Inorganic Chemistry

ARTICLE

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Complexation Abilities of Neuropeptide Gamma toward Copper(II) Ions and Products of Metal-Catalyzed Oxidation

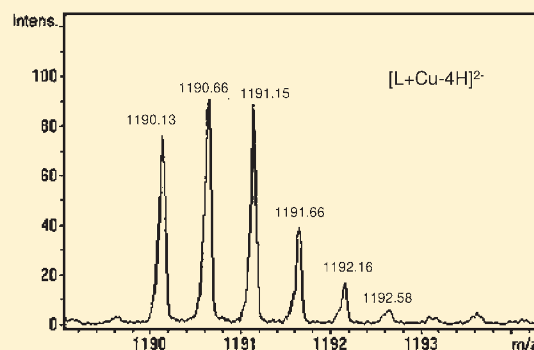
Marta Pietruszka,† Elżbieta Jankowska,‡ Teresa Kowalik-Jankowska,*,† Zbigniew Szewczuk,† and Maria Smużyńska‡

†Faculty of Chemistry, University of Wrocław, Joliot-Curie 14, 50-383 Wroclaw, Poland

‡Faculty of Chemistry, University of Gdansk, Sobieskiego 18, 80-952 Gdansk, Poland

S Supporting Information

ABSTRACT: The stability constants, stoichiometry, and solution structures of copper(II) complexes of neuropeptide gamma (NPG) (D[1]-A-G-H[4]-G-Q-I-S-H[9]-K-R-H[12]-K-T-D-S−F-V-G-L-M[21]-NH_2) and acethyl−neuropeptide gamma (Ac−D[1]-A-G-H[4]-G-Q-I-S-H[9]-K-R-H[12]-K-T-D-S-F-V-G-L-M[21]-NH_2) were determined in aqueous solution. For both peptides the additional deprotonations were observed; therefore, the potentiometric data calculations for NPG were only made in 2.5−7.4 pH range. For Ac−NPG one additional deprotonation was observed, likely hydroxy group of Ser residue, and the potentiometric data calculations in the 2.5−10.5 pH range may be performed. The potentiometric and spectroscopic data (UV−vis, CD, EPR) for the neuropeptide gamma show that a D[1] residue stabilizes significantly the copper(II) complexes with 1N {NH_2,β-COO^-}, 2N {NH_2,β-COO^-,N_{Im}}, and 3N {NH_2,β-COO^-,2N_{Im}} coordination modes as the result of coordination through the β-carboxylate group. The Ac−NPG forms with the copper(II) ions the 3N {3N_{Im}} complex in a wide 4.5−7.5 pH range. At higher pH deprotonation and coordination of the sequential amide nitrogens occur. Metal-catalyzed oxidation of proteins is mainly a site-specific process in which amino acids at metal-binding sites to the protein are preferentially oxidized. To elucidate the products of the copper(II)-catalyzed oxidation of NPG and Ac−NPG the liquid chromatography−mass spectrometry method (LC-MS) and the Cu(II)/H_2O_2 as a model oxidizing system were employed. For solutions containing a 1:4 peptide−hydrogen peroxide molar ratio oxidation of the methionine residue to methionine sulphone was observed. For the 1:1:4 Cu(II)−NPG−H_2O_2 system oxidation of two His residues and cleavage of the G[3]-H[4] and R[11]-H[12] peptide bonds were detected, supporting involvement of His[4] and His[12] in binding of the copper(II) ions. Oxidations of three histidine residues to 2-oxohistidines and fragmentations of Ac−NPG near the His (H[4], H[9],H[12]) residues support participation of the histidyl−imidazole nitrogen atoms in coordination of the metal ions.



INTRODUCTION

Tachykinins comprise a family of structurally related bioactive peptides that participate in the regulation of diverse biological processes.[1] The members of mammalian tachykinin consist of substance P, neurokinin A, neuropeptide K, neurokinin B, and neuropeptide gamma.[2] All these peptides share the common C-terminal consensus sequence Phe-X-Gly-Leu-Met-NH_2. This C-terminus "message" domain is considered to be responsible for activating the receptor.[3] Also, the characteristic C-terminal pentapeptide sequence of the tachykinins is similar to the 25−35 fragment of amyloid-beta, a peptide having a major role in Alzheimer's disease.[4]

Neuropeptide gamma (NPG) is a 21 amino acid residue peptide containing the amino acid sequence of neurokinin A at it is C terminus.[5] The amino acid sequence of this peptide (NPG) is as follows: Asp[1]-Ala-Gly-His[4]-Gly-Gln-Ile-Ser-His[9]-Lys-Arg-His[12]-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met[21]-NH_2. NPG was first isolated from rabbit small intestine extracts,[6] and it is encoded by only one of the four mRNAs generated from the primary transcript of the preprotachykinin (PPT) A gene, called γ-PPT.[7] Extensive studies have provided the evidence that NPG is involved in many biological activities[8] like broncoconstriction, vasodepression, increase in heart rate, and stimulation of salivary secretion. It has a physiological role as a regulator of endocrine functions and can act on the hypothalamo−pituitary−gonadal axis to regulate functions related to reproduction and modulate the regulation of growth hormone secretion.[3] Tachykinins are neurotransmitter and/or neuromodulator substances in both the central nervous system (CNS) and the peripheral nervous system (PNS).[9] NPG is present in several peripheral tissues as well as in the brain, although at lower concentration than neurokinin A (NKA).[10]

Received: February 11, 2011
Published: July 19, 2011

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Copper is one of the most prevalent biological transition metals by all organisms from bacteria to humans,[11] and several studies have reported a rise in the levels of brain copper from youth to adulthood.[12] Copper can also catalyze the production of reactive oxygen species (ROS) such as hydroxyl radicals in the Fenton reaction.[13] ROS would initiate the oxidative damage of many biological targets and can play an important factor in many diseases such as cardiovascular disease, cancer, Parkinson's disease, inflammation, and rheumatoid arthritis.[14]

The metal-ion-catalyzed oxidation (MCO) of biomolecules (nucleic acids, proteins) is thought to involve formation of $OH^{\bullet}$ by means of the Fenton reaction when iron is the metal ion. The reaction involves reduction of Fe^{3+} or Cu^{2+} by a suitable electron donor such as ascorbate or mercaptane. Fe^{2+} and Cu^{+} ions bound to specific metal-binding sites on proteins react with hydrogen peroxide to generate $OH^{\bullet}$.[15,16] For complexes with peptides generation of hydroxyl radicals was observed.[17] It is assumed that H_2O_2 reduces peptide−Cu^{2+} to peptide−Cu^{+}; this is followed by reaction of Cu^{+} with hydrogen peroxide to give $OH^{\bullet}$.[18,19]

Metal-catalyzed oxidation (MCO) of proteins or peptides is mainly a site-specific process in which only one or a few amino acids at metal-binding sites on the protein are preferentially oxidized.[20−24] Oxidation of proteins by MCO can lead to oxidation of amino acid residue side chains, cleavage of peptide bonds, and formation of covalent protein−protein cross-linked derivatives. Results of the studies indicate that the most common pathway for oxidation of simple aliphatic amino acids involves hydroxyl-radical-mediated abstraction of hydrogen atom to form a carbon-centered radical at the α position of the amino acid or amino acid residue in the polypeptide chain.[25]

The present paper reports the results of the combined spectroscopic and potentiometric studies on the copper(II) complexes of NPG and its *N*-acethyl derivative and also presents the products of the copper(II)-catalyzed oxidation on the base of binding sites of the peptides to copper(II) ions at pH 7.4. We demonstrate the relationship between the binding sites of copper(II) ions and prediction of oxidation products for the ligands studied on the base the abilities of metal-catalyzed oxidation of the amino acids residues and obtained molecular ions in MS spectra after oxidation.

MATERIAL AND METHODS

Synthesis of the Peptides. Syntheses of neuropeptide gamma (NPG) Asp[1]-Ala-Gly-His[4]-Gly-Gln-Ile-Ser-His[9]-Lys-Arg-His[12]-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met[21]-NH_2 and its acethyl derivative (Ac−NPG) were carried out using Millipore 9050 peptide synthesizer and continuous-flow methodology.[26−28] A polystyrene/polyethylene glycol copolymer resin (TentaGel R RAM, Rapp Polymere) was used as a solid support. The N-terminal amino group in peptide Ac−NPG was acetylated using 1 M acetylimidazole in dimethylformamide (DMF). Both peptides were cleaved from the resin and deprotected by treatment with a mixture containing 94% trifluoroacetic acid, 2.5% water, 2% triisopropylsilane (TIS), and 1.5% 1,2-ethanedithiol (EDT). The cleavage reaction was carried out for 1.5 h at room temperature.

The crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Luna C_8 semipreparative column (21.2 × 250 mm, 5 μm, Phenomenex). The purity of the peptides was confirmed by analytical RP-HPLC using a C_8 Kromasil column (4.6 × 250 mm, 5 μm) and a 30 min linear gradient of 5−80% acetonitrile (ACN) in 0.1% aqueous trifluoroacetic acid as a mobile phase, and their structure was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Analytical data were as follows: NPG, R_T = 14.2 min, MS $[L + H]^+$ = 2319.8 Da, calcd 2321.6 Da; Ac−NPG, R_T = 9.4 min, MS $[L + H]^+$ = 2363.1 Da, calcd 2363.6 Da.

The purity of the peptides was checked, and the exact concentration of their stock solutions was determined by the Gran method.[29]

Potentiometric Measurements. Stability constants for the proton and Cu(II) complexes were calculated from pH-metric titrations carried out in an argon atmosphere at 298 K using a total volume of 1.5−2 cm^3. Alkali was added from a 0.250 cm^3 micrometer syringe which was calibrated by both weight titration and titration of standard materials. Experimental details: ligand concentration 1.1×10^{-3} mol dm^{-3}, metal-to-ligand molar ratio 1:1.1; ionic strength 0.10 M (KNO_3); $Cu(NO_3)_2$ used as the source of the metal ions; pH-metric titration on a MOLSPIN pH-meter system using a Russell CMAW 711 semimicro combined electrode calibrated in concentration using HNO_3,[30] number of titrations = 2; method of calculation SUPERQUAD.[31] The samples were titrated in the pH region 2.5−10.5. Standard deviations (values) quoted were computed by SUPERQUAD and refer to random errors only. They are, however, a good indication of the importance of the particular species involved in the equilibria. Calculations of the potentiometric data for NPG and the 1:1.1 metal-to-ligand molar ratio were performed to pH 7.4. At higher pH additional deprotonations of the peptide were observed in comparison to those predicted, and good fitting of the experimental data to those calculated at pH higher (above 7.4) was impossible. Neuropeptide gamma contains four anchoring sites: a N-terminal amine group and three histidyl-His[4], -His[9], and -His[12] residues; therefore, the polynuclear complexes may be formed. Titrations of the ligands in the presence of various equivalents of copper(II) were performed; however, precipitation at pH ca. 4.5−5 was observed. At higher pH (above 9) the solutions became to be clear; however, calculations of the potentiometric data were impossible. Although in an equimolar metal-to-ligand ratio polynuclear complexes can be present, mononuclear complexes are dominating.[32,33] It should be mentioned that the presence of 10% of the complexes in solution may be unidentified by potentiometry. Moreover, the fitting parameters of the calculated and experimental data for the mononuclear complexes (χ^2 and σ) are quite satisfied for the Cu(II)−NPG (28.66; 7.11) and Cu(II)−Ac−NPG (10.81; 7.04) systems.

Spectroscopic Measurements. Solutions were of similar concentrations to those used in potentiometric studies. Absorption spectra (UV−vis) were recorded on a Cary 50 "Varian" spectrophotometer in the 850−300 nm range. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter in the 750−250 nm range. 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 potentiometric data. Electron paramagnetic resonance (EPR) spectra were performed in an ethylene glycol−water (1:2, v/v) solution at 77 K on a Bruker ESP 300E spectrometer equipped with the ER 035 M Bruker NMR gaussmeter and the HP 5350B Hewlett-Packard microwave frequency counter at the X-band frequency (∼9.45 GHz). The spectra were analyzed using Bruker's WIN-EPR SimFonia software, version 1.25. Copper(II) stock solution was prepared from $Cu(NO_3)_2 \cdot 3H_2O$. Although potentiometric data calculations for the polynuclear complexes cannot be performed the spectroscopic measurements at pH 10.5 for the solutions containing 1:1, 2:1, and 3:1 for both peptides and also 4:1 for NPG metal-to-ligand molar ratios were carried out.

ESI-MS Measurement. Mass spectra were obtained on a Bruker Micro TOF-Q spectrometer (Bruker Daltonik, Bremen, Germany) equipped with Apollo II electrospray ionization source. The mass spectrometer was operated in the positive- or negative-ion mode. The instrumental parameters were as follows: scan range m/z 400−2300, dry gas−nitrogen, temperature 200 °C, reflector voltage 1300 V, detector

Table 1. Global Formation Constants (log β) for NPG and Ac−NPG and Comparable Peptides at 298 K and I = 0.10 M (KNO_3)

peptide/log β	HL	H_2L	H_3L	H_4L	H_5L	H_6L	H_7L	H_8L	H_9L
NPG[a]	7.64 ± 0.01	14.50 ± 0.01	20.71 ± 0.01	26.16 ± 0.01	29.81 ± 0.01	32.36 ± 0.01			
1−16H[c]	9.96	17.89	24.84	31.38	37.10	41.47	45.37	48.52	51.18
DMG-NH_2[d]	10.91	21.10	30.93	40.04	47.57	54.01	59.96	65.30	
G_5H[e]	8.00	14.87	17.73						
Ac−NPG[b]	10.30 ± 0.01	20.20 ± 0.01	29.19 ± 0.01	36.22 ± 0.01	42.48 ± 0.01	47.83 ± 0.01	51.70 ± 0.01	54.37 ± 0.02	
HuPrP (84−114)[f]	10.68	20.96	30.94	40.22	46.99	53.18	58.82		
Cap 43 (30 a.a.)[g]	6.88	13.14	18.85	23.61	27.61	31.86	35.25		
Ac−HAAHVVH[h]	6.92	13.27	19.03						

[a] Number of experimental points 155, χ^2 29.92, σ 3.54. [b] Number of experimental points 118, χ^2 5.84, σ 5.77. [c] DAEFRHDSGYEVHHQK-NH_2, ref 38. [d] AKRHHGYKRKFH-NH_2, ref 37. [e] GGGGGH, ref 50. [f] Ac−PHGGGWGQGGGTHSQWNKPSKPKTNMKHMAGA-NH_2, ref 32. [g] Ac−TRSRSHTSEGTRSRSHTSEGTRSRSHTSEG-NH_2, ref 41. [h] Ac−HAAHVVH-NH_2, ref 40.

voltage 1920 V. The samples (NPG and 1:1 Cu(II)−NPG molar ratio) were dissolved in water, and the pH value was adjusted by addition of concetrated of NaOH or HNO_3 and infused at a flow rate of 3 μL/min. The instrument was calibrated externally with the Tunemix mixture (Bruker Daltonik, Germany) in quadratic regression mode. In MS/MS mode, the quadruple was used to select the precursor ions, which were fragmented in the hexapole collision cell, generating product ions that were analyzed by the orthogonal reflectron TOF mass analyzer. For CID MS/MS measurements, the collision energy over the hexapole collision cell was set to 21 eV and argon was used as collision gas.

Materials Used in the Oxidation Process. Deionized and triply distilled water was used, and the MOPS buffer at pH 7.4 (Sigma-Aldrich, MOPS 3-(*N*-morpholino)propanesulfonic acid)[34] was treated with Chelex 100 resin (sodium form, Sigma-Aldrich) to remove trace metals. Hydrogen peroxide was purchased from Fluka (Perhydrol, 30%), and ethylenediaminetetraacetic acid (EDTA) and $Cu(NO_3)_2$ were purchased from POCH. Stock solutions (0.10 M) of EDTA and hydrogen peroxide in MOPS buffer were prepared.

Oxidation of the Neuropeptide Gamma and *N*-Acethyl Derivative, Liquid Chromatography−Mass Spectrometry Analysis. Copper(II)-catalyzed oxidation of the peptide in the presence of hydrogen peroxide was monitored by analytical RP-HPLC on a Varian ProStar 240 station using an XTerra C 18 4.6 × 150 mm column (Waters) at a 30 min linear gradient of 5−100% B, where A used 0.1% aqueous trifluoroacetic acid (TFA) and B used 0.1% TFA in 80% acetonitrile (ACN). A reaction mixture (0.2 cm^3) containing 5 × 10^{-4} M peptide and a metal-to-ligand molar ratio of 1:1.1 in a 0.02 M MOPS was incubated at 37 °C for 24 and 48 h in the presence of hydrogen peroxide at a metal to hydrogen peroxide molar ratio of 1:4 for NPG and Ac−NPG. The reaction was started by addition of hydrogen peroxide solution, which was freshly prepared. After incubation, the reaction was stopped by addition of EDTA to a final complex at an EDTA molar ratio of 1:5. The chelating agent EDTA inhibits oxidation of the peptide by removing Cu(II) from the peptide. Oxidized and digested peptides were desalted on 10 μL ZipTipC18 columns (Omnix, Varian). The columns were prepared by wetting with 50% acetonitrile and equilibrated with 0.1% trifluoroacetic acid. Each sample was loaded onto a ZipTip column. The column was washed with 0.1% TFA to remove salts, and then the peptides were eluted with 0.1% formic acid in 80% acetonitrile. The obtained samples were then the subject of LC-ESI-MS analysis. Acetonitrile, water, and formic acid of LC/MS grade were purchased from Sigma. Positive-ion electrospray mass spectrometric analysis was carried out using a Shimadzu ion trap time-of-flight mass spectrometer (LC-MS IT TOF) at unit resolution. The source temperature was 200 °C; the electrospray voltage was −1700 V. Separation and mass analysis of oxidized and digested peptides were carried out using a Phenomenex Jupiter Proteo90A analytical column (2 × 150 mm, 4 μm) with a linear gradient of 0−30% B for 12.5 min followed by a gradient of 30−100% B for 7.5 min (buffer A, 0.2% formic acid/water; buffer B, 0.2% formic acid/ACN; flow rate, 0.2 mL/min). The injection volume was 80 μL, and the temperature in which the analysis proceeded was 40 °C. Data was acquired and analyzed using LC Solution software provided by Shimadzu.

RESULTS AND DISCUSSION

Protonation Constants. Global formation (log β) and protonation (log K) constants of the neuropeptide gamma and its *N*-acethyl derivative have been determined by potentiometric titrations, and the data is presented in Tables 1 and 2. The ligands contain two aspartic acids and three histidine residues. The log K values of the protonations of carboxylate function of Asp side chain are found to be in the range of 2.55−3.87.[35,36] For both ligands three log K values (7.03−5.35, Table 2) fall in the range of basicity of the imidazole ring.[37−41] In both ligands the difference of the three log K values (around 0.60 log units) is rather small, suggesting that protonation of the histidine residues takes place in overlapping processes. Therefore, these constants are very likely macroconstants containing contributions from the protonations of three histidines. For neuropeptide gamma with a free N-terminal amino group, the protonation constant log K = 7.64 (Tables 1 and 2) corresponds very well to protonation of the amino nitrogen and agrees with those for peptides containing the aspartic acid residue in the N-terminal position.[35,37,38,42] For the neuropeptide gamma with increasing pH above 7.4 the fitting of the experimental to calculated potentiometric data was impossible. Above this pH the MS spectra (see below) may suggest replacement of the labile protons of the neuropeptide gamma by the sodium ions.

For the acethyl derivative of neuropeptide gamma at pH above 7 the next protonation constants were observed (Tables 1 and 2). Deprotonation of the lysyl ammonium groups takes place in the pH range from 9 to 11, and two values from the range 8.99−10.30 obtained for the *N*-acethyl derivative of neuropeptide gamma are close to those of other lysine-containing peptides.[33,43,44] For the N derivative of neuropeptide gamma additional deprotonation with a log K value above 9 was detected by potentiometry as it was also observed for neurokinin A[45] and the fragment of neuropeptide gamma.[46] It is most likely that deprotonation of the hydroxyl −OH group of the Ser residue may take place as it had been earlier suggested from the MS/MS analysis for the neurokinin A.[45] It should be mentioned that to clarify this additional deprotonation in the tachykinins studies of modyfied neurokinin A (modification Ser/Ala) are performed.

Table 2. Protonation Constants (log K) for NPG and Ac−NPG and Comparable Peptides at 298 K and I = 0.10 M (KNO_3)

peptide/log K	NH_2-Lys, O^- Ser	O^- Tyr	NH_2 terminal	His	His	His	CO_2^- Asp	CO_2^- Asp	CO_2^- Asp	CO_2^- Asp
NPG			7.64	6.86	6.21	5.45	3.65	2.55		
1−16H[a]		9.96	7.93	6.95	6.54	5.72	4.37	3.90	3.15	2.66
DMG-NH_2[a]	10.91, 10.19, 9.83	9.12	7.52	6.44	5.95	5.34				
G_5H[a]			8.00	6.87			2.86			
Ac−NPG	10.30, 9.90, 8.99			7.03	6.26	5.35	3.87	2.67		
HuPrP (84−114)[a]	10.68, 10.29, 9.98, 9.28			6.77	6.19	5.64				
Cap 43 (30 a.a.)[a]				6.88	6.26	5.71	4.76	4.34	3.91	3.39
HAAHVVH[a]				6.92	6.35	5.76				

[a] References as in Table 1.

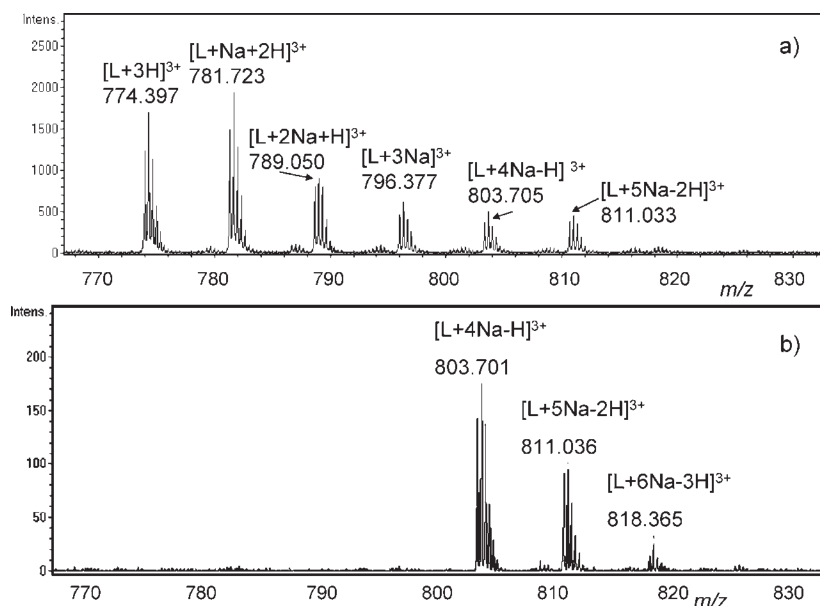


Figure 1. Distribution of peaks corresponding to sodiated species of the neuropeptide gamma recorded in the positive-ion mode at pH 9.2 (a) and 11.6 (b). The m/z range corresponding to the triply charged ions is shown.

The preliminary potentiometric data suggest that it is most likely that the serine residue in tachykinins studied may be deprotonated. The results for the modyfied peptides will be finished and published soon. It seems that the main role in the properties of the tachykinins may have the D-S sequence (where the hydrogen bonds are likely) and the His residues like to those of serine proteases.

The ESI-MS spectra of the peptide recorded at pH 4.6−9.2 (spectrum recorded in pH 9.2 is shown in Figure 1A) are very similar and contain a set of peaks corresponding to triply protonated (m/z 774.397) and oligosodiated forms containing 1−5 sodium atoms: $[L + Na + 2H]^{3+}$, $[L + 2Na + H]^{3+}$, $[L + 3Na]^{3+}$, $[L + 4Na - H]^{3+}$, and $[L + 5Na - 2H]^{3+}$. Although concentrations of the Na ions are similar in all MS experiments, a further increase of the pH dramatically changes the distribution of the sodiated species in the spectrum (Figure 1b). The peaks corresponding to ions containing less than four sodium atoms were not present in the spectrum, but $[L + 4Na - H]^{3+}$ and $[L + 5Na - 2H]^{3+}$ were still present. Even $[L + 6Na - 3H]^{3+}$ was detected in the spectrum. This may suggest that the neuropeptide gamma deprotonates very easily at pH above 9.2 and four protons can be replaced by sodium ions. The CID of the triply protonated $[L + 3H]^{3+}$ resulted in formation of triply charged a and b ions in the m/z range below the precursor (Figure 1S, Supporting Information). The CID-MS spectrum of the triply charged sodiated species $[L + H + 2Na]^{3+}$ is similar to the one obtained for $[L + 3H]^{3+}$, but a and b ions are shifted by 14 units, resulting in formation of disodiated fragments. The most abundant series of peaks corresponding to double sodiated a_n and b_n (n = 16−21) triply charged ions of the doubly charged monosodiated species were observed, which suggests that both sodium ions are situated on the N-terminal part (D^1-S^{16}) of the peptide molecule.

The possibility that the Na ion can be moved along the peptide backbone during CID of sodiated peptide ion cannot be excluded. Reasearchers R. P. Grese and M. L. Gross performed a CID experiment on monosodiated peptide ions.[47] However, the authours observed that one Na ion can be moved from the C-terminus toward the N-terminal residues.

Potentiometry detects a range of Cu(II) complexes with the formation constants reported in Table 3. The values of log K^* are the protonation-corrected stability constants which are useful to compare the ability of ligands to bind a metal ion,[48] and they are given in Table 4. Table 1S (Supporting Information) presents calculated deprotonation constants for amide protons (pK values) in copper(II) complexes with the ligands studied. Spectroscopic properties of the major complexes are given in Table 5.

 dx.doi.org/10.1021/ic2002942 |Inorg. Chem. 2011, 50, 7489–7499

Table 3. Stability Constants of Copper(II) Complexes of NPG and Ac−NPG and Comparable Peptides at 298 K and I = 0.10 M (KNO_3)

peptide/log β	CuH_6L	CuH_5L	CuH_4L	CuH_3L	CuH_2L	CuHL	CuL	$CuH_{-1}L$	$CuH_{-2}L$	$CuH_{-3}L$	$CuH_{-4}L$
NPG[a]					20.98 ± 0.01	15.65 ± 0.02	8.83 ± 0.02	1.53 ± 0.02			
1−16H[c]			35.99	31.49	26.22	20.12	12.63	4.10	−5.21	−15.28	
DMG-NH_2[c]	59.03	54.00	48.31	41.90	34.67	26.36	17.14	7.26	−3.06	−14.05	
G_5H[c]						12.29	8.11	2.63	−5.06	−15.67	
Ac−NPG[b]		46.31 ± 0.03	41.79 ± 0.01	36.11 ± 0.01	29.60 ± 0.01	22.28 ± 0.02	14.66 ± 0.01	5.58 ± 0.02	−3.76 ± 0.02	−13.92 ± 0.03	−24.34 ± 0.02
HuPrP(84−114)[c]	57.51	52.20	46.69	40.13	33.44	25.45	16.24	6.50	−3.92	−14.62	−25.65
Cap43 (30 a.a.)[c]				22.04	17.39	12.72	7.12	0.86	−6.04	−13.92	−23.65
HAAHVVH[c]					17.17	12.84	7.80	0.71	−6.93	−15.14	

[a] Number of experimental points 80, χ^2 28.66, σ 7.11. [b] Number of experimental points 120, χ^2 10.81, σ 7.04. [c] References as in Table 1.

Table 4. Calculated log K^{*a} Values for Copper(II) Complexes with NPG and Ac−NPG and Comparable Ligands

peptide/log K^*	2N {NH_2, COO^- or CO, N_{Im}}	3N {NH_2, COO^- or CO, $2N_{Im}$}	4N {NH_2, N^-, 2 N_{Im}}
NPG	−5.18	−10.51	−17.77
1−16H[b]	−5.61	−10.88	−17.52
DMG-NH_2[b]	−6.27	−11.30	
G_5H[b]	−6.76		

peptide/log K^*	3N {$3N_{Im}$}	4N {N^-,$3N_{Im}$}	4N {$2N^-$,$2N_{Im}$}	4N {$3N^-$,N_{Im}}
Ac−NPG	−11.72	−18.23	−18.52	−19.88
HuPrP(84−114)[b]	−12.13	−18.69	−18.61	−20.41
Cap43 (30 a.a.)[b]	−11.73	−17.99	−18.01	−19.63
HAAHVVH[b]	−11.23	−18.32	−18.50	−20.90

[a] log K^* = log β (CuH_jL) − log β (H_nL) (where the index j corresponds to the number of protons in the ligand coordination to the metal ion and n corresponds to the number of protons coordinated to the ligand). [b] References as in Table 1.

Copper(II) Complexes. The potentiometric data for the Cu(II)−neuropeptide gamma system was calculated at pH 7.4 (above this pH the calculations for the ligand cannot be performed). According to potentiometric and spectroscopic results, NPG forms with copper(II) ions the CuH_2L, CuHL, CuL, and $CuH_{-1}L$ complexes (Table 3, Figure 2). Coordination of the metal ion starts at pH around 3.5, and the CuH_2L complex is formed. The d−d transition energy of 668 nm and the EPR parameter g_{II} = 2.290 are consistent with 2N coordination (Table 5).[38,49] The low value of A_{II} (143 G) may reflect deformation of the complex plane expected when a macrochelate ring is formed. The low values of A_{II} were observed for complexes containing, in the peptide chain, the histidine residue in a position higher than third.[38,49,50] The value of log K^* for this complex is higher by about 1−1.5 log units compared to those of DMG-NH_2[37] and G_5H (−6.76)[50] peptides (Table 4) but is comparable to that of 1−16H peptide[38] containing the aspartic acid residue in the first position of the peptide sequence. Stabilization of 2N complex results from bonding of the β-carboxylate oxygen of Asp[1] residue in the coordination plane forming a six-membered chelate ring and may suggest the 2N {NH_2, β-COO^-, N_{Im}} coordination mode. Stabilization of the copper(II) complexes by coordination of the β-COO^- group of the Asp residue in a different position of the peptide chain is well documented.[35,51] With increasing pH, the CuHL species are formed and the pK value for deprotonation of the CuH_2L complex is 4.93 (Table 1S, Supporting Information). This value may correspond to deprotonation and coordination of a second imidazole nitrogen to Cu(II) ion with formation of the 3N complex with a {NH_2, β-COO^-, $2N_{Im}$} binding mode.[50,52,53] Lack of transitions in CD spectra for the complexes formed in the 3.5−7.5 pH range may support coordination of the imidazole nitrogen of the histidine residues than amide nitrogens (Table 5).[38,49,52] It is indirect proof for coordination of the metal ion by the side chain imidazole residues, which are rather far from the chirality centers of the molecules. The log K^* value for the CuHL complex (−10.51, Table 4) is comparable to that of the 1−16H peptide (−10.88), suggesting the same coordination mode of copper(II) ions in the complexes formed. The next deprotonation (resulting in the CuL species) with a pK value 6.82 (Table 3) has no effect on EPR parameters and may be due to deprotonation of the noncoordinated third histidine residue (6.86 in free ligand, Table 2). With increasing pH above 7.0 the shift of the d−d transition energy to higher values (λ_{max} 542 nm) may indicate deprotonation and coordination (CuL → $CuH_{-1}L$) of first amide nitrogen. Formation of the 4N {NH_2, N^-, $2N_{Im}$} complex from the 3N {NH_2, β-COO^-, $2N_{Im}$} species requires reorganization of the donor set and/or structure around the metal-ion bonding; therefore, high pK_1(amide) values (7.30 and 7.49, respectively) for NPG and 1−16H[38] are observed (Table 1S, Supporting Information) in comparison to those of DMG-NH_2 (6.41)[37] and G_5H (5.48).[50] The EPR parameters, A_{II} 159−160 G and g_{II} 2.275, suggest the 3N binding mode for the CuHL and CuL complexes; however, low A_{II} values indicate that these complexes assume a distored geometry with a significant deviation from planarity.

Protection of the terminal amino group prevents the existence of the coordination modes observed for the neuropeptide gamma, and thus, the anchoring groups available for coordination of metal ion are the imidazole nitrogens of the side chains of the histidyl residues. *N*-Acethyl-neuropeptide gamma (Ac−NPG) starts coordination of the metal ion at pH 3.5 with

Table 5. Spectroscopic Data for Copper(II) Complexes of NPG and Ac−NPG

		UV−vis		CD		EPR	
ligand/species	pH	λ (nm)	ε (M^{-1} cm^{-1})	λ (nm)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	A_{II} (G)	g_{II}
NPG							
CuH_2L {NH_2, βCOO^-, N_{Im}}	4.7	668[a]	72			143	2.290
CuHL {NH_2, βCOO^-,$2N_{Im}$}	6.1	576[a]	66			159	2.275
CuL, $CuH_{-1}L$ {NH_2, βCOO^-, $2N_{Im}$} and {NH_2, N^-,$2N_{Im}$ }	7.0	542[a]	94			160	2.273
Ac−NPG							
CuH_4L {$2N_{Im}$}	5.5	657[a]	22			174	2.285
CuH_3L {$3N_{Im}$,}	6.2	630[a]	42			175	2.278
CuH_2L {$3N_{Im}$, N^-}	6.7	583[a]	57			174	2.275
CuL,CuH_1L,CuH_2L, CuH-$_3$L, CuH-$_4$L {N_{Im}, $3N^-$}	10.0	528[a]	113	621[a]	+0.927	202	2.192
				490[a]	−0.561		
				359[b]	−0.061		
				312[c]	+1.239		

[a] d−d transition. [b] N_{Im} → Cu(II) charge transfer transition, [c] N^-_{amide} → Cu(II) charge transfer transition

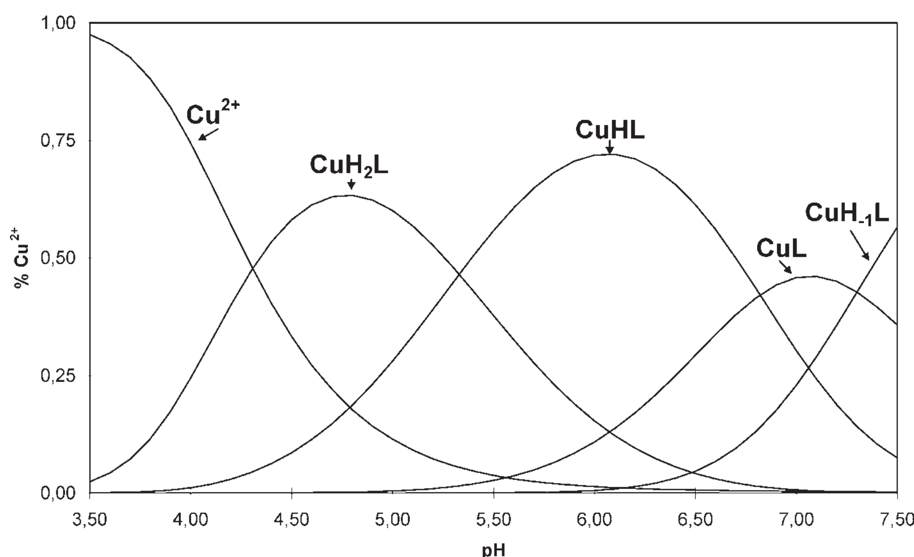


Figure 2. Species distribution diagram for the Cu^{2+}−NPG system at a 1:1 metal-to-ligand molar ratio; [Cu(II)] = 0.001 M.

formation of the CuH_5L complex in which one of the imidazole nitrogens is deprotonated and coordinated (Figure 3). Spectroscopic data for the CuH_4L complex, the d−d transition band at 657 nm, and the EPR parameters A_{II} 174 G and g_{II} 2.285 (Table 5) strongly support the 2N complex with two imidazole nitrogens involved in metal-ion coordination.[54,55] With increasing pH the 3N {3 N_{Im}} type coordination is supported by the visible absorption with a λ_{max}^{d-d} value of 630 nm (Table 5), the predicted value being 634 nm.[56] It should be mentioned that the first well-structured CD spectra can be observed only in parallel with formation of the 4N {N_{Im}, $3N^-$} complex. In the case of CuH_4L, CuH_3L, and CuH_2L complexes the lack of well-structured CD spectra is the consequence of the macrochelate structure, while for the CuHL complex the low concentration and overlapping species makes the assignment of CD spectra more difficult (Table 5). According to the species distribution diagram (Figure 3) the CuL complex in a wide 7−10.5 pH range is formed. The presence in the CD spectrum of N_{Im} → Cu(II) at 359 nm, at 312 nm N(amide) → Cu(II) charge transfer transitions, EPR parameters A_{II} = 202 G, g_{II} = 2.192, and d−d transition at 528 nm (absorption spectrum) (Table5) support the 4N {N_{Im}, $3N^-$} coordination mode.[50,52−55] With increasing pH above 9 the spectroscopic parameters of the complexes formed are similar to each other, suggesting the same binding mode. The p*K* values for deprotonation of the CuL → $CuH_{-1}L$ → $CuH_{-2}L$ → $CuH_{-3}L$ complexes are equal to 9.08, 9.34, and 10.42, respectively, and are comparable to those for the Lys and Ser residues in metal-free ligand (8.99, 9.90, and 10.30, Table 2). The $CuH_{-4}L$ complex was also suggested from the potentiometric data calculations. However, the spectral parameters of this species cannot be obtained (Figure 3). Formation of the $CuH_{-4}L$ species in similar systems was observed,[54] and it can be the effect of deprotonation of the pyrrolic N(1)H group of imidazole or an increase of coordinated amide nitrogen donors around the copper(II) ion.

The log *K** values of the 1N to 4N complexes of the Cu(II)−Ac−NPG system are similar to those obtained for the acethyl derivatives of the peptides containing three histidine

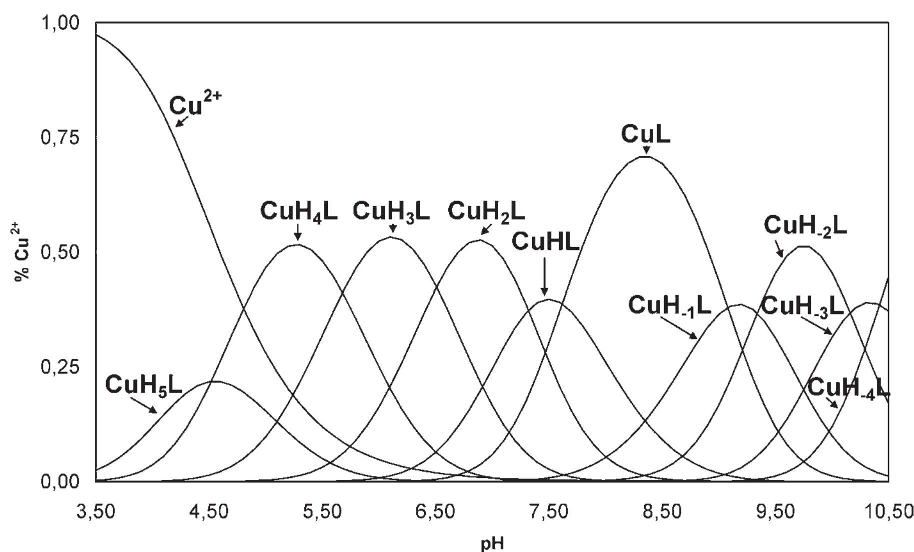


Figure 3. Species distribution diagram for the Cu^{2+}—Ac—NPG system at a 1:1 metal-to-ligand molar ratio; [Cu(II)] = 0.001 M.

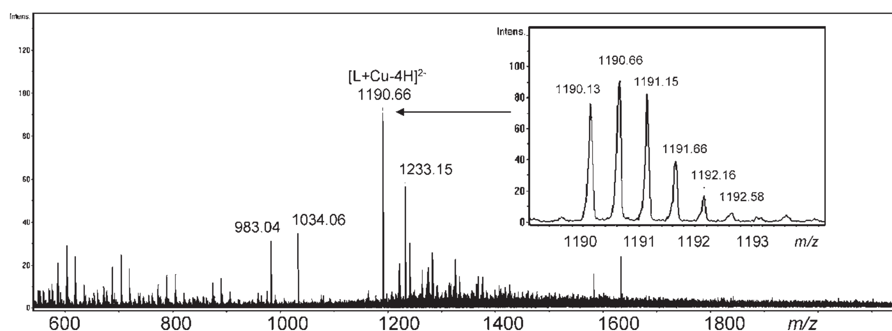


Figure 4. ESI-MS spectrum for the neuropeptide gamma complexed with Cu(II), recorded in the negative-ion mode (pH 9.5).

residues (Table 4), suggesting the same coordination modes in the complexes formed.

Although potentiometric studies for the polynuclear complexes cannot be performed (precipitation in 4.5—9 pH range) the spectroscopic measurements for the solutions containing a metal-to-ligand molar ratio of 2:1, 3:1 for both peptides, and also 4:1 for the NPG at pH 10.5 were performed (Figure 2S, Supporting Information). As presented in Table 2S (Supporting Information) at this pH polynuclear complexes with 4N {NH_2, $3N^-$} or {N_{Im}, $3N^-$} coordination modes are formed. It should be also mentioned that deprotonation of the neuropeptide gamma at pH above 7.4 and pH above 9 of *N*-acethyl-NPG does not have any influence on the complexes formed. These deprotonations do not change the coordination properties of the ligands studied toward copper(II) ions.

The obtained ESI-MS spectrum for the neuropeptide gamma recorded in the negative-ion mode (Figure 4) shows a dominant signal for the $[Cu + L - 4H]^{2-}$ deprotonated doubly charged form (1190.66 m/z) at pH 9.5, whereas the corresponding singly or triply charged species were absent. ESI—MS has been used in a wide variety of fields to study formation, stoichiometry, and speciation of complexes of metals and organic ligands.[57] Our results may indicate a strong tendency of the analyzed copper complex to deprotonation. The EPR parameters A_{II} 196—205 G, g_{II} 2.185—2.200, d—d transitions at 524—550 nm (absorption spectra), and the presence in CD spectra, the $N_{Im} \rightarrow$ Cu(II) at 350—352 nm, and N^-(amide) $\rightarrow$ Cu(II) at 307—314 nm charge transfer transitions, clearly indicate formation of the 4N complexes.

Copper(II)-Catalyzed Oxidation of the Neuropeptide Gamma and Its *N*-Acethyl Derivative. It has been proposed that oxidative stress plays a role in the tissue damage that is associated with various diseases as diabetes[58] and also with aging[59] and cancer.[60,61] The reduced metabolism that occurs with aging is mostly due to an increased concentration of metal ions in the human body.[62] The plasma concentration of copper[63] and iron[64] increases with age, and these metals readily catalyze reactions related to formation of hydrogen peroxide and free radicals such as the superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($OH^\bullet$). Oxidation is one of the major chemical degradation pathways for protein.[65,66] Selective damage to particular residues present in peptides and proteins can arise from the binding of a metal ion, or other initiating species, at a particular site on a peptide or protein.[25] Evidence has been presented for the formation of radicals at specific sites on a number of proteins including catalase,[67,68] bovine serum albumin,[69] β-amyloid precursor protein,[70] porcine myofibrillar protein,[71] parathyroid hormone,[72] as well as small peptides.[73−75]

Side chains of cysteine (Cys), methionine (Met), tryptophan (Trp), histidine (His), and tyrosine (Tyr) residues are prone to oxidation, in that order. Met oxidation forms Met sulfoxide (MetO) and, under extreme conditions, sulfone.[76,77] Oxidative

dx.doi.org/10.1021/ic2002942 |Inorg. Chem. 2011, 50, 7489–7499

alteration of Met to Met(O) is reversed by the methionine sulfoxide reductases (Msr). A major biological role of the Msr systems is regulation of the protein function.[78] Histidine is a very important amino acid residue critical for the function of many enzymes. His oxidation predominantly forms oxo-histidine but also forms a variety of other oxidation products, depending on the oxidation conditions.[66,72]

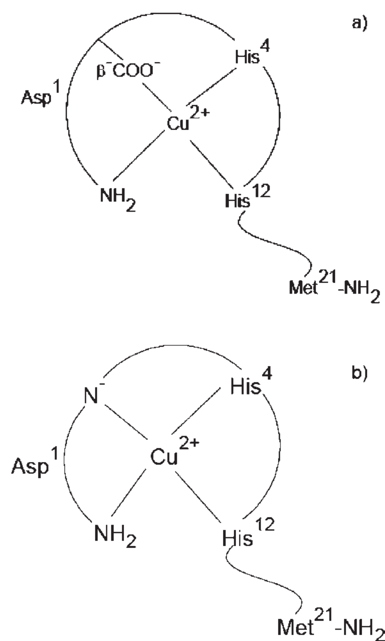
Metal-catalyzed oxidation (MCO) reactions can lead to protein oxidation in a site-specific manner in which only a few amino acids at the metal-binding sites are preferentially oxidized.[79,80] Therefore, the individual amino acids involved in metal binding to a protein can be modified to their metal-catalyzed oxidation products.

It is assumed that for the Cu(II) complexes with peptides the hydrogen peroxide reduces Cu(II)−peptide to Cu(I)−peptide and by reaction of Cu(I) with hydrogen peroxide the $OH^•$ radicals are formed.[19] Spectroscopic data for Cu(II)−neuropeptide gamma and Cu(II)−*N*-acethyl-neuropeptide gamma in MOPS buffer at pH 7.4 (data not shown) is similar to that obtained in aqueous solutions at pH 7.4 (Figures 2 and 3). At pH 7.4 neuropeptide gamma forms the CuL and $CuH_{-1}L$ complexes in equilibrium with the 3N $\{NH_2,\beta\text{-}COO^-,2N_{Im}\}$ and 4N $\{NH_2,N^-,2N_{Im}\}$ coordination modes (Scheme 1), respectively. For the *N*-acethyl derivative of neuropeptide gamma at pH 7.4 the CuH_2L and CuHL complexes dominate with 4N $\{3N_{Im},N^-\}$ and $\{2N_{Im},2N^-\}$ binding modes, respectively (Figure 3 and Table 5).

The chromatograms for the peptides alone, with Cu(II) ions only, with hydrogen peroxide and with Cu(II)−H_2O_2 indicate that the solutions containing peptides alone and with the copper(II) ions only with a 1:1 Cu(II)−peptide molar ratio were not changed in comparison to the peptides alone before incubation (data not shown). Methionine is one of the most reactive amino acids and is oxidized to methionine sulfoxide and sulphone.[76,77,81] LC-MS analysis of the neuropeptide gamma and its derivative revealed the presence of oxidized peptides after 24 h incubation at 37 °C at a 1:4 peptide−hydrogen peroxide molar ratio. For the 1:4 NPG−H_2O_2 system in a chromatographic fraction eluting at 8.3 and 9.9 min triply charged molecular ions $[L + 3H]^{3+}$ with m/z 779.8 and 785.1 Da are present, respectively (Table 3S, Supporting Information), while for Ac−NPG−H_2O_2 triply charged molecular ions were detected with m/z 793.7 and 799.1 Da in the fractions eluting at 9.6 and 10.9 min, respectively (Table 4S, Supporting Information). These peptides displayed molecular masses +16 and +32 Da higher than the respective native sequences. These changes in the molecular masses are indicative of addition of one and two oxygen atoms and may correspond to formation of the methionine sulfoxide and sulphone, respectively.

When the copper(II) ions are added to the NPG−H_2O_2 system at a complex-to-hydrogen peroxide 1:4 molar ratio, further modifications of the peptide occur. The chromatographic peak eluting at 12.1 min (Table 3S, Supporting Information) and in the MS spectra occurring at m/z 575.3 Da may correspond to the fourtly charged molecular ion of the peptide after alkoxy radical modification of the D^1 residue and oxidation of methionine to sulphone and two histidine residues to 2-oxo-histidines. The peptides containing the methionine residue in the peptide chain have a loss of 64 Da (CH_3SOH), and it is diagnostic for the formation and presence of methionine sulphoxide.[82] The A^2−M^{21} fragment of neuropeptide gamma was also observed with the modifications: oxidized two histidine residues to

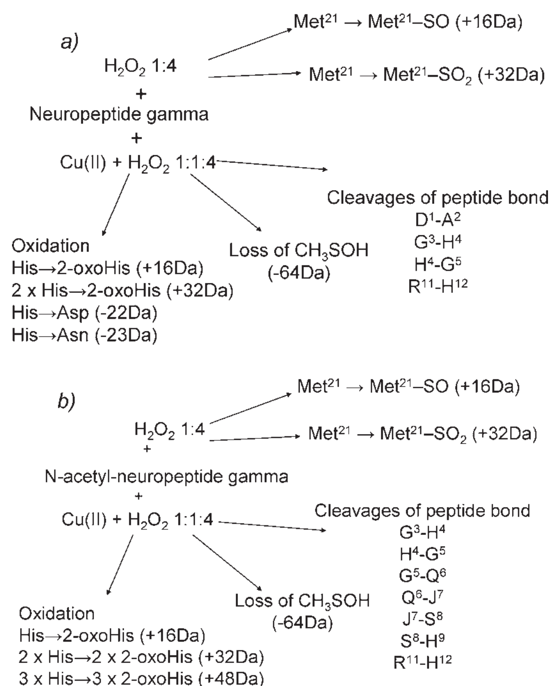
Scheme 1. Binding Sites of the Copper(II) Ions in the CuL (a) and $CuH_{-1}L$ (b) Species Formed in the 1:1 Cu(II)−NPG System at pH 7.4



2-oxo-histidines and loss of CH_3SOH fragment. Mass spectrometry for the chromatographic fraction eluted at 10.7 min yielded a fourth $[L + 4H]^{4+}$charged molecular ion of 548.3 Da, supporting the presence of the A^2−M^{21} fragment. Cleavage of the peptide bond between D^1−A^2 supports involvement of the N-terminal part of the neuropeptide gamma in the binding of the metal ions (Table 3S, Supporting Information). Mass spectrometry for the chromatographic fraction eluted at 12.4 min yielded triply and fourthly charged molecular ions of 648.0 and 486.3 Da, respectively, which may be assigned to the G^5-M^{21} fragment, supporting the presence of a His^4 residue around the copper(II) ions.[83] Cleavage of the peptide bond between R^{11}-H^{12} was also detected by mass spectrometry. For the chromatographic fraction eluting at 13.6 min, the doubly charged molecular ion with m/z 568.8 Da observed may correspond to the H^{12}−M^{21} fragment. Other molecular ions of copper(II)-catalyzed oxidation products for the neuropeptide gamma were also detected, and modifications of this peptide in Table 3S (Supporting Information) are proposed and in Scheme 2a presented. For the Cu(II)−neuropeptide gamma−H_2O_2 1:1:4 molar ratio system cleavage of the peptide bond near His^4 and His^{12} residues was observed. It is suggested from the spectroscopic studies that at pH 7.4 in MOPS buffer the copper(II) ions are coordinated by two His residues. Therefore, knowledge of the oxidation products may suggest that likely His^4 and His^{12} residues are involved in coordination of the copper(II) ions.

The 1:4 complex−H_2O_2 molar ratio was also used for oxidation of the Cu(II)−Ac−NPG system in the presence of hydrogen peroxide. Mass spectrometry for the chromatographic fraction eluting at 11.4 min (Table 4S, Supporting Information) revealed the $[L + 3H]^{3+}$, $[L + 4H]^{4+}$, and $[L + 5H]^{5+}$ molecular ions with m/z 815.0, 611.3, and 489.3 Da (Figure 5). The masses of these oxidation products are +16, +32, and +48 Da higher than the oxidized parent peptide containing methionine sulphone. It may support coordination of three histidine residues to the metal

Scheme 2. Schematic Representation of the Products of Copper(II)-Catalyzed Oxidation for the Neuropeptide Gamma (a) and *N*-Acethyl-Neuropeptide Gamma (b)



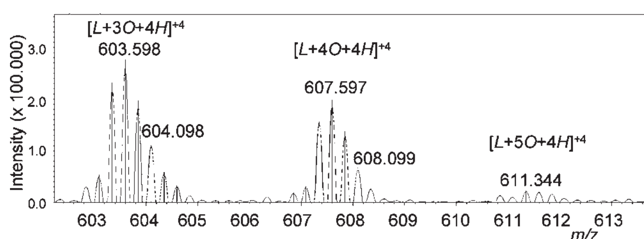


Figure 5. MS spectrum of Ac–neuropeptide gamma after Cu(II)-catalyzed oxidation of the chromatographic fraction eluting at a retention time of 11.4 min. The products of modified Ac–NPG peptide with oxidation of Met to sulphone and one, two, and three His residues oxidation to 2-oxo-His are detected.

ions.[82,84] The fragmentations by cleavage of the peptide bonds near the His residues were also observed. The triply charged molecular ions with *m/z* 677.0 and 519.7 Da are present in fractions eluting at 11.4 and 15.7 min, respectively. These masses correspond to the fragments H^4-M^{21} with the loss of CH_3SOH and H^9-M^{21}, respectively (Table 4S, Supporting Information). Mass spectrometry for the chromatographic fraction eluted at 16.4 min yielded $[L + H]^+$ and $[L + 2H]^{2+}$ molecular ions of 1084.5 and 542.7 Da, which may be assigned to the H^{12}-M^{21} fragment with loss of CH_3SOH from oxidized peptide containing the methionine sulfoxide. The molecular ions for the Ac–neuropeptide gamma after Cu(II)-catalyzed oxidation of the 1:1 complex–hydrogen peroxide system are proposed in Table 4S (Supporting Information) and presented in Scheme 2b. As it is seen in Table 4S (Supporting Information) the Q^6–M^{21}, I^7–M^{21}, and S^8–M^{21} fragments of the Ac–NPG peptide after Cu(II)-catalyzed oxidation were also detected. It means that the oxidation conditions (complex–hydrogen peroxide molar ratio, time of reaction) were sufficient to cleavage further peptide bonds from the binding sites of copper(II) ions. At lower complex–hydrogen peroxide molar ratios or/and reaction time the products of oxidation were not well seen (HPLC measurements). It seems that the presented conditions for oxidation of Ac–NPG are optimum.

■ CONCLUSIONS

The extesive involvement of tachykinins and their receptors in a wide range of biological functions has made them prime targets for physiological and pharmacological investigations. These peptides seem to display great diversity in their involvement with a number of the central nervous system (CNS) disorders.[85]

Copper is an essential trace element that plays a central role in the biochemistry of every living organism. The chemical properties which make copper biologically useful can also potentially lead to toxic effects, namely, copper-incuced oxidative stress. Excess copper has been known to be a potent oxidant, causing generation of ROS in the cells.[86]

Neuropeptide gamma starts the coordination of Cu(II) ions from the N-terminal amino group of the Asp^1 residue. Coordination of the β-carboxylate oxygen of the D^1 residue to metal ions stabilizes the 1N $\{NH_2,\beta\text{-}COO^-\}$, 2N $\{NH_2,\beta\text{-}COO^-,N_{Im}\}$, and 3N $\{NH_2,\beta\text{-}COO^-,2N_{Im}\}$ complexes. Potentiometric studies for the neuropeptide gamma may be performed at pH 7.4. At higher pH additional deprotonations of the peptide occur, as supported by potentiometry. However, the spectroscopic studies indicate that at higher pH (above 9) the mono- as well as polynuclear complexes may be formed. At physiological 7.4 pH the equilibrium of the 3N $\{NH_2,\beta\text{-}COO^-,2N_{Im}\}$ and 4N $\{NH_2, N^-,2N_{Im}\}$ complexes is suggested. Introduction of the acethyl group at the N-terminal amino group changes the coordination mode of the peptide to copper(II) ion. Three histidine (H^4, H^9, H^{12}) residues coordinate the metal ions, and at physiological 7.4 pH an equilibrium of 4N $\{3N_{Im},N^-\}$ and $\{2N_{Im},2N^-\}$ complexes occurs. With the increase of pH the amide functions are involved in metal binding.

For both peptides the methionine residue is converted to methionine sulphone in the reaction with hydrogen peroxide. For the Cu(II)–peptide–H_2O_2 1:1:4 molar ratio systems the histidine residues are oxidized to 2-oxo-histidines. For the neuropeptide gamma oxidation of two histidines was detected, while for the *N*-acethyl-neuropeptide gamma three histidine residues were oxidized. It suggests coordination of copper(II) ions by two and three histidyl imidazole-N donor atoms, respectively. Under the experimental conditions NPG undergoes fragmentation by cleavage of the G^3-H^4 and R^{11}-H^{12} peptide bonds, indicating involvement of the H^4 and H^{12} histidine residues in the copper(II) binding sites. For the Ac–NPG the cleavages G^3-H^4, S^8-H^9, and R^{11}-H^{12} peptide bonds were observed, supporting participation of the H^4, H^9, and H^{12} histidine residues in coordination of the metal ions. It should be mentioned that neuropeptide gamma is a peptide which is very sensitive to the oxidizing agents. Hydrogen peroxide may oxidize the methionine residue to sulphone, while in the copper(II)–neuropeptide gamma–hydrogen peroxide 1:1:4 molar ratio system further modifications of the peptide are observed. Oxidation of His residues to 2-oxo-histidines and many cleavages of the peptide bonds were detected near the binding sites.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
*E-mail: TerKow@wchuwr.chem.uni.wroc.pl.

ACKNOWLEDGMENT

Financial support from the Polish State Committee for Scientific Research KBN Grant NN204 249534 is gratefully acknowledged.

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