Journal of Inorganic Biochemistry 104 (2010) 831–842

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Coordination abilities of neurokinin A and its derivative and products of metal-catalyzed oxidation

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ARTICLE INFO

Article history:
Received 21 January 2010
Received in revised form 23 March 2010
Accepted 26 March 2010
Available online 11 April 2010

Keywords:
Neurokinin A
Copper(II) complexes
Stability constants
Copper(II)-catalyzed oxidation
Products of oxidation

ABSTRACT

The classical tachykinins, substance P, neurokinin A and neurokinin B are predominantly found in the nervous system where they act as neurotransmitters and neuromodulators. Significantly reduced levels of these peptides were observed in neurodegenerative diseases and it may be suggested that this reduction may also result from the copper(II)-catalyzed oxidation. The studies of the interaction of copper(II) with neurokinin A and the copper(II)-catalyzed oxidation were performed. Copper(II) complexes of the neurokinin A (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) and acetyl-neurokinin A (Ac-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) were studied by potentiometric, UV–Vis (UV–visible), CD (circular dichroism) and EPR spectroscopic methods to determine the stoichiometry, stability constants and coordination modes in the complexes formed. The histidine residue in first position of the peptide chain of neurokinin A coordinates strongly to Cu(II) ion with histamine-like {NH_2, N_{Im}} coordination mode. With increasing of pH, the formation of a dimeric complex $Cu_2H_2L_2$ was found but this dimeric species does not prevent the deprotonation and coordination of the amide nitrogens. In the Ac-neurokinin A case copper(II) coordination starts from the imidazole nitrogen of the His; afterwards three deprotonated amide nitrogens are progressively involved in copper coordination. To elucidate the products of the copper(II)-catalyzed oxidation of the neurokinin A and Ac-neurokinin A, liquid chromatography-mass spectrometry (LC–MS) method and Cu(II)/hydrogen peroxide as a model oxidizing system were employed.
Oxidation target for both studied peptides is the histidine residue coordinated to the metal ions. Both peptides contain Met and His residues and are very susceptible on the copper(II)-catalyzed oxidation.
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1. Introduction

Tachykinins (TKs) are a family of closely related peptides whose best known members are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). For many years, the tachykinins were considered almost exclusively as peptides of neuronal origin. SP and NKA are found in the central nervous supplying a number of peripheral tissues [1,2]. SP and NKA are released from nerve ending at both the spinal cord and the peripheral level and play a role as excitatory neurotransmitters [2,3].

In mammals, tachykinins act as neurotransmitters, paracrine or endocrine factors and neuroimmunomodulators [4] and have roles in the nervous system, gastrointestinal tract [5] and cardiovascular system [6]. Important actions include vasodilatation, plasma extravasation, smooth muscle, contraction secretion, neuronal excitation and processing of sensory information; they also have immune and pro-inflammatory actions [7,8].

Tachykinins are believed to exert most of their actions via the 3 tachykinin receptors that have been cloned and characterized: NK_1, NK_2 and NK_3, which bind preferentially to SP, NKA, and NKB, respectively, although each of these peptides is able to bind each of the receptors albeit with lower affinity [9].

The tachykinin peptides are characterized by a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X represents either an aromatic (Phe, Tyr) or a branched aliphatic (Val, Ile) amino acid. The C-terminal region or the message domain is considered to be responsible for activating the receptor. The divergent N-terminal region or the address domain varies in amino acid sequence and length and is postulated to play role in determining the receptor subtype specificity [10].

Neurokinin A is a decapeptide found in mammalian neuronal tissue, with the sequence His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2. It was first isolated from the porcine spinal cord [11]. The structure of NKA has been strongly conserved during evolution. NKA from reptiles and chicken are identical to mammalian NKA, and the frog, teleost, and alasmobranch NKA isolated differ by only two amino acids [12].

There are a number of studies that have specifically addressed the question of tachykinin levels in Parkinson's disease (PD) [13]. Mauborgne et al. first demonstrated that there were reduced levels (approximately 30%) of substance P in the substantia nigra and pallidum

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doi:10.1016/j.jinorgbio.2010.03.016

in patients with PD [13]. Substance P, neurokinin A, neuropeptide K (NPK), and neurokinin B were measured in both control (neurologically normal) and Huntington's disease patients [14]. The studies suggest that the neuropeptides might be important in the etiology of neurodegenerative disease or might be useful diagnostic markers. The putative role for a variety of neuropeptides in degenerative neurological disorders is the subject of extensive and excellent prior reviews [15–19]. Although there is no direct evidence for a causative role of a particular neuropeptide in a particular neurodegenerative disease, certain neuropeptide levels are relatively consistently reported to be altered in certain disorders.

Oxidative stress is considered a major contributor to the pathogenesis of a number of pathological processes leading to atherosclerosis, inflammatory conditions, multiple system atrophy and several neurodegenerative diseases. There is increasing evidence that oxidative stress results from either excessive reactive oxygen species (ROS) production or compromised anti-oxidant defences [20–23]. In general, the ROS are formed ubiquitously in biological systems by both enzymatic and metal-catalyzed oxidation (MCO) reactions [24,25]. The MCO reaction involves reduction of Fe(III) or Cu(II) by a suitable electron donor such as NADH, NADPH, ascorbate, or mercaptane. Fe(II) and Cu(I) ions bound to specific metal-binding sites on proteins react with H_2O_2 to generate $^{\bullet}OH$ [26,27]. A feature of metal-catalyzed oxidations is the site-specific nature of the reaction, i.e. specific amino acid residues located at the metal-binding sites are generally altered [28]. The amino acid residues that are most susceptible to metal-catalyzed oxidations are His, Arg, Lys, Pro, Met and Cys [28,29]. A factor influencing the susceptibility of these amino acid residues to metal-catalyzed oxidation is their ability to form complexes with metals such as Cu(II) or Fe(III). It is within these complexes that reactive oxygen species are generated, and oxidation of specific amino acid residues occurs in what is referred to as a "caged" process [30].

The present paper reports the results of combined spectroscopic and potentiometric studies on the copper(II) complexes of the neurokinin A and its N-acetyl derivative. The peptides studied here are: neurokinin A (NKA), HKTDSFVGLM-NH_2 and Ac-neurokinin A (Ac-NKA), Ac-HKTDSFVGLM-NH_2. To determine the involvement of the NH_2-amino group in the coordination of metal ions, the N-terminal group was blocked by acetylation. Neurokinin A and Ac-neurokinin A coordination of copper(II) lead to the generation of ROS involving the reduction of the oxidation state of the coordinated Cu (II) to Cu(I) and the oxidation of the peptides in the presence of hydrogen peroxide according to the site-specific mechanism. In this work, we examine the metal-catalyzed oxidation of the neurokinin A and its derivative, by the Cu(II)/H_2O_2 system. We would like to demonstrate the relationship between the binding sites of copper(II) ions and the oxidation products of the studied ligands.

2. Material and methods

2.1. Synthesis of the peptides

Synthesis of peptide amides: His^1-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met^{10}-NH_2 (NKA), Ac-His^1-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met^{10}-NH_2 (AcNKA) was performed on a solid-phase using Fmoc strategy with continuous-flow methodology (9050 Plus Millipore Peptide Synthesizer) on a polystyrene/polyethylene glycol copolymer resin (TentaGel R RAM Resin, substitution 0.18 mmol/g) [31]. Acetylation of the N-terminal amino group was performed on the resin using 1 M acetylimidazole in dimethylformamide (DMF). All peptides were cleaved from the resin and deprotected by 2 h shaking in a mixture containing trifluoroacetic acid, phenol, triisopropylsilane and water (88:5:2:5, v/v).

The resulting crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C_8 semi-preparative Kromasil column (25×250 mm, 7 μm). The purified peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and analytical RP-HPLC using a C_8 Kromasil column (4.6×250 mm, 5 μm) or C_{18} XTerra column (4.6×150 mm, 5 μm). As a mobile phase 30 min linear gradient of 5–100% B, where A: 0.1% aqueous trifluoroacetic acid (TFA), B: 80% acetonitrile (ACN)/H_2O + 0.1%TFA, was used.

Analytical data were as follows: NKA: R_t = 15.4 min (Kromasil), M_w obtained 1133.4, M_w calculated 1133.4, AcNKA: 15.6 min (Kromasil), M_w obtained 1175.6, M_w calculated 1175.4.

2.2. Potentiometric measurements

Stability constants for 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 2 mL. Alkali was added from a 0.250 mL micrometer syringe which was calibrated by both weight titration and the titration of standard materials. Experimental details: ligand concentration 1.5×10^{-3} M; metal to ligand molar ratio 1:1.1 for both peptides; ionic strength 0.10 M (KNO_3); $Cu(NO_3)_2$ was used as the source of the metal ions; pH-metric titration on a MOLSPIN pH-meter system using a Russell CMAW 711 semi-micro combined electrode, calibrated in concentration using HNO_3 [32], number of titrations equal 2; method of calculation SUPERQUAD [33]. 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.

2.3. Spectroscopic measurements

Solutions were of similar concentrations to those used in potentiometric studies. For the water solutions containing copper (II) ions and the studied ligands, the precipitation was observed (in potentiometric studies the electrolyte KNO_3 was used, and the precipitation was not observed). Addition of ethylene glycol to the samples (ethylene glycol–water 1:2, v/v) prevented the precipitation and for neurokinin A the solutions were clear in whole pH range while for Ac-neurokinin A the solubility was lower. The aggregation of the peptide may be important in the solubility of the peptide. A solvent effect on solubility and aggregational properties of polypeptides was observed [34].

Absorption spectra (UV–visible) 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 ethylene glycol–water (1:2, v/v) solution at 77 K on a Bruker ESP 300E spectrometer at the X-band frequency (~9.45 GHz) and equipped with the Bruker NMR gaussmeter ER 035M and the Hewlett-Packard microwave frequency counter HP 5350B. The spectra were analyzed by using Bruker's WIN-EPR SimFonia Software Version 1.25. Copper(II) stock solution was prepared from $Cu(NO_3)_2\times3\ H_2O$.

2.4. ESI-MS measurement

The mass spectra were obtained on a Bruker MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with Apollo II electrospray ionization source with ion funnel. The mass spectrometer was operated in the positive ion mode. Mass resolution 15,000 FWHM. The instrumental parameters were as follows: scan range m/z 400–2300, dry gas–nitrogen, temperature 200 °C, reflector voltage 1300 V, detector voltage 1920 V. The sample was dissolved in water at pH 10.5 with the concentration 10^{-4} M (pH value was adjusted using NaOH) and infused at a flow rate of 3 μL/min. Before analysis the instrument was calibrated externally with the Tunemix™

mixture (Bruker Daltonik, Germany) in quadratic regression mode. The mass accuracy for the calibration was better than 5 ppm. In MS/MS (Tandem Mass Spectrometry Analysis) mode, the quadruple was used to select the precursor ions, which were fragmented in the hexapole collision cell, generating product ions that were subsequently mass analyzed by the orthogonal reflectron TOF mass analyzer. For CID MS/MS (Collision-Induced Dissociation Tandem Mass Spectrometry Analysis) measurements, the collision energy over the hexapole collision cell was set to 21 eV, and argon was used as collision gas.

2.5. Materials used in the oxidation process

Deionized and triply distilled water was used, and the phosphate buffer was treated with Chelex 100 resin (sodium form, Sigma-Aldrich) to remove trace metals. Hydrogen peroxide was purchased from Fluka (Perhydrol, 30%) and EDTA and $Cu(NO_3)_2$ were purchased from POCH. 0.10 M stock solutions of EDTA and hydrogen peroxide in a phosphate buffer (pH 7.4) were prepared.

2.5.1. Oxidation of the neurokinin A and its acetyl-derivative and LCMS analysis

Copper(II)-catalyzed oxidation of peptide in the presence of hydrogen peroxide was monitored by analytical RP-HPLC on Varian ProStar 240 station using XTerra C_{18} 4.6×150 mm column (Waters) in 30 min linear gradient 5–100% B, where A: 0.1% aqueous TFA, B: 0.1% TFA in 80% ACN. A reaction mixture (0.5 cm^3) containing 5×10^{-4} M of peptide and metal to ligand molar ratio 1:1.1 in a 0.1 M phosphate buffer (pH 7.4) was incubated at 37 °C for 24 h in the presence of hydrogen peroxide at metal to hydrogen peroxide molar ratio 1:1 and 1:2. The reaction was started by the addition of hydrogen peroxide, which was freshly prepared. After incubation, the reaction was stopped by the addition of EDTA to a final complex to EDTA molar ratio 1:5. The chelating agent EDTA inhibits the oxidation of the peptide by removing Cu(II) from the peptide. Oxidized and digested peptides were lyophilized, dissolved in 0.1% trifluoroacetic acid and desalted on 10 µl ZipTipC18 columns (Omnix, Varian). The columns were prepared by wetting with 50% acetinitrile and equilibrated with 0.1% trifluoroacetic acid. Each sample was loaded onto ZipTip column, the column 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 0–30% B in 12.5 min followed by gradient 30–100% B in 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 were acquired and analyzed using LC Solution software provided by Shimadzu.

3. Results and discussion

3.1. Protonation constants

Calculated protonation constants for the studied and comparable ligands are given in Table 1. Neurokinin A and its N-acetyl derivative contain five and four, respectively, dissociable protons. Deprotonation of the lysyl group takes place at around pH 9–10, but for both peptides additional deprotonation with pK value 9–10 was observed (Table 1).

For neurokinin A the protonation constants of the: N-terminal amino group (log K = 7.41), the imidazole nitrogen of the His residue (log K = 5.43) are comparable to those of the peptides containing the N-terminal His residue (HSDGI-NH_2, HGG, HAA, Table 1). For N-acetyl derivative of neurokinin A the protonation constant of the imidazole nitrogen of the His residue is about one order of magnitude higher in comparison to those of the peptides with free-amine group, but these values are comparable to those of the peptides containing N-blocked amine group (Table 1). The protonation constants the β-carboxylate of the Asp residue in both peptide amides differ only slightly, and they are close to those expected for comparable peptides [35,36].

Table 1
Protonation constants for neurokinin A and Ac-neurokinin A and comparable peptides at 298 K and I = 0.10 M (KNO_3).

Peptide/logβ	HL	H_2L	H_3L	H_4L	H_5L
Neurokinin A	**9.72 ± 0.02**	**19.05 ± 0.01**	**26.46 ± 0.02**	**31.89 ± 0.02**	**35.61 ± 0.02**
HSDGI-NH_2[a]	7.43	13.00	16.54		
HGG[b]	7.32	12.77	15.86		
HAA[b]	7.44	13.02	16.30		
Ac-neurokinin A	**10.11 ± 0.01**	**19.25 ± 0.01**	**25.61 ± 0.02**	**29.53 ± 0.02**	
Ac-HGGGWGQ-NH_2	6.41				
Ac-HGGG[d]	6.59	9.96			
Ac-GGGH[d]	7.03	9.79			
log K	NH_2-Lys and OH-Ser		NH_2-terminal	N_{Im}	COO^-
Neurokinin A	**9.72**	**9.33**	**7.41**	**5.43**	**3.72**
HSDGI-NH_2			7.43	5.57	3.54
HGG			7.32	5.45	3.09
HAA			7.44	5.58	3.28
Ac-neurokinin A	**10.1**	**19.14**		**6.36**	**3.92**
Ac-PHGGGWGQ-NH_2				6.41	
Ac-HGGG				6.59	3.37
Ac-GGGH				7.03	2.76

a ref. [51], b ref. [50], c ref. [54], d ref. [57].

Potentiometry detects a range of Cu(II) complexes with the formation constants reported in Table 2. 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 [37], and they are given in Table 3. The spectroscopic properties of the major complexes are given in Table 4.

3.2. Determination of the additional deprotonation site in neurokinin A and its derivative

Nonenzymatic intramolecular reactions can result in the deamidation, isomerization, and racemization of protein and peptide asparaginyl and aspartyl residues via succinimide intermediates [38]. This five-membered succinimide ring is unstable under physiological conditions and is hydrolyzed to give a mixture of aspartyl and isoaspartyl peptides. This post-translation modification is suspected to contribute to the aging of proteins and to protein folding disorders such as Alzheimer's disease. This modification occurs spontaneously under physiological conditions whose rate is affected by both its amino acid sequence and three-dimensional structure [39]. It should be mentioned that this additional deprotonation is also observed for the fragments of neuropeptide gamma (tachykinin family group) where the HKTDSFVGLM sequence exists [40]. The presence of the TDS-sequence in these peptides may also suggest the formation of the lactones and their alkaline hydrolysis [41]. The MS spectra of the neurokinin A and its acetyl derivative do not show molecular ions [M+2H–H_2O] with changing of pH suggesting lack of the formation of the lactone or succinimide rings in the peptides studied. The obtained ESI-MS spectrum for the Ac-neurokinin A (Fig. 1) shows

Table 2
Stability constants of copper(II) complexes of neurokinin A, Ac-neurokinin A and comparable peptides at 298 K and $I = 0.10$ M (KNO_3).

Peptide/logβ	CuH_3L	CuH_2L	CuHL	CuL	$CuH_{-1}L$	$CuH_{-2}L$	$CuH_{-3}L$	$CuH_{-4}L$	$Cu_2H_2L_2$ or $Cu_2H_{-2}L_2$
Neurokinin A	**31.88 ± 0.02**	**27.90 ± 0.01**		**12.86 ± 0.02**	**4.01 ± 0.03**	**−5.06 ± 0.02**	**−14.82 ± 0.02**	**−25.53 ± 0.03**	**44.86 ± 0.03**
HSDGI-NH_2[a]			12.47	8.69		−4.01			8.63
HGG[b]				8.46		−6.85			7.24
HAA[b]				8.57		−6.37			7.09
Ac-neurokinin A		**23.83 ± 0.02**	**17.84 ± 0.02**	**10.82 ± 0.02**	**2.27 ± 0.03**	**−7.20 ± 0.05**	**−16.79 ± 0.05**		
Ac-PHGGGWGQ-NH_2[c]				3.69	−2.80	−8.99	−17.97	−28.92	
Ac-HGGG[d]				4.20	−2.40	−9.36	−18.28		
Ac-GGGH[d]				4.56	−2.35	−9.03	−17.45		

[a,b,c,d] as in Table 1.

a dominant signal for the monosodiated doubly charged form (599.285 m/z), whereas the monosodiated singly charged species is almost absent. Peaks corresponding to diprotonated (588.295 m/z) and oligosodiated forms are also present on the spectrum. The most abundant peaks at 588 m/z and 599 m/z, corresponding to the $[M+2H]^{2+}$ ion and its monosodiated counterpart $[M+H+Na]^{2+}$ was chosen as a precursor for the fragmentation by CID. The fragmentation pattern of the doubly protonated peptide (Fig. 2a) is dominated by doubly protonated a and b fragments (according to nomenclature developed by Roepstorff and Fohlman [42]). No y series, containing C-terminal tail were observed. This is due to the presence of basic amino acid residues (that are accessible for protonation) on N-terminal side of the peptide only. In contrast to the CID spectrum obtained for fragmentation of the double protonated peptide, $[M+2H]^{2+}$, sodiated ions become the major type of the fragment ions resulted from the CID of the doubly charged monosodiated species $[M+H+Na]^{2+}$. This suggest that the sodium salts are relatively stable in the performed CID conditions. It is known that structure of the sodiated peptide ions may affect the CID MS/MS fragmentation pattern. Alkali metal ions interact selectively with polar functional groups to form a chelate coordination structure of the peptide–metal ion complex and can form fixed charges of the peptide backbone. MS/MS analysis of metaliated peptides is known method of localization of the peptide groups involved in interaction with the metal [43]. Recently, Bensadek and co-workers found that the fragmentation of the sodiated peptides gives rise to sodium cationized y ions, since the sodium cation is chelated at the C-terminus of tested peptides [44]. In this paper we performed the CID analysis of the doubly charged monosodiated species $[M+H+Na]^{2+}$ to estimate possible binding site of sodium ion to the peptide. We noticed a striking difference in the fragmentation patterns between sodiated and non-sodiated peptide ions (Fig. 2a and b) suggesting that the presence of the sodium cation selectively interacts with the peptide. The CID of the doubly charged monosodiated species $[M+H+Na]^{2+}$ resulted in the formation of sodiated y ions in the m/z range above the precursor and a characteristic a_n/b_n ($n = 6$–10) pair in the low m/z range. Only double charged sodiated a_{6-10} and b_{6-10} double charged ions in the in the range of m/z 376–600 were detected during CID of the doubly charged monosodiated species $[M+H+Na]^{2+}$, what suggests, that the metal ion is localized in a polar group of one of six amino acid residues situated on N-terminal peptide part. On the other side, formation of singly charged sodiated y_7–y_9 ions suggests that the sodium adduct is located rather on the C-terminal heptapeptide. The presence of the single charged non-sodiated b_2 and b_3 ions at 308.17 m/z and 409.22 m/z, respectively further confirms that the sodium ion is not localized on the first three N-terminal residues. The identified fragments presented above indicate that the sodium adduct may be attached to Ser^5 or Asp^4 side chains. These suggestions are in agreement with previous investigations performed by Feng and co-workers [45]. The authors studied lithium and sodium ion binding energies of N-acetyl and N-glycyl amino acids and concluded that the presence of a coordinating group (e.g., –OH, $–CO_2H$) in the amino acid side chain can significantly increase the lithium and sodium binding energies. However, a sodiated singly charged peak at 674.32 m/z corresponds to $[y_6Na]^+$ fragment, that does not contain the aspartic acid residue. This may indicate that Ser^5 present in $[y_6Na]^+$ fragment may be particularly responsible for interaction with the sodium ion. A transfer of the sodium ion from Asp^4 carboxyl group to y_6 fragment containing Ser^5 during CID is rather doubtable, and therefore the sodium ion should be attached to the serine residue before the fragmentation. As can be expected, no peak corresponding to the $[y_5Na]^+$ fragment is present in the spectrum, confirming that sodium ion is attached to the serine residue only in $[y_6Na]^+$ fragment. These data may suggest a deprotonation of the hydroxyl-OH group of the Ser residue in the

Table 3
Calculated log K^* and deprotonation constants for amide protons (pK) values for Cu(II) complexes with studied and comparable ligands.

Peptide/log K^*	1 N {N_{Im} or NH_2}	2 N{N_{Im}, NH_2}	3 N dimeric{NH_2, N^-,CO, N_{Im}}	3 N{NH_2, $2N^-$,CO or COO^-}	4 N{NH_2, $3N^-$}
Neurokinin A	**−0.01**	**−3.99**	**−18.92**	**−13.60**	**−22.45**
HSDGI-NH_2	−0.53	−4.31	−17.37		
HGG		−4.31	−18.30	−14.17	
HAA		−4.45	−18.95	−13.81	
AAAAA-NH_2[a]				−16.44	−24.41
log K^*[b]		1 N{N_{Im}}	2 N{N_{Im}, N^-}	3 N{N_{Im}, $2N^-$}	4 N{N_{Im}, $3N^-$}
Ac-neurokinin A		**−1.78**	**−7.77**	**−14.79**	**−23.34**
Ac-HGGGWGQ-NH_2		−2.72	−9.21	−15.40	−24.38
Ac-HGGG		−2.39	−8.99	−15.95	−24.87
Ac-GGGH		−2.47	−9.38	−16.06	−24.48
		pK_1	pK_2	pK_3	
Ac-neurokinin A		**5.99**	**7.02**	**8.55**	
Ac-HGGGWGQ-NH_2		6.49	6.19	8.98	
Ac-HGGG		6.60	6.96	8.92	
Ac-GGGH		6.91	6.68	8.42	

[a] ref. [53], [b] $K^* = \log\beta(CuH_jL) - \log\beta(H_nL)$ where the index j corresponds to the number of the protons in the coordinated ligand to metal ion and n corresponds to the number of protons coordinated to ligand.

Table 4
Spectroscopic data for the Cu(II) complexes of neurokinin A and Ac-neurokinin A.

Species	Absorption			CD		EPR	
	pH	λ (nm)	ε (M^{-1} cm^{-1})	λ (nm)	Δε (M^{-1} cm^{-1})	$A_{\|\|}$	$g_{\|\|}$
H_2N-HKTDSFVGLM-NH_2							
CuH_3L	3.5					163	2.299
{NH_2 or N_{Im}}							
CuH_2L	5.5	654[a]	55	694[a]	+0.397	189	2.238
{NH_2, N_{Im}}				323[d]	−0.229		
$Cu_2H_2L_2$	7.5	598[a]	98	690[a]	+0.335	Dimer	
{NH_2, N^-, CO, N_{Im}}				564[a]	−0.060		
				345[d]	−0.133		
				282[bc]	−0.077		
CuL	8.5	584[a]	106				
{NH_2, $2N^-$, CO}							
$CuH_{-1}L$, $CuH_{-2}L$,	Above	516[a]	58	530[a]	−0.989	202	2.179
$CuH_{-3}L$	9.0			311[e]	+0.563		
{NH_2, $3N^-$}				273[b]	−2.300		
Ac-HKTDSFVGLM-NH_2							
CuH_2L	5.0	732[a]	16	313[d]	+0.049	164	2.297
{N_{Im},}							
CuHL	6.5	660[a]	[f]	597[a]	−0.107	171	2.242
{N_{Im}, N^-}				309[e]	+0.043		
				255[c]	+0.246		
CuL	8.0	600[a]	[f]	584[a]	+0.219	186	2.229
{N_{Im}, $2N^-$}				348[d]	+0.036		
				304[e]	+0.062		
				256[c]	−0.235		
$CuH_{-1}L$, $CuH_{-2}L$,	Above	540[a]	[f]	547[a]	−0.372	205	2.182
$CuH_{-3}L$	9.0			311[e]	+0.148		
{N_{Im}, $3N^-$}				255[c]	+1.022		

[a] *d–d* transition.
[b] $NH_2 \rightarrow Cu(II)$ charge transfer transition.
[c] N_{Im} (π2) → Cu(II) charge transfer transition.
[d] $N_{Im} \rightarrow Cu(II)$ charge transfer transition.
[e] N-(amide) → Cu(II) charge transfer transition.
[f] ε cannot be estimated because of higher base line, the solutions were not too clear.

ligands studied. It seems that the reason of the low protonation constant of the hydroxyl OH group of the serine may be presence of the His, Asp and Ser residues (like in serine proteases) and the secondary structure of the peptide. Moreover, it should be also mentioned that this additional deprotonation has not any influence on the coordination abilities of the neurokinin A and its derivative.

3.3. Copper(II) complexes

According to potentiometric and spectroscopic results, the neurokinin A forms with Cu(II) ions the CuH_3L, CuH_2L, CuL, $CuH_{-1}L$, $CuH_{-2}L$, $CuH_{-3}L$, $CuH_{-4}L$ and $Cu_2H_2L_2$ complexes (charges omitted for simplicity, Table 2). Two protonation constants of the Lys residue and, most likely, hydroxy group of Ser residue were detected, therefore, the 1 N {NH_2, CO or N_{Im}} complex will be the CuH_3L species (Scheme 1). This complex at pH 3–5 is formed (Fig. 3) with EPR parameters $g_{||} = 2.299$ and $A_{||} = 163$ G (Fig. 4a, Table 4). In CD spectra the *d–d* absorption band is not observed for this complex (concentration of copper(II) about 0.001 M) and it is in agreement with the previous findings of Sigel and Martin, which state that ability of metal ions to sense asymmetry on a nearby side chain is transmitted in the decreasing order: N^-(amide) > NHCO > COO^- > NH_2 [46]. The next species, CuH_2L, is detected in the wide 3.5–7.5 pH range as a major complex (Fig. 3). The EPR parameters $g_{||} = 2.238$ and $A_{||} = 189$ G (Fig. 4b), the *d–d* transition energy at 654 nm and the presence in CD spectra of the $N_{Im} \rightarrow Cu(II)$ charge transfer transition at 323 nm are consistent with the histamine-like coordination {NH_2, N_{Im}} of the neurokinin A to copper(II) ions (Scheme 1) [47–51]. Coordination of histidine normally gives charge transfer transitions at *c.a.* 330 nm [$\pi_1(N_{Im}–Cu(II))$]. The magnitude and precise energy of the charge transfer transitions for an imidazole nitrogen to Cu(II) are very sensitive to the position of the ring plane relative to the complex plane, and thus to the possibility of its rotation [52]. The value of log K^* for 2 N {NH_2, N_{Im}} complex of neurokinin A is comparable to those of the peptides containing the histidine residue in the first position of the peptide chain (H^1) (Table 3).

With increasing pH above 6.5 in the CD spectra the $N_{Im} \rightarrow Cu(II)$ charge transfer transition at 345 nm is present but the EPR signal

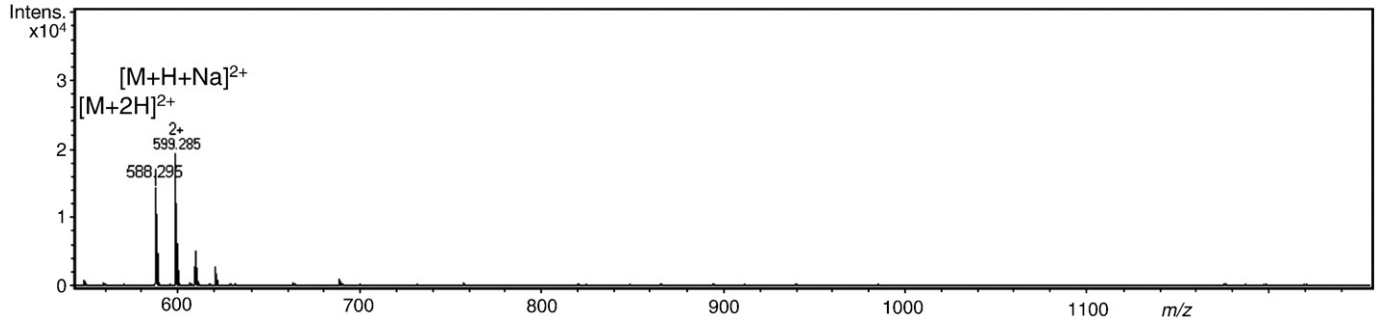


Fig. 1. Mass spectrum of Ac-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 at pH 10.5.

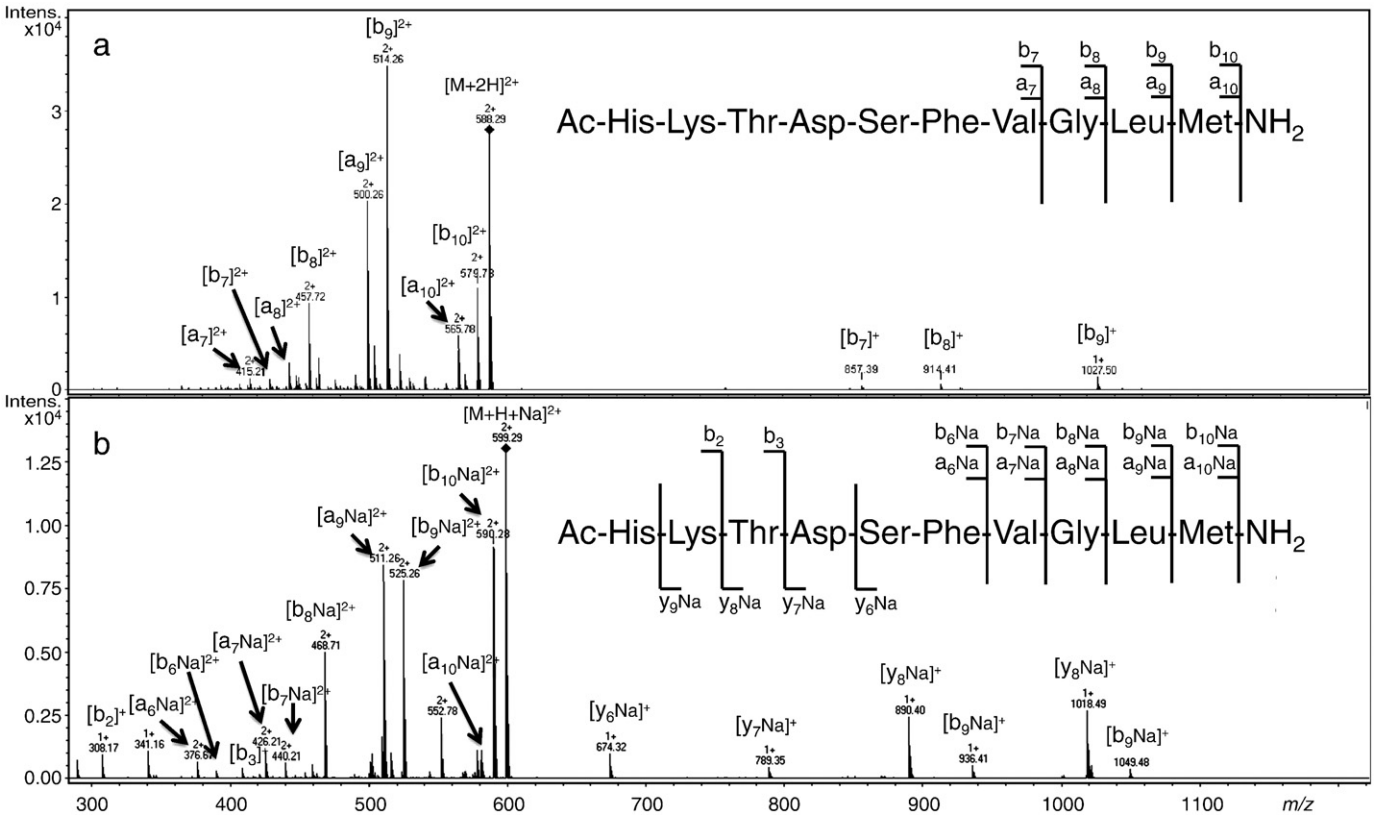
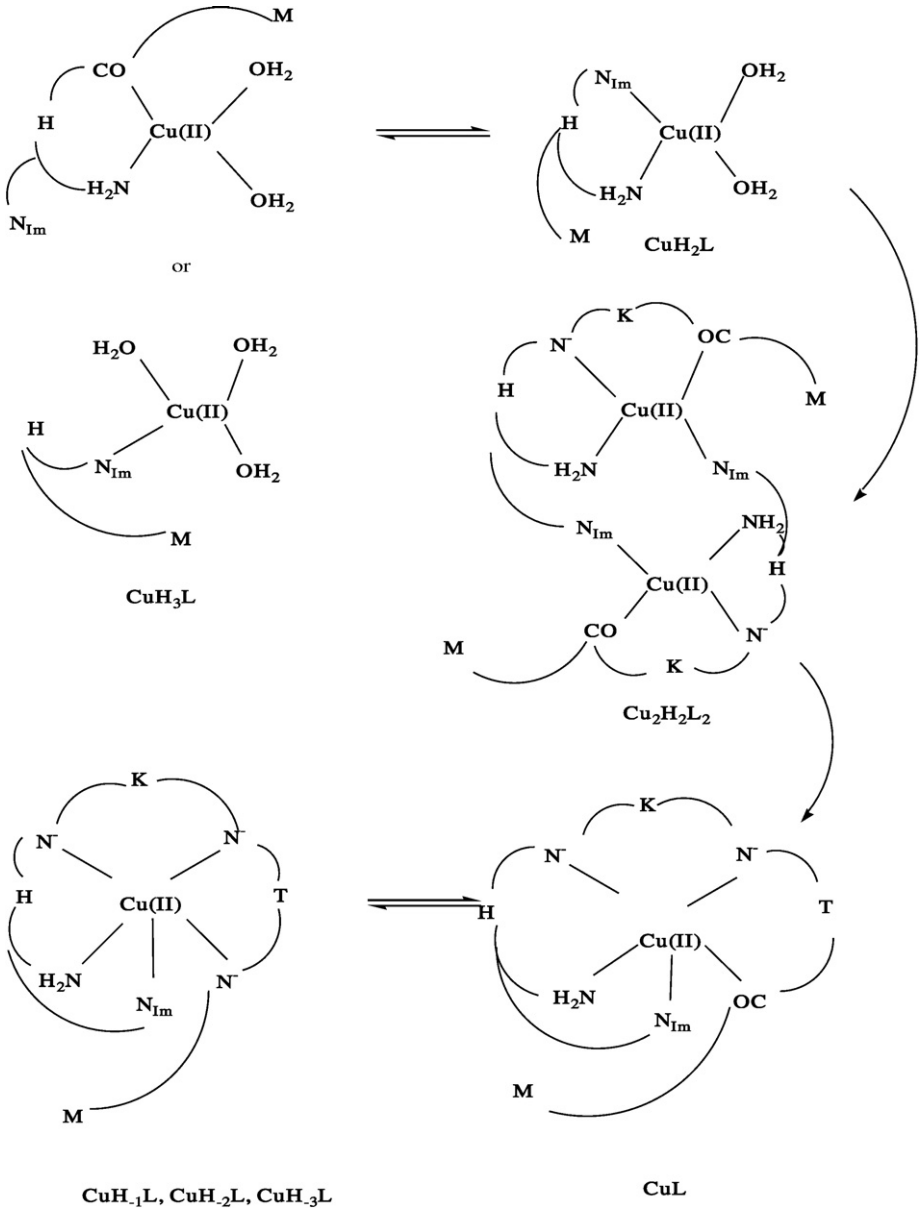


Fig. 2. MS/MS spectrum of doubly charged peptide Ac-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 (parent ion *m/z* 589.29) (a). MS/MS spectrum of the doubly charged monosodiated peptide Ac-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 ion at *m/z* 599.29. $[y_nNa]^+$ denotes y_n ions in which a proton has been exchanged for a sodium cation.

intensity is reduced indicating a possible antiferromagnetic interaction among copper(II) ions in the complex formed (Table 4). In pH range 6.5–8.5 (Fig. 3), the dimeric $Cu_2H_2L_2$ species is formed through Gly-Gly-like coordination, and the imidazole nitrogen acts as bridging ligand (Scheme 1). The structure of the imidazole-bridging dimer has been proposed for HG [47], HM [48], HH [49], HGG and HAA [50] and HSDGI-NH_2 [51]. Above pH 8, the band diagnostic of imidazole nitrogen binding is no longer observed and the CuL complex with {NH_2, $2N^-$, CO} coordination mode is formed (Scheme 1). The coordination of the ligand in the Cu(II)–neurokinin A system at pH above 8.5 follows by sequential deprotonation and coordination of neighboring peptide nitrogen with the formation of 4 N {NH_2, $3N^-$} complex (Scheme 1). The *d–d* energy at 516 nm, the parameters of CD spectra and $g_{II} = 2.179$, $A_{II} = 202$ G correspond very well to the 4 N {NH_2, $3N^-$} coordination mode (Fig. 4c, Table 4). These parameters of UV–Vis, CD and EPR are not altered in basic solution suggesting the same coordination binding in the $CuH_{-1}L$, $CuH_{-2}L$ and $CuH_{-3}L$ complexes. Value of log K^* for the dimeric complex of neurokininA is comparable to those of the HGG and HAA and about 1.5 log units lower in comparison to that of HSDGI-NH_2 (Table 3). The value of log K^* for 3 N {NH_2, $2N^-$,CO or COO^-} species of neurokinin A is comparable to those of the HGG and HAA, and about three orders of magnitude higher compared to that of pentaalanine amide (Table 3) [53]. This stabilization of the CuL complex of neurokinin A may proceed from the additional coordination of the histidine side-chain residue in axial position as it was suggested for HGG and HAA [50]. The stabilization of the 4 N {NH_2, $3N^-$} complex by about two orders of magnitude is also observed in comparison to that of pentaalanine amide (Table 3). This stabilization may also result from the axial interaction of the histidine residue in the $CuH_{-1}L$ complex (Scheme 1).

Six metal complex species can be fitted to the experimental titration curves obtained for the Cu(II)–Ac-neurokinin A system: CuH_2L, CuHL, CuL, $CuH_{-1}L$, $CuH_{-2}L$, $CuH_{-3}L$ (Table 2). The protection of the N-terminal nitrogen of His[1] dramatically changes the coordination, which begins at a higher pH than that discussed above (Fig. 5). The coordination of the histidine side chain instead of the amine N would yield 7,5,5-membered fused chelate rings, resembling that of the prion protein octarepeat metal-binding site [54,55]. This type of structure has later been confirmed by the X-ray diffraction analysis of the copper(II) complex of Ac-His-Gly-Gly-Gly-Trp-NH_2, a segment of the octarepeat region [56]. Since then a number of N-protected peptides with histidine at the N-terminus have been shown to have similar type of coordination [50,57,58]. The anchoring site at the imidazole nitrogen is metal-bound in the CuH_2L species (Scheme 2). This complex undergoes stepwise deprotonation processes giving CuHL, CuL, $CuH_{-1}L$, $CuH_{-2}L$, $CuH_{-3}L$ complexes. The major species in the "physiological" pH, 6–9 is the 3 N {N_{Im}, $2N^-$} (CuL) complex. The *d–d* transition energy at 600 nm, the EPR parameters $g_{II} = 2.229$, $A_{II} = 186$ G and the presence in the CD spectrum of charge transfer transitions $N_{Im} \rightarrow Cu(II)$ at 348 nm and $N^-(amide) \rightarrow Cu(II)$ at 304 nm (Table 4) indicate a 3 N complex with {N_{Im}, $2N^-$} coordination mode. With increasing of pH, the shift of the absorption maximum to 540 nm and the EPR parameters $g_{II} = 2.182$, $A_{II} = 205$ G suggest coordination of the additional nitrogen donor atom with the formation of the 4 N $CuH_{-1}L$, {N_{Im}, $3N^-$} complex (Scheme 2). The values log K^* for 1 N, 2 N, 3 N and 4 N copper(II) complexes of Ac-neurokinin A are higher by about 1, 1.5, 0.7 and 1 orders of magnitude, respectively, in comparison to those of Ac-PHGGGWGQ-NH_2 (Table 3). It should be mentioned that the coordination of the histidine side chain in the $CuH_{-3}L$ complex of Ac-HGGG peptide yields 7,5,5-membered fused chelate rings, while in the $CuH_{-3}L$ complex of Ac-GGGH 5,5,6-membered chelate rings. However, the log K^* values for the 1 N-to-4 N complexes of Cu(II)–Ac-HGGG and Cu(II)–Ac-GGGH systems and the deprotonation constants for amide protons (pK values) of their Cu(II) complexes are similar to each other (Table 3).

The CD spectra in the range 190–300 nm of metal-free NKA and Ac-NKA and complexed ligands at pH 7 indicate an unordered



Scheme 1. Schematic structures of the Cu(II)–neurokinin A complexes formed with increasing of pH.

structure of peptides with time (aging process) at this pH (data not shown).

3.4. Oxidation of neurokinin A and its derivative

The oxidation of protein causes the modification of amino acids, protein aggregation, and protein fragmentation [27,28]. The mechanism of generation for the active species from Cu(II)/peptide/H_2O_2 has been considered (reactions (1)–(3)) [59,60].

$$\text{peptide} - \text{Cu(II)} + H_2O_2 \rightarrow \text{peptide} - \text{Cu(I)} + O_2^{-}\bullet + 2H^{+} \quad (1)$$

$$\text{peptide} - \text{Cu(II)} + O_2^{-}\bullet \rightarrow \text{peptide} - \text{Cu(I)} + O_2 \quad (2)$$

$$\text{peptide} - \text{Cu(I)} + H_2O_2 \rightarrow \text{peptide} - \text{Cu(II)} + {}^{\bullet}OH + OH^{-} \quad (3)$$

In accord with a site-specific mechanism [28,61], suggesting that the active oxygen species are formed on or near the metal-binding sites of the protein and react predominantly with amino acid residues of close proximity the spectroscopic measurements in a 0.1 M phosphate buffer at pH 7.4 were made (data not shown). The spectroscopic data for the Cu(II)–neurokinin A and Cu(II)–Ac-neurokinin A in phosphate buffer suggest that the coordination modes of these peptides to copper(II) ions are similar to those obtained in aqueous solution at pH 7.4. For the Cu(II)–neurokinin A system, the $Cu_2H_2L_2$ complex dominates at pH 7.4 (Fig. 3) with the coordination of copper (II) ions by the nitrogen atoms of amine group and imidazole of histidine (H^1) and atoms of amide nitrogen and oxygen of carbonyl group of the lysine residue (K^2). For the Cu(II)–Ac-neurokinin A system at pH 7.4 the CuL complex exists (Fig. 5) with the 3 N {N_{Im}, $2N^{-}$} coordination binding. Therefore, the modification of the peptides at N-terminal amine group should be expected.

The chromatograms of the neurokinin A and Ac-neurokinin A after 24 h incubation at 37 °C for the peptide alone, with Cu(II) only, hydrogen peroxide and with Cu(II)–H_2O_2 indicate that for the solution containing peptide alone and the copper(II) ions with 1:1 peptide to copper(II) molar ratio were not changed in comparison to the peptide alone before incubation (Fig. 6). For the solution containing the peptide–hydrogen peroxide for both systems at 1:1 or 1:2 molar ratio a new peak on the chromatogram was detected. Liquid chromatography/mass spectrometry

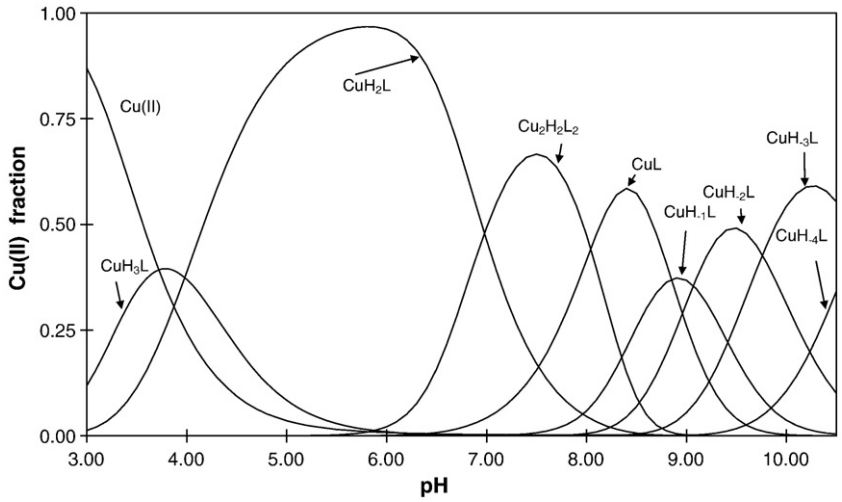


Fig. 3. Species distribution curves for Cu(II) complexes of neurokinin A. Cu(II) to peptide molar ratio 1:1, [Cu(II)] = 0.001 M.

(LC–MS) was the primary analytical tool utilized in the product analysis of the oxidation reactions. Liquid chromatography (LC) was employed to monitor and quantitate the reaction products, while mass spectrometry enabled the structural identification. The desalted reaction mixture of peptide–hydrogen peroxide systems contains two major components, one corresponding to the Met sulfoxide (t_R = 10.5 min for neurokinin A, t_R = 11.6 for Ac-neurokinin A, Tables 5 and 6, respectively) and one corresponding to unoxidized peptide (t_R = 12.3 min and 13.4 min for neurokinin A and Ac-neurokinin A, Tables 5 and 6, respectively). The doubly charged molecular ions with *m/z* 575.2 Da and 596.8 Da for neurokinin A and its acetyl-derivative, respectively, were present in mass spectra (Tables 5 and 6). The oxidation of the methionine residue to the methionine sulfoxide by hydrogen peroxide alone [62,63] and by metal-catalyzed oxidation was observed [64–66].

When the copper(II) ions are alone with peptides the modifications of these peptides don't occur (Fig. 6), but when the copper(II) ions with hydrogen peroxide are together then further oxidation of the peptides is observed (Tables 5 and 6). The exposure of the peptides to Cu(II) and H_2O_2, resulted in an efficient conversion of the peptide His residues into asparagines, aspartic acid, aspartylurea, formylasparagine [67], and 2-oxo-His [68–70]. It is suggested that the sequence and conformation are important parameters controlling the metal-catalyzed oxidation of His [71]. For the Cu(II)–neurokinin A system, mass spectrometry of the LC fraction eluted at 13.3 min yielded a doubly charged molecular ion with mass of 583.4 Da supporting the oxidation of the His residue to 2-oxo-His or further oxidation of Met sulfoxide to sulfone (Table 5). The loss of sulfinic acid (CH_3SOH, 64 Da) from the oxidized methionine residue is diagnostic for the oxidized methionine [65]. The masses of $[M+H]^+$ and $[M+2H]^{2+}$ molecular ions at 1085.5 and 542.7 Da, respectively, observed for the LC fraction eluting at 13.3 min may be assigned to the peptide with loss of CH_3SOH from the peptide with oxidized methionine to sulfoxide (Table 5). The involvement of the histidine in binding of copper(II) ions is supported by the cleavage of the H^1–K^2 peptide bond. Mass spectrometry for the LC fraction eluted at 13.0 min yielded masses of $[M+H]^+$ and $[M+2H]^{2+}$ molecular ions at 997.5 and 498.7 Da, respectively (Table 5, Fig. 7). These masses may be assigned to K^2–M^{10} fragment. Under experimental conditions the other molecular ions of copper(II) catalyzed oxidation products for neurokinin A were also detected and the modifications of this neuropeptide are proposed in Table 5.

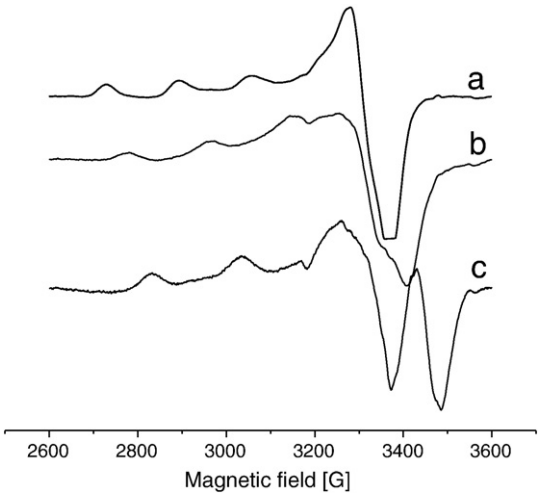


Fig. 4. X-band EPR spectra of 1:1.1 Cu(II)–neurokinin A frozen solution at 77 K at pH 3.5 (a), 5.5 (b) and 10.0 (c).

When the sample of Cu(II)–Ac-neurokinin A–H_2O_2 1:1:1 and 1:1:2 molar ratio after incubation was loaded onto ZipTip column to remove salts and was eluted with 0.1% formic acid in 80% acetonitrile the precipitation of the oxidized Ac-neurokinin A was observed. However, the obtained sample was the subject of LC-ESI-MS analysis (Table 6). Mass spectrometry of the LC fraction eluted at 14.6 min yielded a mass of 1207.5 Da supporting the oxidation of the His residue to 2-oxo-His or further oxidation of Met sulfoxide to Met sulfone (Table 6). A peak of the doubly charged $[M+2H]^{2+}$ molecular ion occurring at *m/z* 577.4 Da in the LC fraction eluting at 14.9 min (Table 6) may correspond to the peptide containing the oxidized histidine residue to aspartic acid. The fragmentation products obtained by cleavage of the peptide bonds near the His residue were also observed supporting the involvement of this residue in binding of copper(II) ions (Table 6). It is well known that physicochemical properties of a protein can be significantly altered upon covalent modifications. The data for proteins suggest that modification of amino acid residues (especially His) leads to alternations in secondary and tertiary structures, and may increase the exposure of the hydrophobic surface area which results in consequent noncovalent aggregation and precipitation [65,72,73].

4. Conclusions

Neurokinin A contains the histidine residue in the first position of the peptide sequence (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-

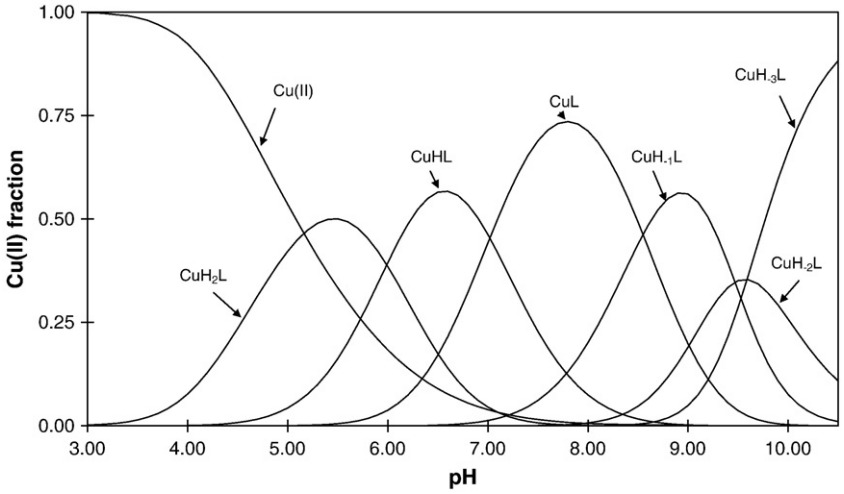
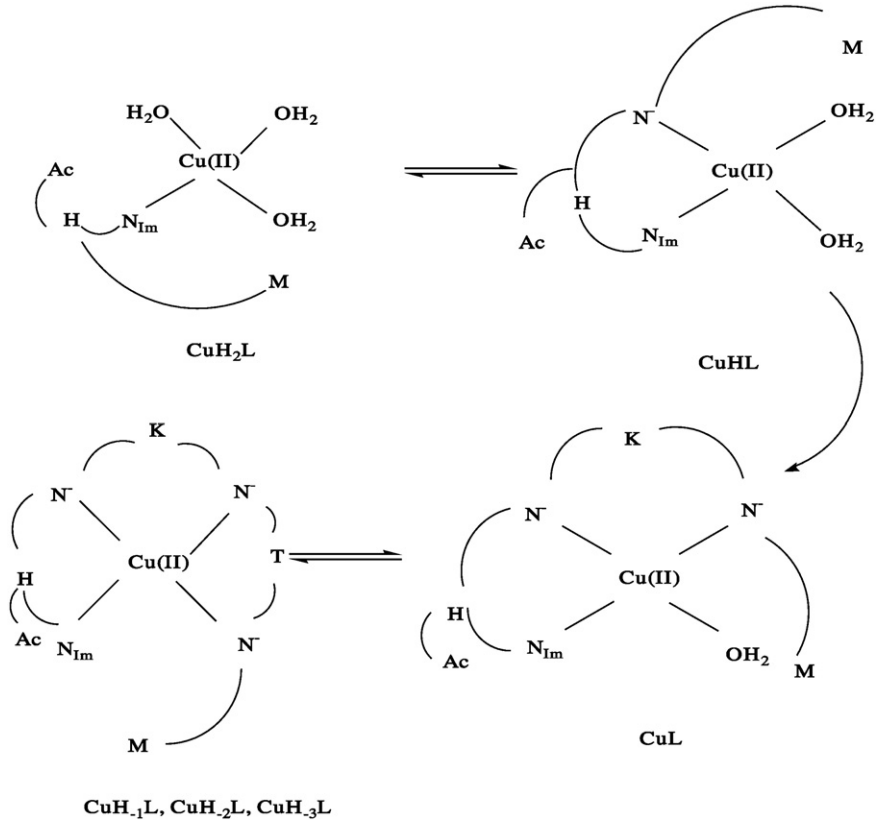


Fig. 5. Species distribution curves for Cu(II) complexes of Ac-neurokinin A. Cu(II) to peptide molar ratio 1:1, [Cu(II)] = 0.001 M.

NH_2) and forms monomeric and dimeric complexes of copper(II) ions. The CuH_2L complex with the histamine {NH_2, N_{Im}} type coordination mode predominates at low and wide 3.5–7.5 pH range. In the pH 6.5–8.5 range the formation of a dimeric complex $Cu_2H_2L_2$ was found in which the coordination of copper(II) ions is glycylglycine-like, while the fourth coordination site is occupied by the imidazole nitrogen atom, forming a bridge between two copper(II) ions. The formation of this dimeric species does not prevent the deprotonation and coordination of the sequential amide nitrogens and at pH above 8.0 the 4 N {NH_2, $3N^-$} complex is formed. The coordination of the histidine side chain instead of the amine NH_2 nitrogen yields 7,5,5-membered fused chelate rings for the $CuH_{-1}L$ complex of Cu(II)–Ac-neurokinin A system. The copper(II) ion is coordinated by the imidazole nitrogen atom of histidine and deprotonated amide nitrogens from the next Lys, Thr and Asp residues.

For the neurokinin A and its acetyl derivative the additional deprotonation at pH 9–10 was detected. The MS/MS experiments suggest a deprotonation of hydroxy group of Ser residue.

For the neurokinin A and Ac-neurokinin A-hydrogen peroxide systems at 1:1 and 1:2 molar ratio the oxidation of the methionine residue (M^{10}) to methionine sulfoxide was observed. For the both studied peptides the coordination of the copper(II) ions by the



Scheme 2. Binding modes of the species formed in the copper(II)–Ac-neurokinin A system.

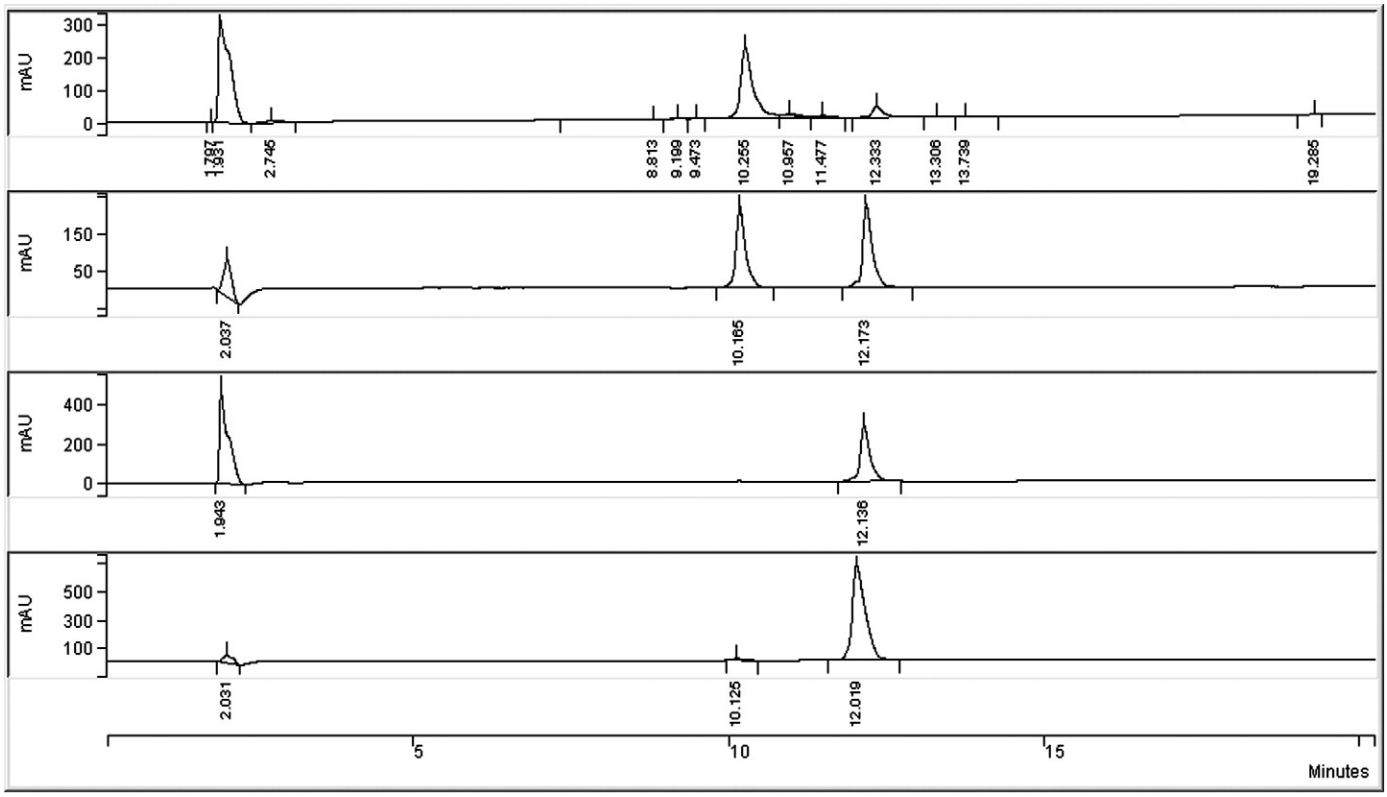


Fig. 6. Chromatograms of the neurokinin A after 24 h incubation at 37 °C for the peptide alone (first from bottom), peptide with copper(II) (1:1 molar ratio) (second from bottom), peptide with hydrogen peroxide (1:2 molar ratio) (second from top) and peptide with copper(II) and hydrogen peroxide (1:1:2 molar ratio) (first from top). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of peptide and Cu(II) 0.50 mM. Incubation at 37 °C.

histidine occurs, therefore, in the Cu(II)–peptide–hydrogen peroxide 1:1:1 and 1:1:2 molar ratio systems, oxidation of histidine to 2-oxo-histidine may be suggested. Fragmentations by the cleavage of the peptide bonds near the His residue were also detected.

Hydrogen peroxide is produced *in vivo* [74] and if both H_2O_2 and copper(II) ions are available *in vivo*, then radicals will form. The dominant risk factor associated with the neurodegenerative diseases is increasing age. Several studies in animals and humans have reported a

Table 5
Products of oxidized neurokinin A (HKTDSFVGLM-NH_2) in Cu(II)–neurokinin A-H_2O_2 system analyzed by LCMS spectra.

Determination of peptide modification	Charge	MW_{calcd} (Da)	t_R(min)				MW_{obsd} (Da)		
		14.6	10.3	10.5	11.9	12.3	13.0	13.3	13.9
Neurokinin A	1	1133.6					1133.6		
	2	567.8					567.8		
Neurokinin A:H_2O_2 (1:1)									
Oxidation of Met → sulfoxide	2	575.8		575.2					
	3	384.2		383.8					
Cu(II):neurokinin A:H_2O_2 (1:1:1)									
Oxidation of Met → sulfoxide	1		1149.6	1149.6					
	2		575.8	575.3					
Oxidation of Met → sulfoxide and His → 2-oxo-His or Met → sulfone	2	583.8						583.4	
Oxidation of Met → sulfone, cleavage of the H–K peptide bond (K^2–M^{10})	2	515.2				516.8			
Oxidation of Met → sulfoxide, cleavage of the H–K peptide bond (K^2–M^{10})	1	1012.5						1011.4	
	2		507.2					506.3	
Cleavage of H^1–K^2peptide bond (K^2–M^{10})	1		996.5				997.5	993.4	
	2	499.2					498.7	498.7	
Loss of CH_3SOH fragment from peptide with oxidized Met → sulfoxide	1	1085.6						1085.5	
	2	543.8						542.7	
Oxidation of Met → sulfoxide, loss of CH_3SOH fragment, decarboxylation of D^4 and deamination of H^1	1	1040.6					1038.5		
	2	521.3					519.7		
Oxidation of Met → sulfoxide and His → 2-oxo-His or Met → sulfone, decarboxylation of D^4 and deamination of H^1	2	561.3						560.7	559.8
Cleavage of D^4–S^5peptide bond,S^5–M^{10} fragment	1	652.1							652.4

Table 6
Products of oxidized Ac-neurokinin A (Ac-HKTDSFVGLM-NH_2) in Cu(II)–Ac-neurokinin A–H_2O_2 system analyzed by LCMS spectra.

Determination of peptide modification	Charge	MW_{calcd} (Da)	t_R (min)		MW_{obsd} (Da)		
			11.6	13.4	14.6	14.9	15.1
Ac-Nurokinin A	1	1175.4		1175.6			
	2	588.7		588.8			
Ac-neurokinin A:H_2O_2 (1:1 and 1:2)							
Oxidation of Met → sulfoxide	1	1191.4	1192.6				
	2	596.7	596.8				
Cu(II):Ac-neurokinin A:H_2O_2 (1:1:1)							
Oxidation of Met → sulfoxide and	1	1207.4			1207.5		
His → 2-oxo-His or Met → sulfone	2	604.7			604.8	603.4	
Oxidation of His → Asp	2	577.7				577.4	
Cleavage of T^3–D^4peptide bond,(D^4–M^{10})	1	768.0				767.4	
Cu(II):Ac-neurokinin A:H_2O_2 (1:1:2)							
Oxidation of Met → sulfoxide and	1	1207.4			1207.6		
His → 2-oxo-His or Met → sulfone	1	604.7			604.4		
Cleavage of K^2–T^3peptide bond,(T^3–M^{10})	1	856.1					855.3
Oxidation of Met → sulfone, decarboxylation of D4							

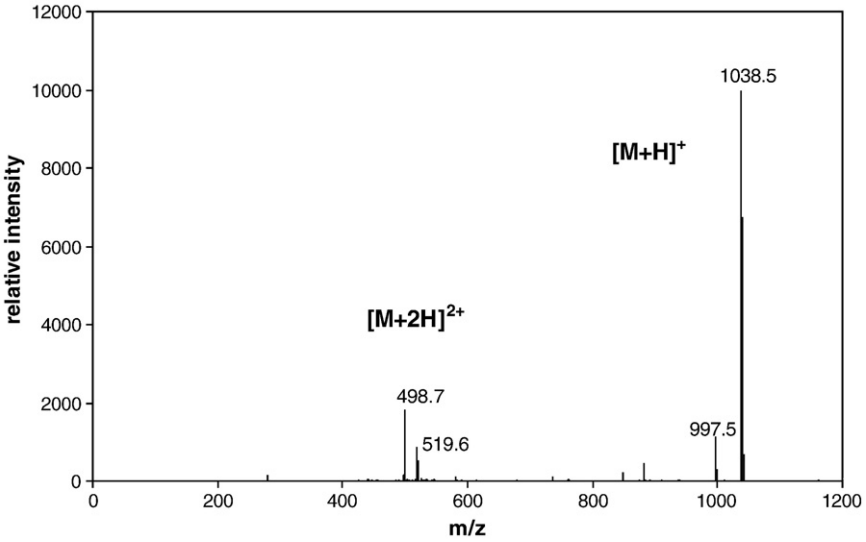


Fig. 7. Mass spectrum of the LC fraction eluted at 13.0 min for the Cu(II)–NKA–H_2O_2 1:1:1 molar ratio system after 24 h incubation. Solution contained 0.1 M phosphate buffer (pH 7.4); concentration of the neurokinin A, Cu(II) and hydrogen peroxide 0.50 mM. Incubation at 37 °C.

rise in the levels of brain copper from youth to adulthood [75]. It is well-understood that the ability of metal ions, for example copper, to accept and donate electrons can lead to radical formation, reactive oxygen species (ROS) and oxidative attack of tissue components contributing to disease and perhaps aging itself [76,77].

Acknowledgment

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

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