II) ions were determined by spectroscopic methods (UV-Vis, CD and EPR). Potentiometric measurements were used to calculate the stability constants and stoichiometry of the resulting complexes with pH change. In addition, mass spectrometry was used to confirm the stoichiometry of copper(II)-peptide complexes. Short peptides allow to determine the importance of individual amino acid residues in the coordination process.

2. Experimental

2.1. Materials

The *N*- and *C*-terminally protected peptides **Ac-HGHE-NH$_2$** (**P^{I}**), **Ac-GHEH-NH$_2$** (**P^{II}**), **Ac-HEHQ-NH$_2$** (**P^{III}**) and **Ac-EHEH-NH$_2$** (**P^{IV}**) were purchased from KareBayBiochem (USA). Copper(II) nitrate ($Cu(NO_3)_2$), Potassium nitrate (KNO_3) were obtained from Sigma-Aldrich Co, (Germany). Ethylene Glycol ($HOCH_2CH_2OH$) was purchased from Baker Analyzed, (USA). Carbonate-free solution of Sodium hydroxide (NaOH) was received from Fluka, (Switzerland) while the Nitric acid (HNO_3) was purchased from MerckKGaA, (Germany). Calibration buffers (pH = 4.01 and 9.21) were obtained from Mettler-Toledo GmbH, (Germany).

2.2. Potentiometry

The potentiometric measurements of the studied peptides (**Ac-HGHE-NH$_2$** (**P^{I}**), **Ac-GHEH-NH$_2$** (**P^{II}**), **Ac-HEHQ-NH$_2$** (**P^{III}**) and **Ac-EHEH-NH$_2$** (**P^{IV}**)) and their copper(II) complexes were carried out in an argon atmosphere and at a constant temperature (298 K) on a 905 Titrando Methrom. Solutions were prepared in distilled water (Simplicity UV, Millipore with 18.2 MΩ resistance) with addition of HNO_3 to receive appropriate pH value. Changes of pH were monitored with an InLab Semi-Micro pH electrode form Mettler-Toledo GmbH. All measurements were performed at constant ionic strength (0.1 M KNO_3) in the pH range from 2.5 to 8.0 and 0.1 M NaOH as a titrant. The concentration of ligands was 2 mM or 2.5 mM and the total volume of samples was 2 ml. In the complexes the metal to ligand molar ratio was 1:1 and 1:2. The exact concentrations and purities of the ligands were determined by the Gran method [29]. The SUPERQUAD program was used for the stability constant calculations [30]. The standard deviation values were referring to random errors only. In general, titrations of the respective ligand in the presence of various equivalents of Cu(II) were analyzed in batch calculations, in which all titration curves are fitted at the same time with one model. The formation reaction equilibrium of ligands with protons and copper(II) ions ale given in Eq. (1)

```
1   MKKLLLLTAI LSSGLYAASI DHIQTYSPDY LANQAQTGMI NGVSPYYNPA ALGRLEKGKY
61   LHAGFQFAHG HEKMSYKGKE HKANLNQAIP NIALTFVDDK GATFFNFGGL AGGGKLRYDG
121 VSGVDVLTDL AQFKPLGIYD KGSSLTGSNK YEQITLGRAF NIDDKLSFSV AARVVHGTRR
181  LNGNLNIGAN PTAQYKQEKA QQVAQEVSKA VDAATQGKGL SAAQIAAIKT KKTNEALAKL
241 QQRVNDLSQN GLSGDIDSKR EAWGYGFQLG VNYKVNDKLN LAARYDSRIK MNFKAKGHEH
301 QLETTDILKQ TIGLSTFYPQ YTINSKIRRD LPAILSVGAS YKVADNYLVS TTANYYFNHQ
361  AKMDRVTTFG EHEHGRDYKN GWEIAVGNEY KLNDKFTLIG SLNYANTGAK TASYNDTEYA
421 LNSFTLGGGI RYQYDESLAI TASVAHFIYQ GAEGNFKEKY GVTENQKYKK EITAVGLSVT
481 KKF
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Fig. 1. The one-letter code of amino acid sequence of P1 protein from *Fusobacterium nucleatum* bacteria. The His residues are marked.

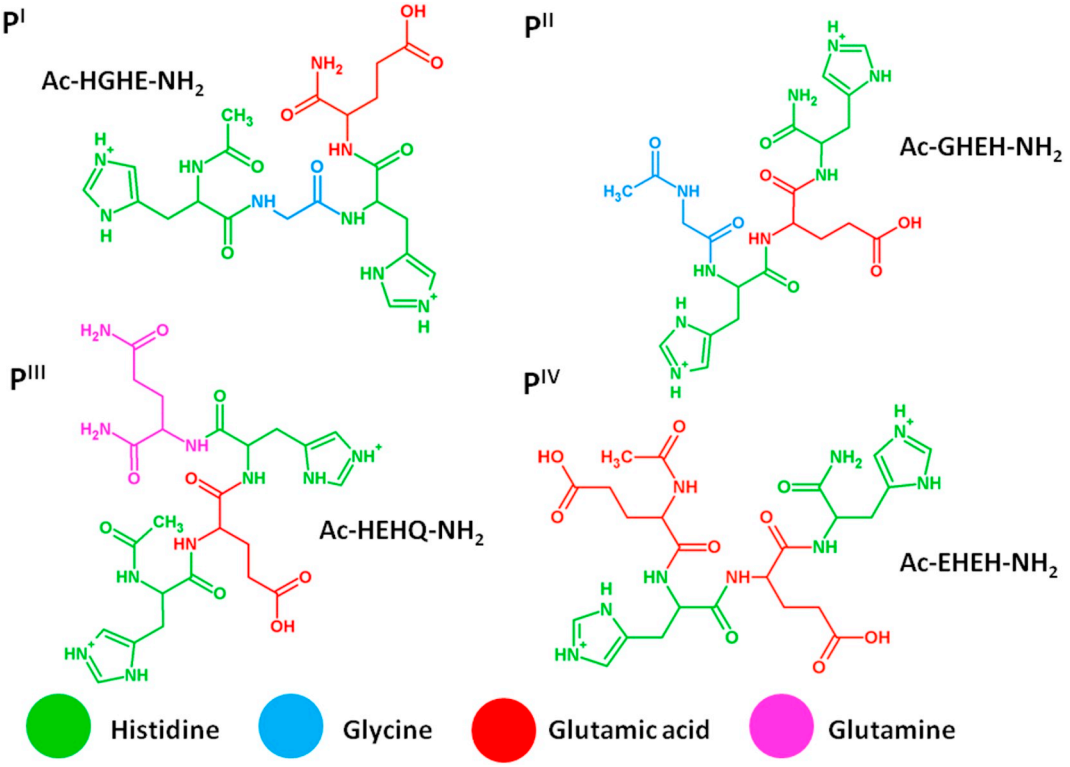

Fig. 2. The structural patterns of the tested peptides studied.

$$pCu + qH + rL = CupHqLr \quad (1)$$

in which L are the peptides under study. The stability constant β_{pqr} is defined in Eq. (2):

$$\beta_{pqr} = \frac{[Cu_pH_qL_r]}{[Cu]^p[H]^q[L]^r} \quad (2)$$

2.3. Spectroscopic measurements

2.3.1. UV–Vis

The electronic absorption spectra were recorded on a Cary 60 spectrophotometer (Agilent Technologies) over the spectral range of 300–900 nm. The concentration of solutions as well as the metal to ligand molar ratio was similar as in the potentiometric studies. The spectra of copper(II) complexes were recorded in the pH range from 3 to 8 using 1 cm cuvette. The spectra were scanned at the pH and stoichiometric M:L values adequate to obtain the maximum formation of the particular species but also in this condition other species coexist with given ε values determined in a smaller concentration. The ε values for a specific pH are average values that include all complex forms with corresponding ε values in this pH. The addition of a small amount of HNO_3 and NaOH was used to change the pH of the solution and the changes were monitored with an InLab Semi-Micro pH electrode form Mettler-Toledo GmbH.

2.3.2. CD

Circular dichroism (CD) spectra were recorded on Jasco J-715 spectropolarimeter (JASCO) in the 220–800 nm spectral range using 0.1 and 1 cm cuvettes. The concentration of samples and metal to ligand molar ratio were the same as those used in UV–Vis method. CD spectra were expressed in terms of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (ε_L and ε_R are molar extinction coefficients for the left and right polarized light, respectively).

2.3.3. EPR

Electron paramagnetic resonance (EPR) spectra of copper(II) complexes were recorded on Bruker spectrophotometer (Bruker ELEXSYS E500 CW-EPR) at the X-band frequency (9.5 GHz). The concentration of samples and metal to ligand molar ratio were the same as those used in UV–Vis and CD methods. The spectra were recorded at the maximum concentration of the particular species obtained from potentiometric data and also in these conditions other species coexist in a smaller concentration. Ethylene glycol was added as a cryoprotectant to each sample (30% of sample volume). Samples were frozen and the measurements were carried out in liquid nitrogen at 77 K. EPR parameters were determined by simulation of the experimental spectra (Bruker's WIN-EPR SimFonia software, version 1.25).

2.4. Mass spectrometry

The mass spectra (MS) were obtained on a Bruker MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with Apollo II electrospray ionization source. The measurements of ligands and their copper(II) complexes were carried out in the positive ion mode. The instrumental parameters were as follows: scan range *m/z* 0–2500, nitrogen was used as a dry gas, temperature 200 °C, reflector voltage 1300 V and detector voltage 1920 V. The concentration of all studied systems was 1 mM and metal to ligand molar ratios 1:1 and 1:2. The samples were prepared in water and infused at a flow rate of 3 µl/min. The instrument was calibrated externally with Tunemix™ mixture (Bruker Daltonik, Germany), before each experiment.

3. Results and discussion

Potentiometric studies were used to calculate the stepwise and cumulative protonation constants (Table 1). All tested ligands contain two histidyl residues and one or two glutamyl residues. For this reason the **Ac-HGHE-NH$_2$** (**P^{I}**, 69–72 sequence of P1), **Ac-GHEH-NH$_2$** (**P^{II}**, 297–300 sequence of P1), **Ac-HEHQ-NH$_2$** (**P^{III}**, 298–301 sequence of P1) peptides behave like H_3L type of ligand, whereas the last one **Ac-EHEH-NH$_2$** (**P^{IV}**, 371–374 sequence of P1, Fig. 1) is H_4L. The lowest pK_a values for the **P^{I}**, **P^{II}**, **P^{III}** and **P^{IV}** peptides belong to the carboxyl groups

Table 1
Formation and deprotonation (logβ and pK_a, respectively) constants for studied ligands.

Species	Ac-HGHE-NH_2 P^I	Ac-GHEH-NH_2 P^{II}	Ac-HEHQ-NH_2 P^{III}	Ac-EHEH-NH_2 P^{IV}
		logβ[a]		
HL	6.95(1)	6.88(1)	6.82(1)	7.00(3)
H_2L	13.10(1)	13.04(1)	12.90(1)	13.24(1)
H_3L	17.04(1)	17.04(1)	16.81(1)	17.67(1)
H_4L	–	–	–	21.36(1)
		pK_a[b]		
COOH	–	–	–	3.69
COOH	3.94	4.00	3.91	4.43
His	6.15	6.16	6.08	6.24
His	6.95	6.88	6.82	7.00

[a] $\beta(H_nL) = [H_nL]/[L][H^+]^n$; standard deviations on the last digit are given in parentheses.
[b] $pK_a = \log\beta(H_nL) - \log\beta(H_{n-1}L)$.

from the side chain of glutamic acid. The highest and intermediate values correspond to the histidyl imidazole nitrogens. Detailed data are consistent with the literature and are presented in the Table 1 [2,18].

All tested ligands formed mononuclear (metal to ligand molar ratio 1:1) and bis-complexes (metal to ligand molar ratio 1:2) in aqueous solution in the presence of copper(II) ions. Formation of this type of complexes was confirmed by calculations based on potentiometric measurements and mass spectrometry. The coordination process of copper(II) ions by peptide ligands was similar in all cases. The stability and deprotonation constants for mononuclear and bis-complexes are provided in Tables 2 and 3, respectively.

In order to be able to fully characterize the mononuclear and bis-complexes, both potentiometric measurements as well as the spectroscopic studies were carried out in two molar ratios of metal to ligand (1:1 and 1:2). For this reason the coordination process will be described separately. In the case of mononuclear complexes, the analysis of potentiometric data revealed the occurrence of the same complex forms: CuL, $CuLH_{-2}$ and $CuLH_{-3}$. For the $\mathbf{P^I}$ peptide (Fig. 2), the $CuLH_2$ species was additionally detected (Fig. 3A) and CuLH complex for $\mathbf{P^I}$, $\mathbf{P^{II}}$ and $\mathbf{P^{IV}}$ peptide (Fig. 3A, B and D).

The coordination process began at the low pH value by anchoring copper(II) ion by imidazole nitrogen atom [4,12,14]. The deprotonation constants of the $CuLH_2$ complex ($CuLH_2 \rightleftarrows CuLH + H^+$) for $\mathbf{P^I}$ peptide is equal 4.36 (Table 2) and corresponds to the deprotonation to the carboxylate group of glutamic acid [31,32]. The appearance of the $CuLH_2$ form for $\mathbf{P^I}$ peptide suggests that the deprotonation of the carboxyl group of the glutamic acid residue occurs after binding of copper (II) ion. The $\mathbf{P^I}$ peptide is H_3L type of ligand (Table 1), and the first complex is $CuLH_2$ species. It should be noted that the $CuLH_2$ complex is

Table 2
Stability Constants and Calculated Deprotonation Constants (logβ and pK_a, respectively) of mononuclear copper(II) complexes with the studied ligands.

Species	Ac-HGHE-NH_2 P^I	Ac-GHEH-NH_2 P^{II}	Ac-HEHQ-NH_2 P^{III}	Ac-EHEH-NH_2 P^{IV}
		logβ[a]		
$CuLH_2$	15.59(4)	–	–	–
CuLH	11.23(2)	11.16(1)	–	11.78(4)
CuL	6.76(1)	6.78(1)	6.52(1)	6.98(1)
$CuLH_{-2}$	−5.40(1)	−6.06(1)	−6.34(1)	−6.25(1)
$CuLH_{-3}$	−12.17(2)	−13.31(3)	−13.82(2)	−13.66(2)
		pK_a[b]		
$pK_{a\ 2/1}$	4.36	–	–	–
$pK_{a\ 1/0}$	4.47	4.38	–	4.80
$pK_{a\ -2/-3}$	6.77	7.25	7.48	7.41

[a] $\beta(CuH_nL) = [CuH_nL]/[Cu][L][H^+]^n$; standard deviations on the last digit are given in parentheses.
[b] $pK_a = \log\beta(CuH_nL) - \log\beta(CuH_{n-1}L)$.

Table 3
Stability Constants and Calculated Deprotonation Constants (logβ and pK_a, respectively) of bis-complexes with copper(II) ions and studied ligands.

Species	Ac-HGHE-NH_2 P^I	Ac-GHEH-NH_2 P^{II}	Ac-HEHQ-NH_2 P^{III}	Ac-EHEH-NH_2 P^{IV}
		logβ[a]		
CuL_2H	17.1(1)	17.62(2)	–	18.15(2)
CuL_2	12.11(1)	12.35(1)	11.38(2)	12.29(1)
CuL_2H_{-1}	5.13(8)	5.79(1)	4.72(3)	5.44(4)
CuL_2H_{-2}	−0.82(2)	−1.10(1)	–	−2.12(1)
CuL_2H_{-3}	–	−9.12(2)	–	–
		pKa[b]		
$pK_{a\ 1/0}$	4.99	5.27	–	5.86
$pK_{a\ 0/-1}$	6.98	6.56	6.66	6.85
$pK_{a\ -1/-2}$	5.95	7.00	–	7.56
$pK_{a\ -2/-3}$	–	8.02	–	–

[a] $\beta(CuH_nL) = [CuH_nL]/[Cu][L][H^+]^n$; standard deviations on the last digit are given in parentheses.
[b] $pK_a = \log\beta(CuH_nL) - \log\beta(CuH_{n-1}L)$.

formed at pH < 4 for the $\mathbf{P^I}$ peptide (Fig. 3A). At the same time, the carboxyl group of the glutamic acid and imidazole nitrogen of the second histidine residue are protonated (complex $CuLH_2$, Table 1). The situation is different for the $\mathbf{P^{II}}$, $\mathbf{P^{III}}$ and $\mathbf{P^{IV}}$ peptides, which are an H_3L ($\mathbf{P^{II}}$ and $\mathbf{P^{III}}$) and H_4L ($\mathbf{P^{IV}}$) type of ligands. For the $\mathbf{P^{II}}$ and $\mathbf{P^{IV}}$ peptide the first emerging is the CuLH species, while for $\mathbf{P^{III}}$ peptide the first complex is CuL. According to the Fig. 3B, C and D these complexes (CuLH and CuL) are formed at pH above 4. At this pH for $\mathbf{P^{II}}$ and $\mathbf{P^{IV}}$ peptides only the imidazole nitrogen of second His residue is protonated and the CuLH complexes are formed (Table 1) [4,6,12,14,33]. However, due to the low concentration of these forms in the solution and by overlapping others complexes and copper(II) ions, their spectroscopic analysis was impossible (Fig. 3). What is more, it should be noted that in the case of complex with $\mathbf{P^{IV}}$ peptide, the presence of the CuLH complex in the solution is about 25%. For this CuLH complex of the $\mathbf{P^{IV}}$ peptide, the proton derives from the second uncoordinated imidazole of the histidine residue. This peptide contains two glutamic acid residues. The presence of the CuLH form in a 25% in aqua solution may suggest the participation of the imidazole proton in the hydrogen bond formation between the deprotonated glutamic acid and protonated imidazole of second His residue. Stabilization of this complex in comparison to others studied systems by formation of Glu-His hydrogen bond cannot be excluded. In the pH range from 5 to approximately 6, the CuL complex dominate for all peptides and its maximum concentration is about pH 5.5 (Fig. 3). It should be noted that 5.5 is the pH that is present in some parts of the intestine or oral cavity in which the carcinogenesis process may occurs in the presence of *Fusobacterium nucleatum*. Spectroscopic parameters of the CuL complexes (Table 4) together with the logK* values, clearly indicate that the next imidazole nitrogen is included in the coordination sphere of the copper(II) ions (Tables 4 and 5) [34]. The logK* value is useful for comparing the binding capacities between different ligands [35,36]. The logK* values corresponding to the protonation corrected stability constants of the coordinated groups to the metal ions and protonated groups in the complex [14]. The comparable values logK* for the CuL complex of the studied and analogues peptides are observed, where the following $\{2 \times N_{Im}\}$ coordination mode is suggested. This similarity may suggest no additional interaction (carboxylate group of Glu residue) of peptides with copper(II) ions. The d-d transition bands in UV–Vis (642–685 nm, predicted by Prenesti value 692 nm [37]), the CD as well as EPR parameters $A_{||}$ = 159–165 G, $g_{||}$ = 2.307–2.315 are typical for the systems where the same $\{2 \times N_{Im}\}$ coordination mode is observed (Figs. S1–S4, Table 4) [5]. The parameters of the EPR spectra clearly indicate changes in the coordination environment of the metal ion. It is known that with the coordination of an additional nitrogen donor the $g_{||}$ value decreases and the $A_{||}$ value increases [38,39].

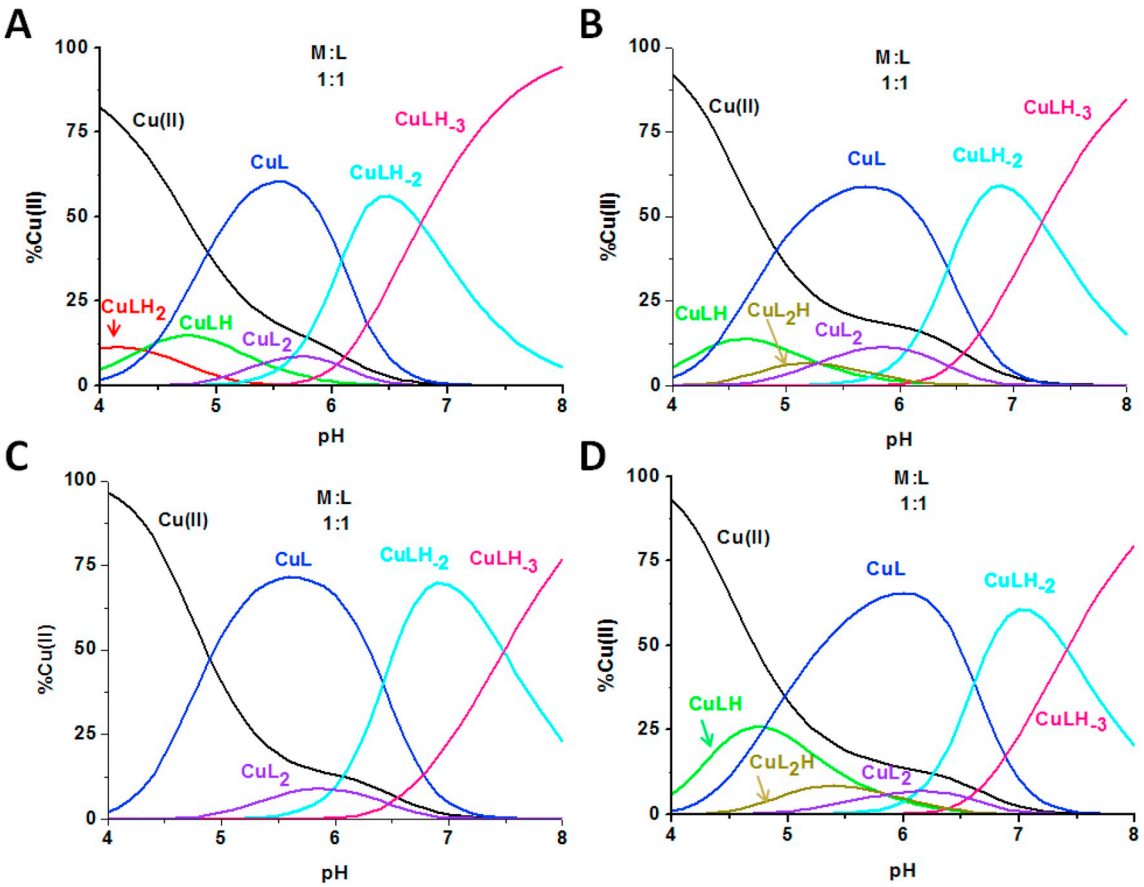

Fig. 3. Species distribution curves for copper(II) complexes with A) **Ac-HGHE-NH$_2$**, B) **Ac-GHEH-NH$_2$**, C) **Ac-HEHQ-NH$_2$** and D) **Ac-EHEH-NH$_2$** (M:L molar ratio 1:1, [Cu(II)] = 2 mM.

With the increase of pH value of the solution a next complex $CuLH_{-2}$ is formed (Fig. 3). The stoichiometry of the complex suggest the following coordination mode $\{2 \times N_{Im}, 2 \times N^{-}_{amide}\}$ [40,41]. High prevalence of this complex allowed on its full spectroscopic characteristics. On the UV–Vis spectra, in the range of d-d transitions, a hipsochromic shift is observed (Figs. 4 and S1, Table 4). In addition, the involvement of a further donor atom in the binding of copper(II) ions was evidenced by charge transfer transitions on CD spectra at $N_{Im}(\pi_1) \rightarrow Cu(II)$, 339–345 nm, $N^{-}_{amide} \rightarrow Cu(II)$ at 275–301 nm and $N_{Im}(\pi_2) \rightarrow Cu(II)$ at 246–263 nm (Fig. S3, Table 4) [42–44]. Moreover, for the $CuLH_{-2}$ complex of the **Ac-HGHE-NH$_2$** peptide (**P^{I}**) in CD spectra only one band at 576 nm is observed in comparison to those observed for the rest peptides containing **HEH** sequence (three bands, Table 4, Figs. 5 and S2). It means that the presence of the glutamic acid in the **HEH** parts of the studied peptides coordinated to copper(II) ions in the $CuLH_{-2}$ species ($\{2 \times N_{Im}, 2 \times N^{-}_{amide}\}$ binding site) gives the complexes with lower symmetry comparison to that for the Cu(II)-**Ac-HGHE-NH$_2$** system [45,46]. However, the presence of one band on the CD spectrum for Cu(II)-**Ac-HGHE-NH$_2$** may also be due by the presence of the glycine residue and the presence of only two His residues opposite to the peptides containing the **HEH** motif that have three chiral backbones (two His residues and one Glu). The EPR parameters also indicated the formation of a 4 N complex (the $A_{||}$ shifts to greater values, $g_{||}$ to lower values due to the increase of the number of nitrogen atoms) [47]. A square based chromophore is expected to show a $g_{||}$ value of 2.200 and $A_{||}$ in the range 180–200 G [39]. However, the difference in values $A_{||}$ and small only in $g_{||}$ for the $CuLH_{-2}$ complex for **P^{I}** and **P^{II}** may suggest a lower symmetry of the complex for the **P^{II}** ligand compared to the **P^{I}** [39]. The smaller hyperfine constant of **P^{II}** could be attributed to a tetrahedral distortion at the copper site due to steric hindrance of the ligand. The obtained parameters are consistent with the literature data for similar systems [47]. Additionally, and very interestingly the logK* values for the $CuLH_{-2}$ complexes showed the stabilization of the Cu(II)-**P^{I}** complex in relation to other complexes, in particular **P^{IV}** where two glutamic acid residues are present. This means the stabilization of the $CuLH_{-2}$ complex (logK*) of the **Ac-HGHE-NH$_2$** peptide by one order of magnitude in comparison to that of the **Ac-EHEH-NH$_2$** peptide (Table 5) [11].

With increasing of pH the $CuLH_{-2}$ complex loses one proton and $CuLH_{-3}$ species is form. The pK_a values (6.77–7.60, Table 2) may indicate the inclusion of the next amide nitrogen in the coordination sphere [2]. This complex form is dominated in the solution from pH 7 for the **P^{I}** complex and from pH 7.5 for the **P^{II}**, **P^{III}** and **P^{IV}** peptide (Fig. 3). The shift to lower wavelength of d-d bands is observed on the UV–Vis spectra also indicating the involvement of the next amide nitrogen in the binding of copper(II) ion. The EPR parameters for the $CuLH_{-3}$ complex $A_{||}$ = 182–190 G, $g_{||}$ = 2.208–2.215 are typical for 4 N $\{N_{Im}, 3 \times N^{-}_{amide}\}$ complexes (Figs. S1 and S4, Table 4) [48]. Interestingly, in the range of d-d transitions on the CD spectra, three Cotton effects for the $CuLH_{-3}$ complexes with **P^{II}**, **P^{III}** and **P^{IV}** peptides, are observed, respectively. This may suggest the formation of the $CuLH_{-3}$ complexes with the **P^{II}**, **P^{III}** and **P^{IV}** peptides with low symmetry in comparison to that with the **P^{I}** peptide, where only one positive Cotton effect is observed or this may result from the presence Gly residue in the **P^{I}** peptide (Figs. 5A, B and S2) [49]. In addition, the logK* values for the $CuLH_{-2}$ and $CuLH_{-3}$ complexes, indicate about one order of magnitude higher stabilization of these complexes for the P1 ligand in comparison to those of other (**P^{II}**, **P^{III}** and **P^{IV}**) ligands (Table 5).

Importantly, the calculation of the potentiometric data could only

Table 4
Spectroscopic parameters for dominant Cu(II) complexes of the **Ac-HGHE-NH$_2$** (**P^{I}**), **Ac-GHEH-NH$_2$** (**P^{II}**), **Ac-HEHQ-NH$_2$** (**P^{III}**) and **Ac-EHEH-NH$_2$** (**P^{IV}**) peptides, (metal to ligand molar ratio 1:1 [Cu(II) = 2 mM], metal to ligand molar ratio 1:2 [Cu(II) = 1 mM]).

Species	Metal to ligand molar ratio 1:1													
	Ac-HGHE-NH$_2$ P^{I}							**Ac-GHEH-NH$_2$ P^{II}**						
	UV–Vis		CD		EPR			UV–Vis		CD		EPR		
	λ [nm]	ε [M^{-1} cm^{-1}]	λ [nm]	Δε [M^{-1} cm^{-1}]	$A_{\|\|}$ [G]/ [cm^{-1}]	$g_{\|\|}$	$g_{\perp}$	λ [nm]	ε [M^{-1} cm^{-1}]	λ [nm]	Δε [M^{-1} cm^{-1}]	$A_{\|\|}$ [G]/ [cm^{-1}]	$g_{\|\|}$	$g_{\perp}$
CuL pH 5.5	670[a]	30	615[a]	0.11	160/ 173	2.313	2.067	685[a]	19	623[a]	0.11	161/ 174	2.314	2.068
CuLH$_{-2}$ pH 6.5–7.0	576[a]	60	576[a] 345[b] 301[c] 263[d]	0.19 0.83 −0.49 0.35	195/ 201	2.207	2.056	557[a]	54	663[a] 564[a] 482[a] 339[b] 276[c,d]	0.06 −0.14 0.36 −1.00 1.73	187/ 192	2.202	2.040
CuLH$_{-3}$ pH 8.0	568[a]	68	576[a] 345[b] 300[c] 264[c,d]	0.21 0.98 −0.66 0.17	190/ 196	2.215	2.044	548[a]	70	663[a] 568[a] 484[a] 339[b] 273[c,d]	0.04 −0.24 0.47 −1.34 2.62	188/ 193	2.202	2.040
	Ac-HEHQ-NH$_2$ P^{III}							**Ac-EHEH-NH$_2$ P^{IV}**						
CuL pH 5.5	642[a]	33	645[a] 332[b] 256[d]	0.07 −0.36 0.72	159/ 172	2.315	2.067	651[a]	23	621[a] 330[b]	0.08 −0.19	165/ 178	2.306	2.066
CuLH$_{-2}$ pH 6.5–7.0	564[a]	65	654[a] 556[a] 485[a] 342[b] 275[c,d]	0.13 −0.29 0.20 −1.28 1.98	–	–	–	552[a]	54	638[a] 549[a] 482[a] 340[b] 275[c,d]	0.11 −0.26 0.22 −1.10 1.62	–	–	–
CuLH$_{-3}$ pH 8.0	552[a]	72	659[a] 554[a] 485[a] 344[b] 275[c,d]	0.14 −0.34 0.24 −1.42 2.18	182/ 188	2.208	2.044	545[a]	64	643[a] 550[a] 481[a] 340[b] 272[c,d]	0.11 −0.34 0.27 −1.37 2.26	188/ 194	2.209	2.043

Species	Metal to ligand molar ratio 1:2													
	Ac-HGHE-NH$_2$ P^{I}							**Ac-GHEH-NH$_2$ P^{II}**						
	UV–Vis		CD		EPR			UV–Vis		CD		EPR		
	λ [nm]	ε [M^{-1} cm^{-1}]	λ [nm]	Δε [M^{-1} cm^{-1}]	$A_{\|\|}$ [G]/ [cm^{-1}]	$g_{\|\|}$	$g_{\perp}$	λ [nm]	ε [M^{-1} cm^{-1}]	λ [nm]	Δε [M^{-1} cm^{-1}]	$A_{\|\|}$ [G]/ [cm^{-1}]	$g_{\|\|}$	$g_{\perp}$
CuL pH 5.0	673[a]	21	636[a]	0.09	165/ 178	2.307	2.071	684[a]	9	659[a]	0.08	159/ 172	2.315	2.065
CuL$_2$ pH 6.0–6.5	620[a]	27	581[a] 347[b] 300[c] 264[c,d]	0.12 0.19 −0.28 0.15	184/ 193	2.247	2.047	637[a]	17	552[a] 312[b]	0.10 −0.13	186/ 195	2.247	2.050
CuL$_2$H$_{-2}$ pH 7.0–7.5	570[a]	47	574[a] 346[b] 300[c] 264[c,d]	0.16 0.63 −0.45 0.12	190/ 197	2.216	2.044	547[a]	43	668[a] 570[a] 485[a] 340[b] 276[c,d]	0.05 −0.09 0.29 −0.84 1.35	188/ 193	2.202	2.039
	Ac-HEHQ-NH$_2$ P^{III}							**Ac-EHEH-NH$_2$ P^{IV}**						
CuL pH 5.0	682[a]	12	619[a]	0.07	162/ 175	2.312	2.066	690[a]	13	625[a]	0.05	159/ 171	2.309	2.060
CuLH$_{-3}$ pH 8.0	547[a]	37	648[a] 555[a] 485[a] 343[b] 275[c,d]	0.10 −0.16 0.12 −0.72 1.04	181/ 187	2.210	2.040	–	–	–	–	–	–	–
CuL$_2$ pH 6.0–6.5	607[a]	19	540[a] 328[b]	0.10 −0.27	188/ 197	2.248	2.051	590[a]	24	600[a] 493[a] 337[b] 267[c,d]	0.07 0.09 −0.45 0.25	187/ 196	2.250	2.055

(*continued on next page*)

Table 4 (*continued*)

Species	Metal to ligand molar ratio 1:2													
	Ac-HGHE-NH$_2$ P^{I}							**Ac-GHEH-NH$_2$ P^{II}**						
	UV–Vis		CD		EPR			UV–Vis		CD		EPR		
	λ [nm]	ε [M^{-1} cm^{-1}]	λ [nm]	Δε [M^{-1} cm^{-1}]	$A_{\|\|}$ [G]/ [cm^{-1}]	$g_{\|\|}$	$g_{\perp}$	λ [nm]	ε [M^{-1} cm^{-1}]	λ [nm]	Δε [M^{-1} cm^{-1}]	$A_{\|\|}$ [G]/ [cm^{-1}]	$g_{\|\|}$	$g_{\perp}$
CuL_2H_{-1} pH 6.5–7.0	563[a]	32	641[a] 555[a] 487[a] 342[b] 275[c,d]	0.08 −0.06 0.11 −0.58 0.68	182/ 188	2.210	2.039	564[a]	35	629[a] 549[a] 487[a] 337[b] 270[c,d]	0.08 −0.04 0.14 −0.66 0.52	–	–	–
CuL_2H_{-2} pH 7.0–7.5	–	–	–	–	–	–	–	543[a]	43	642[a] 549[a] 481[a] 340[b] 270[c,d]	0.10 −0.22 0.18 −0.97 1.04	186/ 192	2.206	2.042

$A_{||}[cm^{-1}] = 0.467 \times g_{||} \times A_{||}[G]$.

[a] d-d transition.

[b] $N_{Im}(\pi_1) \rightarrow$ Cu(II) charge transfer transition.

[c] $N^{-}_{amide} \rightarrow$ Cu(II) charge transfer transition.

[d] $N_{Im}(\pi_2) \rightarrow$ Cu(II) charge transfer transition.

Table 5
Calculated logK* values for copper(II) mononuclear and bis-complexes with **Ac-HGHE-NH$_2$ (P^{I})**, **Ac-GHEH-NH$_2$ (P^{II})**, **Ac-HEHQ-NH$_2$ (P^{III})** and **Ac-EHEH-NH$_2$ (P^{IV})** peptides and similar peptides.

	CuL	$CuLH_{-2}$	$CuLH_{-3}$	CuL_2
	2N	4N	4N	4N
logK*[a]	{2 × N_{Im}}	{2 × N_{Im},2 × N^{-}_{amide}}	{N_{Im},3 × N^{-}_{amide}}	{4 × N_{Im}}
P^{I}	−6.34	−18.50	−19.12	−14.09
P^{II}	−6.26	−19.10	−20.19	−13.73
P^{III}	−6.38	−19.24	−20.64	−14.42
P^{IV}	−6.26	−19.49	−20.68	−14.19
Ac-HGHG-NH$_2$[b]	–	−19.39	−23.35	–
Ac-βAHGH-NH$_2$[c]	−6.83	−20.56	−31.00	–

[a] The logK* = logβ(CuH_iL)-logβ(H_nL) where the i correspond to the number of protons in the coordinated ligand to metal ion and the n correspond to the number protons of the coordinated ligand and released from ligand during complexation.

[b] Ref. [2].

[c] Ref. [32].

be made to pH 8 probably due to the formation of a mixture of different complexes at a higher pH (hydroxy complexes). Spectroscopic measurements, which were conducted over a wider pH range, clearly indicate that copper(II) ions are also bound at a higher pH. An analogous situation has already been described in the literature [48].

As mentioned earlier, the formation of bis-complexes was observed in the solution. The possibility of the formation of such complexes has already been described in the literature for short peptides (for the tripeptides) containing at least one histidyl residue in the amino acid sequence [3,48,50]. In addition, peptides containing a histidyl residue at the first or the second position of the amino acid sequence have a greater ability to form bis-complexes [3]. Other amino acid residues also have an impact on the bis-complexes formation. The increase of the negative charge of the ligand, the presence of a non-coordinating side chains may have the influence on weakening of the coordination capacity, especially in the case of the bis-complexes formation [19].

As shown in Fig. 6, the bis-complexes are formed at around pH 5. Calculations based on potentiometric measurements revealed the formation in solution of the following complexes: CuL_2 and CuL_2H_{-1} for the all of the studied ligands. The CuL_2H and CuL_2H_{-2} complexes are

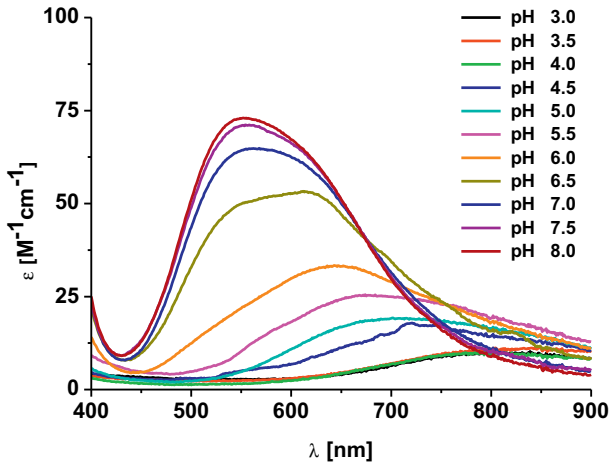

Fig. 4. UV–Vis spectra of the Cu(II)-**Ac-HEHQ-NH$_2$**; metal to ligand molar ratio 1:1, [Cu(II)] = 2 mM.

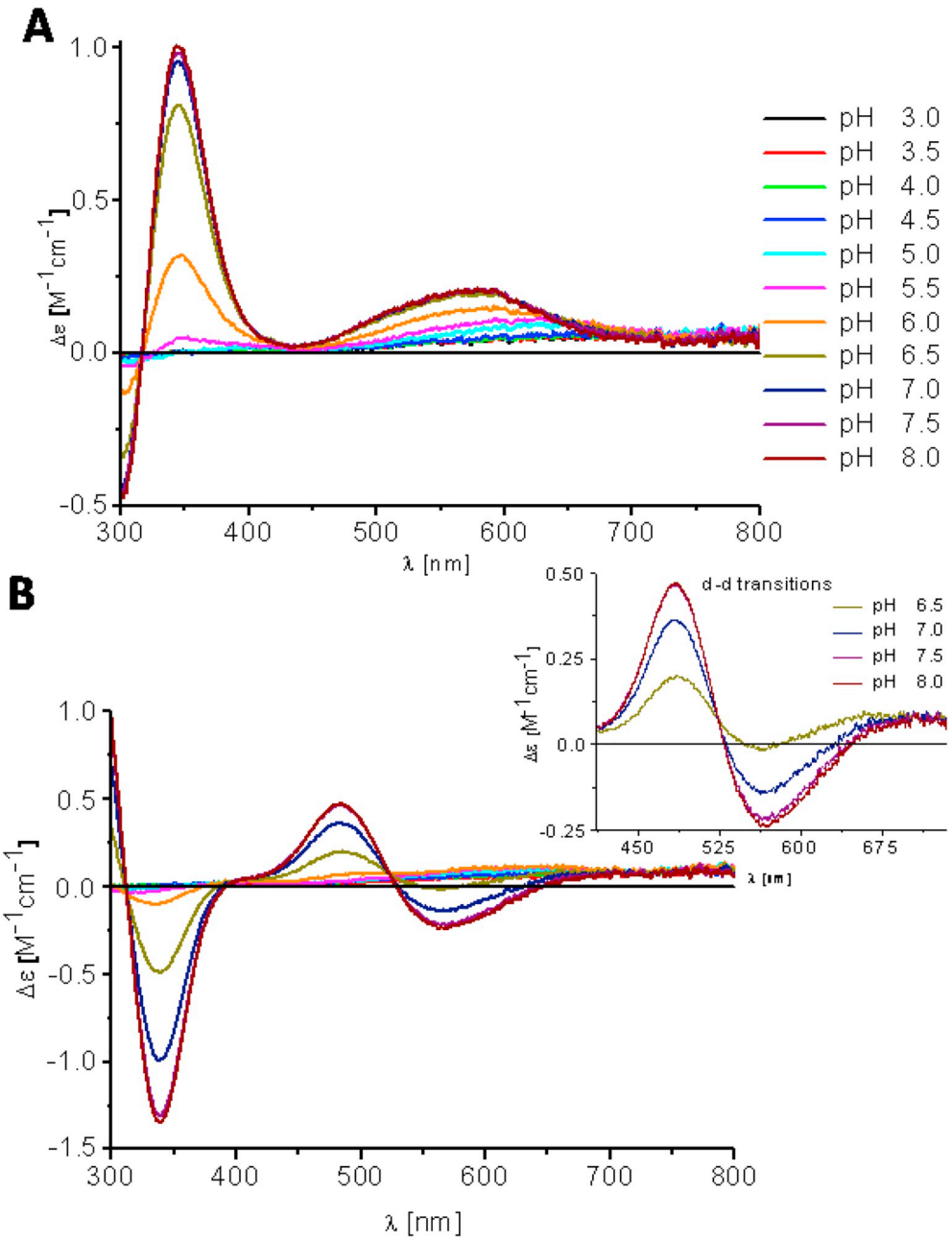

Fig. 5. CD spectra for A) Cu(II)-**Ac-HGHE-NH$_2$** and B) Cu(II)-**Ac-GHEH-NH$_2$** complexes as a function of the pH (metal to ligand molar ratio 1:1, [Cu(II)] = 2 mM).

formed for the **P^{I}**, **P^{II}** and **P^{IV}** peptides while the CuL_2H_{-3} species for the **P^{III}** peptide. The maximum of the first complex (CuL_2H) was observed at pH approximately 5.5. The maximum concentration of the CuL_2H complex is observed for the **P^{IV}** peptide. It may suggest as for CuLH complex of this ligand that stabilization of the CuL_2H complex may results from the formation of hydrogen bond between deprotonated glutamic acid and protonated imidazole of His residue. The stoichiometry of the CuL_2H complex suggest the involvement of imidazole nitrogens in the binding of copper(II) ion and formation {3 × N_{Im}} binding site.

When pH of the solution increases, the CuL_2 complex is formed and dominates at pH around 6 (the pH that is present in some parts of the intestine or oral cavity in which the carcinogenesis process may occurs in the present of *Fusobacterium nucleatum*). The pK_a values (4.99–5.86, Table 3) indicated the involvement of a subsequent imidazole nitrogen in the coordination sphere and the formation {4 × N_{Im}} coordination mode [37,51,52]. Spectroscopic parameters for the CuL_2 complex are typical for the 4 imidazole nitrogen donors around copper(II) ions. The d-d transition energy at 590–637 nm and the EPR parameters $A_{||}$ = 184–188 G, $g_{||}$ = 2.247–2.250 support this suggestion (Table 4, Figs. 7, S5 and S6) [37,53]. Moreover, an additional confirmation indicating the participation of 4 nitrogen donors is hyperfine splitting (9 lines due to hyperfine interaction with four nitrogen nuclei ($I^{14}N$ = 1)) observed on the perpendicular part of the EPR spectrum (Fig. 7, pH 6.4) [48]. The logK* for the 4 N{4 × N_{Im}} complexes for all studied ligands are comparable to each other (Table 5).

With the increase of pH value of the solution the CuL_2H_{-1} complex is formed. The pK_a values for the CuL_2H_{-1} complex formation from CuL_2 species (6.53–6.98, Table 3) clearly indicated that the amide nitrogen is involved in the coordination sphere [2]. The presence of CuL_2H_{-1} species on the distribution diagram of the complexes allowed characterizing this form only for **P^{III}** and **P^{IV}** peptides. The highest concentration of the CuL_2H_{-1} species is observed in solution at pH about 7 (Fig. 6). The resulting donor set may be attributed to 4 N

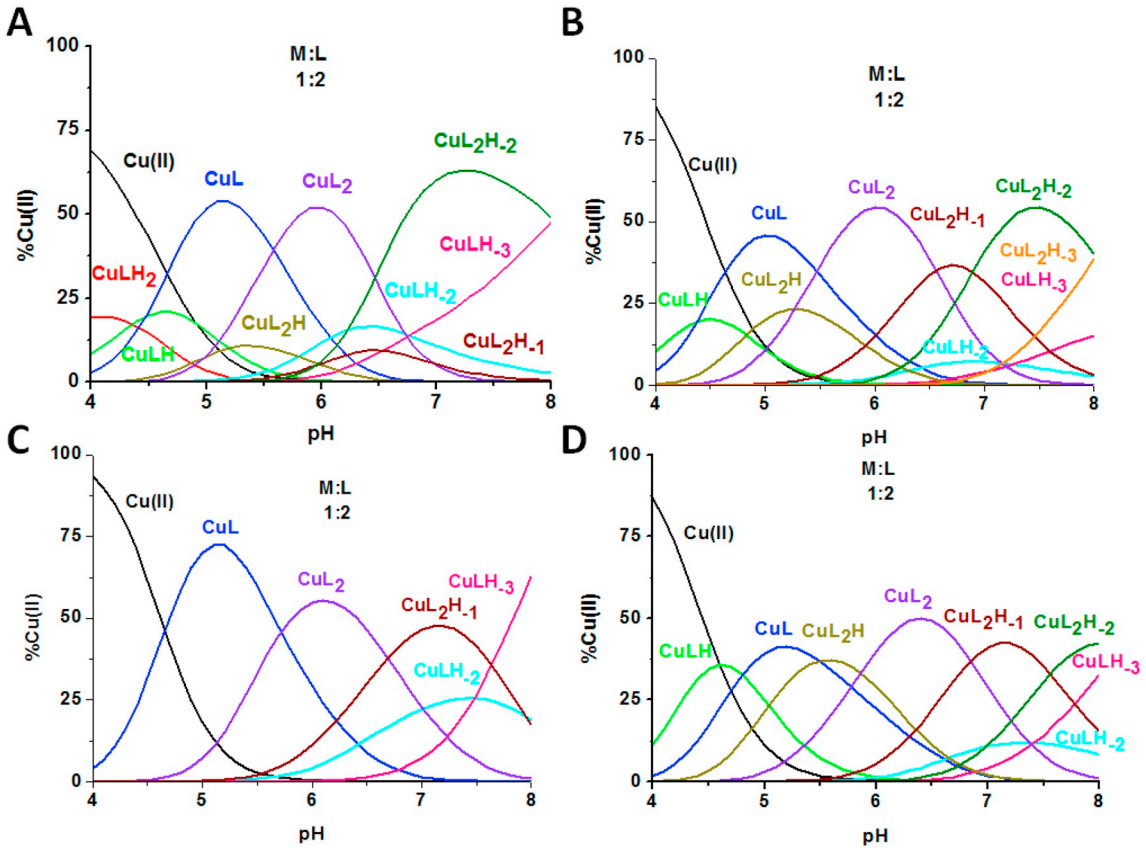

Fig. 6. Species distribution curves for copper(II) complexes with A) **Ac-HGHE-NH$_2$**, B) **Ac-GHEH-NH$_2$**, C) **Ac-HEHQ-NH$_2$** and D) **Ac-EHEH-NH$_2$** (molar ratio M:L 1:2, [Cu(II)] = 1 mM.

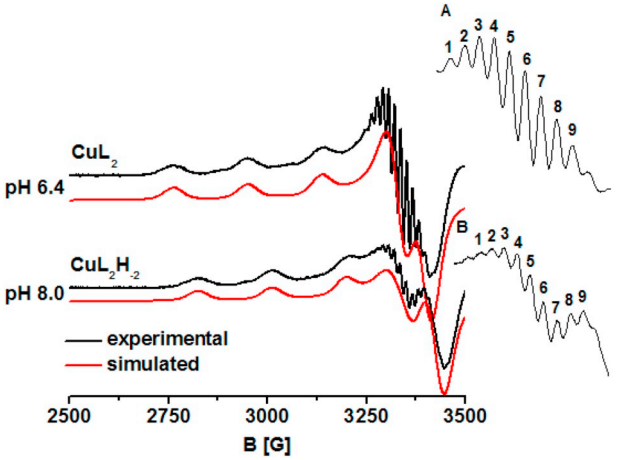

Fig. 7. Selected X-band EPR spectra for Cu(II)-**Ac-EHEH-NH$_2$** (as a representative, M:L molar ratio 1:2, [Cu(II)] = 1 mM). Insert shows the EPR perpendicular region of Cu(II)-4N species obtained at pH A) 6.4 and B) 8.0 for the CuL_2 and CuL_2H_{-2} complexes form, respectively.

$\{3 \times N_{Im}, 1 \times N^-_{amide}\}$ binding site.

The next CuL_2H_{-2} bis-complex is formed in the solution as a result of the deprotonation of the next amide nitrogen. This CuL_2H_{-2} complex is dominated in the pH range from 6 to 8 for the complexes with **P^{I}** and **P^{II}** peptides (Fig. 6). For the **P^{IV}** peptide the maximum of the CuL_2H_{-2} complex is observed around pH 8. Hypsochromic shifts of d-d bands on UV–Vis spectra suggests changes in the coordination sphere around copper(II) ions (Fig. S5). The increase of the charge transition $N^-_{amide} \rightarrow$ Cu(II) band for the CuL_2H_{-2} complexes in comparison to CuL_2 species partially confirmed the contribution of the second amide nitrogen $\{2 \times N_{Im}, 2 \times N^-_{amide}\}$ in the coordination of copper(II) ions (Fig. S8). For the $CuLH_{-2}$ and CuL_2H_{-2} complexes where the 4 N $\{2 \times N_{Im}, 2 \times N^-_{amide}\}$ binding site is suggested, the spectroscopic data are similar (Table 4). The EPR spectra for these $CuLH_{-2}$ and CuL_2H_{-2} complexes (Figs. 7B and S6) confirm this 4 N$\{2 \times N_{Im}, 2 \times N^-_{amide}\}$ coordination mode by the presence of nine hyperfine lines on the perpendicular part of EPR spectrum.

The last CuL_2H_{-3} bis-complex is observed only for **P^{II}** ligand (Fig. 6). The location of this species on the distribution diagram of the complexes prevents its full analysis. The stoichiometry of the CuL_2H_{-3} complex suggests the involvement of a next amide nitrogen in the binding of copper(II) ion, but also suggests the formation of hydroxy complexes.

The ESI-MS method is used to study the formation and stoichiometry of metal complexes [54–56]. For the Cu(II)-**Ac-HGHE-NH$_2$** system mass the signals correspond to the free ligand (m/z = 520.2 Da, z = 1+), equimolar complex ($[CuLH_{-1}]^+$, m/z = 581.1 Da, z = 1+), bis-complex ($[CuL_2H_{-1}]^+$, m/z = 1100.4 Da, z = 1+) and its sodium ($[CuL_2H_{-2}Na]^+$, m/z = 1122.4 Da, z = 1+) and potassium adducts ($[CuL_2H_{-2}K]^+$, m/z = 1138.3 Da, z = 1+), dinuclear complex ($[Cu_2L_2H_{-3}]^+$, m/z = 1161.3 Da, z = 1+) and its sodium ($[Cu_2L_2H_{-4}Na]^+$, m/z = 1183.3 Da, z = 1+) and potassium adducts ($[Cu_2L_2H_{-4}K]^+$, m/z = 1201.2 Da, z = 1+) were also observed. The ESI-MS spectra obtained for the Cu(II)-**Ac-GHEH-NH$_2$** system the MS signals which comes from the free ligand (m/z = 520.2 Da, z = 1+), the equimolar complex ($[CuLH_{-1}]^+$, m/z = 581.1 Da, z = 1+) and bis-complexes ($[CuL_2H_{-1}]^+$, m/z = 1100.4 Da, z = 1+), ($[CuL_2]^{2+}$, m/z = 550.7 Da, z = 2+) were observed. For the Cu(II)-**Ac-HEHQ-NH$_2$** system obtained the mass signals correspond to the free ligand (m/z = 591.3 Da, z = 1+), equimolar complex ($[CuLH_{-1}]^+$, m/z = 652.2 Da, z = 1+, Fig. 8) and bis-complex ($[CuL_2H_{-1}]^+$, m/z = 1242.5 Da, z = 1+). For the Cu(II)-**Ac-EHEH-NH$_2$** system mass

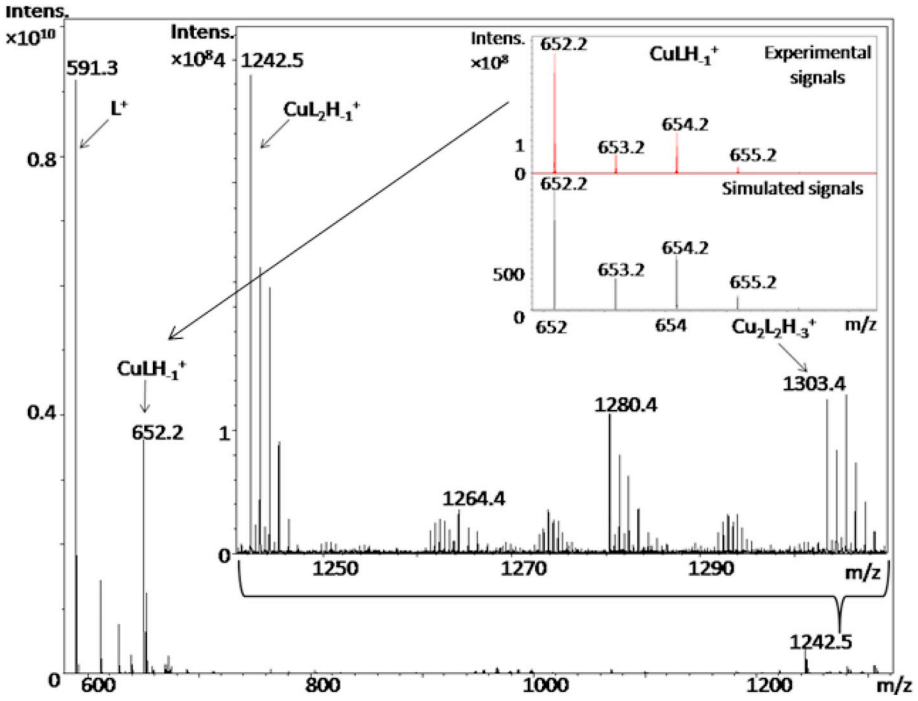

Fig. 8. Mass spectrum for the Cu(II)-**Ac-HEHQ-NH$_2$** complex (metal to ligand molar ratio 1:1, [Cu(II)] = 2 mM). Experimental and simulated spectra for the $[CuLH_{-1}]^+$ (of $\mathbf{P^{III}}$) molecular ion with *m/z* 652.2 Da (above).

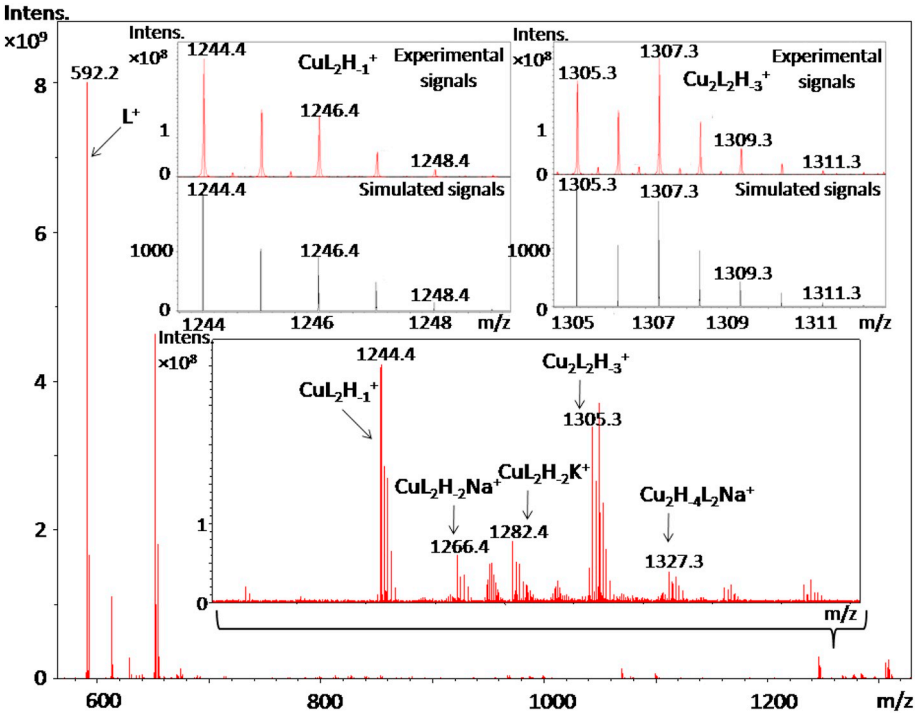

Fig. 9. Mass spectrum for the Cu(II)-**Ac-EHEH-NH$_2$** complex (metal to ligand molar ratio 1:1, [Cu(II)] = 2 mM). Experimental and simulated spectra for the: $[CuL_2H_{-1}]^+$ molecular ion with *m/z* 1244.4 Da (above on the left) and $[Cu_2L_2H_{-3}]^+$ molecular ion with *m/z* 1305.3 Da (above on the right).

spectra signals were observed to the free ligand (*m/z* = 592.2 Da, z = 1+), the equimolar complex ($[CuLH_{-1}]^+$, *m/z* = 653.2 Da, z = 1+), bis-complex ($[CuL_2H_{-1}]^+$, *m/z* = 1244.4 Da, z = 1+) and its sodium ($[CuL_2H_{-2}Na]^+$, *m/z* = 1266.4 Da, z = 1+) and potassium adducts ($[CuL_2H_{-2}K]^+$, *m/z* = 1282.4 Da, z = 1+), dinuclear complex ($[Cu_2L_2H_{-3}]^+$, *m/z* = 1305.3 Da, z = 1+) and its sodium adduct ($[Cu_2L_2H_{-4}Na]^+$, *m/z* = 1327.3 Da, z = 1+). The ESI-MS spectra support the formation of the mononuclear and bis-complexes under the MS experimental conditions. It should be noted that the MS method detects even very low concentrations of the complexes, therefore, not all complexes observed in the MS method were detected in potentiometry. What is important, the stoichiometry of complexes with the use of mass spectrometry is detected in the gas phase. Moreover, the formation of dinuclear complexes was also observed in the conditions of the MS experiments (Fig. 9). On Fig. 8 (MS spectra) the $CuL_2H_{-1}^+$ and $Cu_2L_2H_{-3}^+$ complexes are present.

4. Conclusions

The studied ligands effectively bind copper(II) ions to forming both mononuclear and bis-complexes. The coordination process began around pH 4 by anchoring metal ion through the imidazole nitrogen atom. At the pH of small intestine (5.5), the CuL complex dominates in the solution. The stoichiometry of the CuL complex and spectroscopic data suggest $2\,N\{2 \times N_{Im}\}$ coordination mode of copper(II) ions. At pH 6, the transition metal ion enforced the deprotonation of amide nitrogens and at pH 7 the copper(II) complexes with four nitrogen $4\,N\{2 \times N_{Im}, 2 \times N^{-}_{amide}\}$ binding site are formed. For complexes containing a histidyl residue in the first position the coordination process for the $CuLH_{-3}$ complexes proceed towards

C-terminal. In the $CuLH_{-3}$ species the $4\,N\{N_{Im}, 3 \times N^{-}_{amide}\}$ coordination mode is present and for the **P^{I}** and **P^{III}** this binding mode may occurs with the formation of 7-5-5 chelate rings. If the histidyl residue is in the fourth position for the peptides **Ac-GHEH-NH_2** and **Ac-EHEH-NH_2** the binding process for the $CuH_{-3}L$ species proceeds in the opposite direction (*N*-terminal) with the formation of the 5-5-6 chelate rings. The differences in the stability of the resulting complexes were observed, and the most stable is the CuL and $CuLH_{-1}$ complexes for the **Ac-HGHE-NH_2** ligand. The formation of bis-complexes for the studied ligands is observed. At pH 6–6.5 the CuL_2 complex dominates in solution for all studied ligands. For the peptide **Ac-EHEH-NH_2** (**P^{IV}**) the stabilization of the CuLH and CuL_2H complexes is observed. The formation of the hydrogen bond of the deprotonated carboxyl group and protonated imidazole of His residue takes part in the stabilization of CuLH and CuL_2H complex. The logK* for the studied complexes with the $\{2 \times N_{Im}\}$ and $4\,N\{4 \times N_{Im}\}$ binding sites are comparable to those formed with similar ligands containing two histidine residues [2,32]. The logK* value for the $CuLH_{-2}$ complex with $4\,N\{2 \times N_{Im}, 2 \times N^{-}_{amide}\}$ binding mode for the **Ac-HGHE-NH_2** (**P^{I}**) peptide is about one order of magnitude higher in comparison to that of the **P^{IV}** ligand containing two glutamic acid residues.

Abbreviations

P1 protein	one of the outer-membrane protein from *Fusobacterium nucleatum*
P^{I}	Ac-HGHE-NH_2 peptide
P^{II}	Ac-GHEH-NH_2 peptide
P^{III}	Ac-HEHQ-NH_2 peptide
P^{IV}	Ac-EHEH-NH_2 peptide
UV-Vis	electronic absorption spectroscopy
CD	circular dichroism
EPR	electron paramagnetic resonance
MS	mass spectrometry

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.110819.

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