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Coordination Abilities of a Fragment Containing D^1 and H^{12} Residues of Neuropeptide γ and Products of Metal-Catalyzed Oxidation

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Stoichiometry, stability constants, and solution structures of copper(II) complexes of the (1−2,10−21)NPγ ($D^1-A^2-K^{10}-R-H^{12}-K-T-D-S-F-V-G-L-M^{21}-NH_2$) and Ac−(1−2,10−21)NP$\gamma$ ($Ac-D^1-A^2-K^{10}-R-H^{12}-K-T-D-S-F-V-G-L-M^{21}-NH_2$) fragments of neuropeptide γ were determined in aqueous solution in the pH range 2.5−10.5. The potentiometric and spectroscopic data (UV−vis, CD, EPR) show that an N-terminal Asp residue stabilizes significantly the copper(II) complexes with 1N {NH_2, β-COO^-} and 2N {NH_2, β-COO^-, N_{Im}} coordination modes of the (1−2,10−21)NPγ as the result of coordination through the β-carboxylate group. In a wide pH range of 4−9, the imidazole nitrogen of His[12] is coordinated to form a macrochelate. The (1−2,10−21)NPγ peptide consists of 14 amino acid residues and contains an N-terminal amine group and the histidine residue, and as it is suggested, this fragment is able to bind two equivalents of copper(II) ions. The postmortem studies support the involvement of oxidative stress and the production of reactive oxygen species in neurodegenerative diseases. The susceptibility of proteins to oxidative damage is highly dependent on the specific properties of individual proteins, such as unique sequence motifs, surface accessibility, protein folding, and subcelluar localization. Metal-catalyzed oxidation of proteins is mainly a site-specific process in which one or a few amino acids at metal-binding sites on the protein are preferentially oxidized. To elucidate the products of the copper(II)-catalyzed oxidation of the (1−2,10−21)NPγ and Ac-(1−2,10−21)NPγ fragments of neuropeptide γ, the liquid chromatography−mass spectrometry method and the use of Cu(II)/hydrogen peroxide as a model oxidizing system were employed. For both peptides, the oxidation of the methionine residue to methionine sulfoxide for the solutions containing peptide−hydrogen peroxide was observed. The oxidations of the histidine to 2-oxo-histidine and the methionine sulfoxide to sulfone were detected for the Cu(II)−Ac-(1−2,10−21)NPγ−hydrogen peroxide 1:1:4 molar ratio system. Fragmentations of both peptides near the His residue were observed, supporting the participation of this (His) residue in the coordination of the copper(II) ions.

1. Introduction

Tachykinin peptides are a family of peptides that share the common amino acid sequence of Phe−X−Gly−Leu−Met−NH_2, where X is usually Val or Phe in their C-terminal region.[1] These peptides induce various biological responses such as salivation; vasodilation; hypotension; stimulation of certain immune cells; and contraction of gastrointestinal, respiratory, and urinary smooth muscle.[2−7] A single gene called preprotachykinin A encodes most of these peptides and produces the precursor proteins designated as α-, β-, γ-, and δ-preprotachykinin.[8,9] γ-Preprotachykinin A contains the sequence of neuropeptide γ (NPγ) which is a 21-residue peptide. Neuropeptide γ is an amino terminally extended form of neurokinin A (NKA) and has the sequence Asp−Ala−Gly−His−Gly−Gln−Ile−Ser−His−Lys−Arg−His−Lys−Thr−Asp−Ser−Phe−Val−Gly−Leu−Met−NH_2. NPγ was first isolated from a rabbit intestine[10] and possesses a higher affinity toward the NK-2 receptor of tachykinin than neurokinin A—a "classical" mammalian agonist of this receptor. Nuclear magnetic resonance (NMR) studies have been reported on NPγ in dimethyl sulfoxide (DMSO),[11] in a

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50% trifluorethanol (TFE)/water mixture, and in 200 mM sodium dodecyl sulfate (SDS) micelles.[12] The effect of calcium ions on the conformation of NPγ was also studied using CD spectropolarimetry.[13] The calcium bound conformation of tachykinins has been suggested to be required for receptor activation.[14] The structure–activity relationship of neuropeptide γ derived from mammalian and fish tissue was studied,[15] in which was indicated a critical role of the α-helical structure for receptor binding.

There are many studies that have detailed the alterations in tachykinin levels in a number of neurodegenerative conditions.[16] Substance P, neurokinin A, neuropeptide K, and neurokinin B were measured in both control (neurologically normal) and Huntington's disease brains obtained postmortem. All peptides were significantly reduced in the substantia nigra of Huntington's disease patients compared with the control group.[17] The dominant risk factor associated with neurodegenerative diseases is increasing age. Several studies in animals and humans have reported a rise in the levels of brain copper from youth to adulthood.[18] It is well-understood that the ability of metal ions, for example, Cu and Fe, to accept and donate electrons can lead to radical formation, reactive nitrogen and oxygen species (all termed "ROS"), and oxidative attack of tissue components contributing to disease and perhaps aging itself.[19] One of the major pathological features of a neurodegenerative brain is the presence of extensive oxidative stress, including elevated levels of protein carbonyls, 8-hydroxyguanine, and lipid peroxidation.[20]

The present paper reports the results of combined spectroscopic (UV–vis, CD, and EPR) and potentiometric studies on the copper(II) complexes of the (1–2,10–21)NPγ and Ac-(1–2,10–21)NPγ fragments of neuropeptide γ (NPγ). The fragments studied here are D^1–A^2–K^{10}–R–H^{12}–K–T–D–S–F–V–G–L–M^{21}–NH_2 [(1–2,10–21)NPγ] and Ac–D^1–A^2–K^{10}–R–H^{12}–K–T–D–S–F–V–G–L–M^{21}–NH_2 [Ac-(1–2,10–21)NPγ]. The products of the copper(II)-catalyzed oxidation process for the studied fragments of neuropeptide γ in the presence of hydrogen peroxide at pH 7.4 were investigated. This study was performed in order to determine coordination abilities of the neuropeptide γ fragments and the relationship between the binding mode and the products of metal-catalyzed oxidation. Now, we start to study the interaction of copper(II) ions with neuropeptide γ containing the three histidine residues (D^1–A^2–G–**H^4**–G–Q–I–S–**H^9**–K^{10}–R–**H^{12}**–K–T–D–S–F–V–G–L–M^{21}–NH_2), and at the beginning we studied the fragments of this peptide. These studies may be useful in the interpretations of the results obtained for more complicated systems.

2. Material and Methods

2.1. Synthesis of the Peptides. Synthesis of peptide amides, D^1–A^2–K^{10}–R^{11}–H^{12}–K–T–D–S–F–V–G–L–M^{21}–NH_2 [(1–2,10–21)NPγ] and Ac-D^1–A^2–K^{10}–R–H^{12}–K–T–D–S–F–V–G–L–M^{21}–NH_2 [Ac-(1–2,10–21)NPγ], was performed on a solid phase using the Fmoc strategy with the continuous-flow methodology (9050 Plus Millipore Peptide Synthesizer) on a polystyrene/polyethylene glycol copolymer resin (TentaGel R RAM Resin, substitution 0.18 mmol/g).[21] Acetylation of the N-terminal amino group was performed on the resin using 1 M acetylimidazole in DMF. All peptides were cleaved from the resin and deprotected by 2 h of shaking in a mixture containing trifluoroacetic acid, phenol, triisopropylsilane, and water (88:5:2:5, v/v).

The resulting crude peptides were purified in portions by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C_8 semipreparative Kromasil column (25 × 250 mm, 7 μm). A linear gradient of 0–35%, 0–50%, or 10–50% B was usually applied in the course of purification, where A used 0.1% aqueous trifluoroacetic acid (TFA) and B used 80% acetonitrile (ACN)-H_2O + 0.1% TFA. In some more challenging cases, an isocratic mode was utilized 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, a 30 min linear gradient of 5–100% B was used (A and B defined identically as in preparative chromatography).

Analytical data were as follows: for (1–2, 10–21)NPγ, R_t = 14.9 min (Kromasil), MS = 1603.1 $[M]^+$, M_{calc} = 1603.8; for Ac-(1–2,10–21)NPγ, 12.2 min (XTerra), MS = 1645.9 $[M]^+$, M_{calc} = 1645.9.

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 1.5–2 cm^3. Alkali was added from a 0.250 cm^3 micrometer syringe which was calibrated by both weight titration and the titration of standard materials. Experimental details: ligand concentration, 1.0×10^{-3} M; metal to ligand molar ratio, 1:1.1 for both peptides and 2:1 for (1–2,10–21)NPγ; 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,[22] number of titrations = 2; method of calculation, SUPERQUAD[23] and HYPERQUAD.[24] The samples were titrated in the pH region 2.5–10.5. Standard deviations (values) quoted were computed by SUPERQUAD and HYPERQUAD 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. 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 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

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Table 1. Formation Constants (log β_{pqr}) and Protonation Constants (log *K*) of the (1−2,10−21)NPγ Fragment of Neuropeptide γ, Its N-Acethyl Derivative, and Comparable Peptides (T = 298 K, I = 0.10 M KNO_3)

log β_{0qr}	HL	H_2L	H_3L	H_4L	H_5L	H_6L	H_7L	H_8L
(1−2,10−21)NPγ	**10.88 ± 0.01**	**20.83 ± 0.01**	**30.00 ± 0.01**	**37.41 ± 0.01**	**43.55 ± 0.01**	**47.22 ± 0.01**	**49.75 ± 0.01**	
1−10H[a]	9.87	17.59	24.02	28.35	31.86	34.54		
G_5H[b]	8.00	14.87	17.73					
DAAA[c]	7.69	11.29	14.36					
Ac-(1−2,10−21)NPγ	**10.74 ± 0.01**	**20.41 ± 0.01**	**26.77 ± 0.01**	**30.73 ± 0.01**	**33.84 ± 0.01**			
ScrHuPrPAc-106−126NH_2[d]	10.24	19.85	26.28					
H2B63−93[e]	9.83	19.09	25.32	30.14	34.51	38.39	41.88	
Ac-M^{29}-D^{30}-56[f]	11.04	21.34	31.26	40.33	46.92	51.79	55.88	59.29

log *K*	NH_2-Lys, NH_2-Lys, succinimide	O^--Tyr	NH_2	N_{Im}	COO^-	COO^-	COO^-	COO^-
(1−2,10−21)NPγ	**10.88; 9.95; 9.17**		**7.41**	**6.14**	**3.67**	**2.53**		
1−10H		9.87	7.72	6.43	4.33	3.51	2.68	
G_5H			8.00	6.87	2.86			
DAAA			7.69		3.60	3.07		
Ac-(1−2,10−21)NPγ	**10.74; 9.67**			**6.36**	**3.96**	**3.11**		
ScrHuPrPAc-106−126NH_2	10.24, 9.61			6.43				
H2B63−93	9.83	9.26		6.23	4.82	4.37	3.88	3.49
Ac-M^{29}-D^{30}-56	11.04, 10.30, 9.92	9.07		6.59	4.87	4.09	3.41	

[a] Ref 51, DAEFR**H**DSGY-NH_2. [b] Ref 50, GGGGG**H**. [c] Ref 35. [d] Ref 26, Ac-NGAKALMGG**H**GATKVMVGAAA-NH_2. [e] Ref 25, Ac-NSFVNDIFERIAGEASRLA**H**YNKRSTITSRE-NH_2. [f] Ref 46, Ac-$M^{29}D^{30}$GKTKEGVLZVGSKTKEGVV**H**$^{\mathbf{50}}$GVATVA^{56}-NH_2.

NMR gaussmeter and the HP 5350B Hewlett-Packard microwave frequency counter at the X-band frequency (~9.45 GHz). The spectra were analyzed by using Bruker's WIN-EPR SimFonia software, version 1.25. Copper(II) stock solution was prepared from $Cu(NO_3)_2 \times 3\ H_2O$.

2.4. 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 ethylenediaminetetraacetic acid (EDTA) and $Cu(NO_3)_2$ were purchased from POCH. Stock solutions (0.10 M) of EDTA and hydrogen peroxide in a phosphate buffer (pH 7.4) were prepared.

2.5. Oxidation of the Fragments of Neuropeptide γ and 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 (0.5 cm^3) containing 5×10^{-4} M peptide and a metal to ligand molar ratio of 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 a metal to hydrogen peroxide molar ratio of 1:1 for the (1−2,10−21)NPγ peptide and 1:4 for Ac-(1−2,10−21)NPγ. 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 at an EDTA molar ratio of 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 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. The 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 were acquired and analyzed using LC Solution sofware provided by Shimadzu.

3. Results and Discussion

3.1. Protonation Constants. The C-termini of the peptides were blocked by amidation. The protonation constants (log *K*) of the peptides were calculated by means of potentiometric titrations and are presented in Table 1. These peptide ligands have seven (H_7L) and five (H_5L) protonation sites for the (1−2,10−21)NPγ and Ac-(1−2,10−21)NPγ, respectively. The molecules contain two lysyl residues (K^{10}, K^{13}) and one histidyl residue (H^{12}). From the studies on peptides, it is evident that the high log *K* values (range, 10.88−9.17) can be assigned to the deprotonation of the lysyl ammonium groups.[25,26] The protonations of the two lysyl residues for (1−2,10−21)NPγ and the one for Ac-(1−2,10−21)NPγ, likely because of different conformations of these fragments, take place in overlapping processes, and none of the log *K*(Lys) values can be assigned to any specific amino acids in the sequence. The protonations of these lysyl residues overlap, likely, with the hydrolyze of a five-membered succinimide ring formed between Asp and Ser residues in the HKTDSFVGLM sequence, as is suggested for neurokinin A.[27,28]

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Table 2. Stability Constants (log β_{pqr}) of the Copper(II) Complexes of the Fragments of Neuropeptide γ and Comparable Peptides (T = 298 K, I = 0.10 M KNO_3)

peptide/species/log β	CuH_4L	CuH_3L	CuH_2L	CuHL	CuL	$CuH_{-1}L$	$CuH_{-2}L$	$CuH_{-3}L$
(1−2,10−21)NPγ	**42.22 ± 0.02**	**37.82 ± 0.01**	**31.52 ± 0.01**	**23.72 ± 0.02**	**15.46 ± 0.02**	**6.54 ± 0.02**	**−3.40 ± 0.02**	**−14.06 ± 0.03**
1−10H[a]			23.15	18.11	12.02	3.60	−5.35	−15.48
G_5H[b]				12.29	8.11	2.63	−5.06	−15.67
DAAA[c]					6.04	−0.34	−7.82	−16.88
Ac-(1−2,10−21)NPγ			**24.44 ± 0.01**	**17.94 ± 0.02**	**12.12 ± 0.01**	**3.84 ± 0.01**	**−5.58 ± 0.01**	**−16.01 ± 0.01**
ScrHuPrPAc-106−126NH_2[d]			23.52		11.75	4.40	−5.13	−15.51
H2B63−93[e]			23.74		11.23	2.95	−6.81	−16.25
Ac-M^{29}-D^{30}-56[f]	44.01	37.24	30.76	22.23	12.91	3.11	−7.30	−17.99

binuclear complexes	Cu_2L	$Cu_2H_{-1}L$	$Cu_2H_{-2}L$	$Cu_2H_{-3}L$	$Cu_2H_{-4}L$	$Cu_2H_{-5}L$	$Cu_2H_{-6}L$
(1−2,10−21)NPγ	**21.62 ± 0.04**	**14.68 ± 0.03**	**7.47 ± 0.02**	**−1.04 ± 0.03**	**−10.48 ± 0.04**	**−20.39 ± 0.05**	**−30.72 ± 0.04**

[a, b, c, d, e, f] References and sequence of the peptides as in Table 1.

Table 3. Calculated log K^{*a} Values for Mononuclear Cu(II) Complexes with (1−2,10−21)NPγ and Ac-(1−2,10−21)NPγ Fragments of Neuropeptide γ and Comparable Ligands

coordination mode, log K^{*a}	1N {NH_2, CO or COO^-, or N_{Im}}	2N {NH_2, CO or COO^-, N_{Im}}	3N {NH_2, N^-,CO, N_{Im}}	4N {NH_2, 2N^-, N_{Im}}	4N{NH_2, 3N^-}
(1−2,10−21)NPγ	**−1.33**	**−5.73**	**−12.03**	**−19.83**	**−21.95**
1−10H	−0.87	−5.91	−12.00	−20.42	−22.94
G_5H	−2.58	−6.76	−12.24	−19.93	−23.67
DAAA	−1.65				

log K^{*a}, coordination mode	1N {N_{Im}}	2N {N_{Im}, N^-}	3N{N_{Im},2N^-}	4N{N_{Im},3N^-}
Ac-(1−2,10−21)NPγ	**−2.33**	**−8.83**	**−14.65**	**−22.93**
ScrHuPrPAc-106−126NH_2	−2.76		−14.53	−21.88
H2B63−93	−1.58		−14.09	−22.37
Ac-M^{29}-D^{30}-56	−2.91	−9.68	−16.16	−24.69

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

Proteins are subject to a variety of spontaneous degradation reactions under physiological conditions that can limit their lifetime.[29,30] Aspartyl and asparaginyl residues are particularly susceptible to nonenzymatic degradation because intramolecular succinimide-forming reactions at these sites lead to their isomerization, racemization and, in the case of asparaginyl residues, deamidation and cleavage as well.[31−33] The post-translational modification spontaneously occurs under physiological conditions where the rate is affected by both its amino acid sequence and three-dimensional structure.[34]

For the (1−2,10−21)NPγ peptide with a free N-terminal amino group, the protonation constant log K = 7.41 (Table 1) corresponds very well to protonation of the amino nitrogen, and this value agrees with those for the peptides containing the aspartic acid residue in the N-terminal position.[35,36] The next protonation constant observed for the ligands studied, log K = 6.36 and 6.14, corresponds to protonation of the imidazole nitrogen of the His residue, and these values are comparable with literature values.[37−39] The log K values of the protonations of carboxylate functions of Asp side chains are found to be in the range of 2.53 to 3.96 (Table 1).[35,40]

Table 4. Calculated Deprotonation Constants for Amide Protons (pK) in Copper(II) Complexes

peptide	pK_1	pK_2	pK_3
(1−2,10−21)NPγ	**6.30**	**7.80**	**8.26**
1−10H	6.09	8.42	8.95
G_5H	5.48	7.69	10.61
DAAA	6.38	7.48	9.06
Ac-(1−2,10−21)NPγ	**6.5**	**5.82**	**8.28**
Scr-HuPrPAc-106−126NH_2			7.35
H2B63−93			9.04
Ac-M^{29}-D^{30}-56	6.77	6.48	8.53

3.2. Copper(II) Complexes of the Fragments of Neuropeptide γ. Potentiometry detects a range of Cu(II) complexes with the formation constants reported in Table 2. Table 3 contains the values of log K^*, the protonation corrected stability constants which are useful to compare the ability of various ligands to bind a metal ion.[41,42]

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Table 5. Spectroscopic Parameters of the Major Species Formed in the Copper(II)−(1−2,10−21)NPγ and Cu(II)−Ac-(1−2,10−21)NPγ Systems

	UV−vis		CD		EPR	
ligand/species	λ (nm)	ε ($M^{-1}cm^{-1}$)	λ (nm)	Δε ($M^{-1}cm^{-1}$)	A_{II} (G)	g_{II}
(1−2,10−21)NPγ Mononuclear Complexes						
CuH_3L pH 5.5 {NH_2, COO^-, N_{Im}}	678[a]	38	760[a] 628[a] 384[b]	+0.071 −0.123 −0.020	133	2.301
CuH_2L pH 7.0 {NH_2, N^-, CO, N_{Im}}	610[a]	121	670[a] 502[a] 314bb[b,c]	−0.078 −0.205 +0.665	150	2.239
CuL, $CuH_{-1}L$ $CuH_{-2}L$, $CuH_{-3}L$ {NH_2, $3N^-$} pH 10	507[a]	200	643[a] 506[a] 309[c]	+0.121 −1.056 +0.828	212	2.178
(1−2,10−21)NPγ Dinuclear Complexes						
Cu_2L pH 6.8 {NH_2, N^- and N_{Im}, $2N^-$} or {NH_2, $2N^-$ and N_{Im}, N^-}	637[a]	29	632[a] 485[a] 354[b] 312[c]	+0.081 −0.145 −0.023 +0.407		
$Cu_2H_{-2}L$ pH 7.8 {NH_2, $2N^-$and N_{Im}, $3N^-$} or {NH_2, $3N^-$and N_{Im}, $2N^-$}	571[a]	129	630[a] 488[a] 353[b] 313[c]	+0.312 −0.465 −0.137 +0.566		
$Cu_2H_{-3}L$, Cu_2 $H_{-4}L$, pH 9.3 $Cu_2H_{-5}L$, $Cu_2H_{-6}L$ {NH_2, $3N^-$ and N_{Im}, $3N^-$} pH 9.3	510−550bb[a]	150	632[a] 491[a] 353[b] 313[c]	+0.406 −0.650 −0.182 +0.557		
Ac-(1−2,10−21)NPγ						
CuH_2L pH = 5.5 {N_{Im}}	756[a]	17			155	2.324
CuL pH 7.0 {N_{Im}, $2N^-$}	572[a]	106	534[a] 338[b]	+0.416 −0.718	170 seven (7) lines	2.233
$CuH_{-1}L$, $CuH_{-2}L$ $CuH_{-3}L$ pH 9.0 {N_{Im}, $3N^-$}	540[a]	122	630[a] 490[a] 351[b] 310[c]	+0.808 −1.297 −0.314 +0.977	194 nine (9) lines	2.190

[a] d−d transition. [b] N(Im) → Cu(II) charge transfer transition. [c] N^-(amide) → Cu(II) charge transfer transition, bb − broad band.

Tables 4 and 5 contain the calculated deprotonation constants for amide protons (p*K*) in Cu(II) complexes and spectroscopic properties of major complexes, respectively. Tables 1S and 2S and Figures 1Sa, 1Sb, 1Sc, 2S, and 3S are given in the Supporting Information.

The Ac-(1−2,10−21)NPγ fragment of neuropeptide γ contains only one His[12] anchoring site for copper(II) ions. Therefore, the mononuclear 1:1 complexes are dominated in the solution.[26,43,44] Six metal complex species can be fitted to the experimantal titration curves obtained for the Cu(II)−Ac-(1−2,10−21)NPγ system: CuH_2L, CuHL, CuL, $CuH_{-1}L$, $CuH_{-2}L$, and $CuH_{-3}L$ (charges omitted for simplicity, Table 2). Copper(II) ions start binding to the imidazole of the His residue at pH 4 to form the monodentate species CuH_2L, as is shown in the species distribution diagram (Figure 1). The d−d transition energy at 756 nm and the EPR parameters A_{II} = 155 G and g_{II} = 2.324 are in good agreement with the 1N {N_{Im}} coordination mode.[45,46] The CuH_2L complex loses the proton to give a CuHL species which cannot be detected spectroscopically because of its low concentration and overlap with the CuH_2L and CuL species. With an increasing of the pH above 5.7, the CuL complex is formed with the 3N {N_{Im}, $2N^-_{am}$} coordination mode. This bonding mode is easily seen in the CD, EPR, and absorption spectra (Table 5). The presence of the N_{Im} → Cu(II) charge transfer transition at 338 nm, the d−d energy at 572 nm (Figure 1Sa, Supporting Information), and EPR parameters A_{II} = 170 G and g_{II} = 2.233 correspond to a three nitrogen bonding mode.[25,26,47,48] The appearance of a seven line superhyperfine splitting pattern in the EPR spectrum (data not shown) suggests a 3N coordination mode with three nitrogen atoms placed in an equatorial plane around the copper(II) ion. The CuL complex is present in the solution in a wide 5.5−9.5 pH range (Figure 1). The value of log *K** for the 3N

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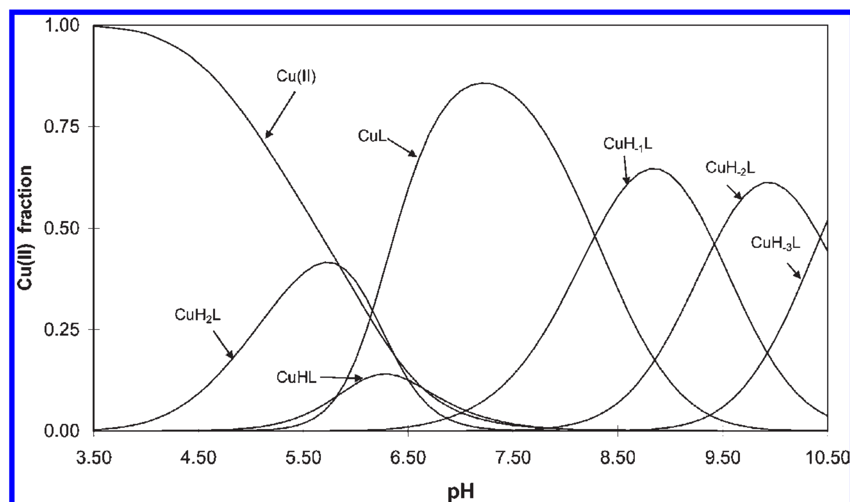

Figure 1. Species distribution curves for Cu(II) complexes of the Ac-(1−2,10−21)NPγ fragment of neuropeptide γ. Cu(II) to peptide molar ratio, 1:1; [Cu(II)] = 0.001 M.

complex of the N-acetylated derivative studied is about 1.5 log units higher compared to those of Ac-M^{29}-D^{30}-56[46] and Ac-GGGH[49], but this value is comparable to those of ScrHuPrPAc-106−126NH_2[26] and H2B63−93 (Table 3).[25] Above pH 7, the CuL complex loses the next amide proton to give a $CuH_{-1}L$ species which has four nitrogen donor centers $\{N_{Im}, 3N^{-}{}_{am}\}$ arranged in a square planar geometry around the central Cu(II) ion (Figure 1). The d−d transition energy at 540 nm and the presence in the CD spectra of the charge transfer transitions $N_{Im} \rightarrow$ Cu(II) and $N_{am}{}^{-} \rightarrow$ Cu(II) at 351 and 310 nm (Figure 1Sa, Supporting Information), respectively, and EPR parameters A_{II} = 194 G and g_{II} = 2.190, suggest the 4N $\{N_{Im}, 3N^{-}{}_{am}\}$ coordination mode (Table 5). The log K^* value calculated for the complex of the ligand studied is about 1.5−1.7 orders of magnitude higher compared to those of Ac-GGGH[49] and Ac-M^{29}-D^{30}-56[46], but this value is comparable to that of the H2B63−93 peptide.[25] In the system studied, the imidazole nitrogen acts as the anchoring group, while three amide nitrogens, i.e., those of His, Arg, and Lys residues, complete the coordination of the copper(II) ion. From pH 9 to 10.5, CD spectra, UV−vis, and EPR parameters are similar to each other, supporting no variation in the coordination mode during the next deprotonations. The deprotonation constants (pK values) $CuH_{-1}L \rightarrow CuH_{-2}L \rightarrow CuH_{-3}L$ equal 9.42 and 10.43 (Table 2) and may correspond to deprotonations of the lysine side chain and the hydrolysis of the succinimide ring (Table 1).

The stability constants of the Cu(II) complexes of the (1−2,10−21)NPγ peptide have been determined by potentiometric titrations by using two different metal-to-ligand ratios (M/L, 1:1 and 2:1). The precipitation of copper(II) hydroxide was never observed at any studied M/L ratios or pH values; therefore, this suggests the formation of mono- and binuclear complexes. This indicates that the number of Cu(II) ions that are bonded by the peptide is equal to the number of anchoring sites that are present in the molecule (N-terminal amine and imidazole nitrogen of the His[12] residue). The stability constants for this system can be calculated by using the HYPERQUAD computer program.[24] To check the computational models, spectroscopic techniques were applied (UV−vis, CD, and EPR spectroscopies). The best fitting of the potentiometric data was obtained by invoking the species listed in Table 2, and the corresponding speciation curves at two different metal-to-ligand ratios are plotted in Figure 2a and b. This model and corresponding speciation curves are in good agreement with the spectroscopic measurements. It is seen from Table 2 that both mono- and dinuclear species can exist in different protonation stages. It is caused by the presence of two lysyl residues and hydrolysis of the succinimide ring formed between Asp−Ser residues. The amine groups of the lysines are not metal-binding sites at any pH value, and their deprotonations reaction takes place in the same pH range as reported for the free ligand (above pH 9). From the speciation curves it is well seen that for the 2:1 metal-to-ligand solution the mononuclear complexes in an amount of 5 to 40% are present to a pH of 7 (Figure 2b).

For the 1:1 metal-to-ligand molar ratio, three complexes (the CuH_3L and CuH_2L and with the same coordination modes the CuL, $CuH_{-1}L$, $CuH_{-2}L$, and $CuH_{-3}L$) may be characterized by spectroscopy. The coordination of the metal ion starts at a pH around 3.5, and the CuH_4L complex is formed. The value of log K^* for this complex is higher by about 1.2 log units compared to that of G_5H,[50] but this value is comparable to those of DAAA[35] and 1−10H[51] (Table 3). This stabilization of 1N species results from the bonding of the β-carboxylate oxygen of the Asp[1] residue in the coordination plane, forming a six-membered chelate ring, and it may suggest the 1N $\{NH_2,\beta\text{-}COO^{-}\}$ coordination mode (Scheme 1). The stabilization of the Cu(II) complexes for the peptides containing the Asp residue in different positions of the peptide chain is well documented.[35,52] With increasing pH, the CuH_3L complex is formed with a maximum concentration at pH 5.5 (Figure 2a). The pK value for the deprotonation of the CuH_4L is 4.4 (Table 2), and this

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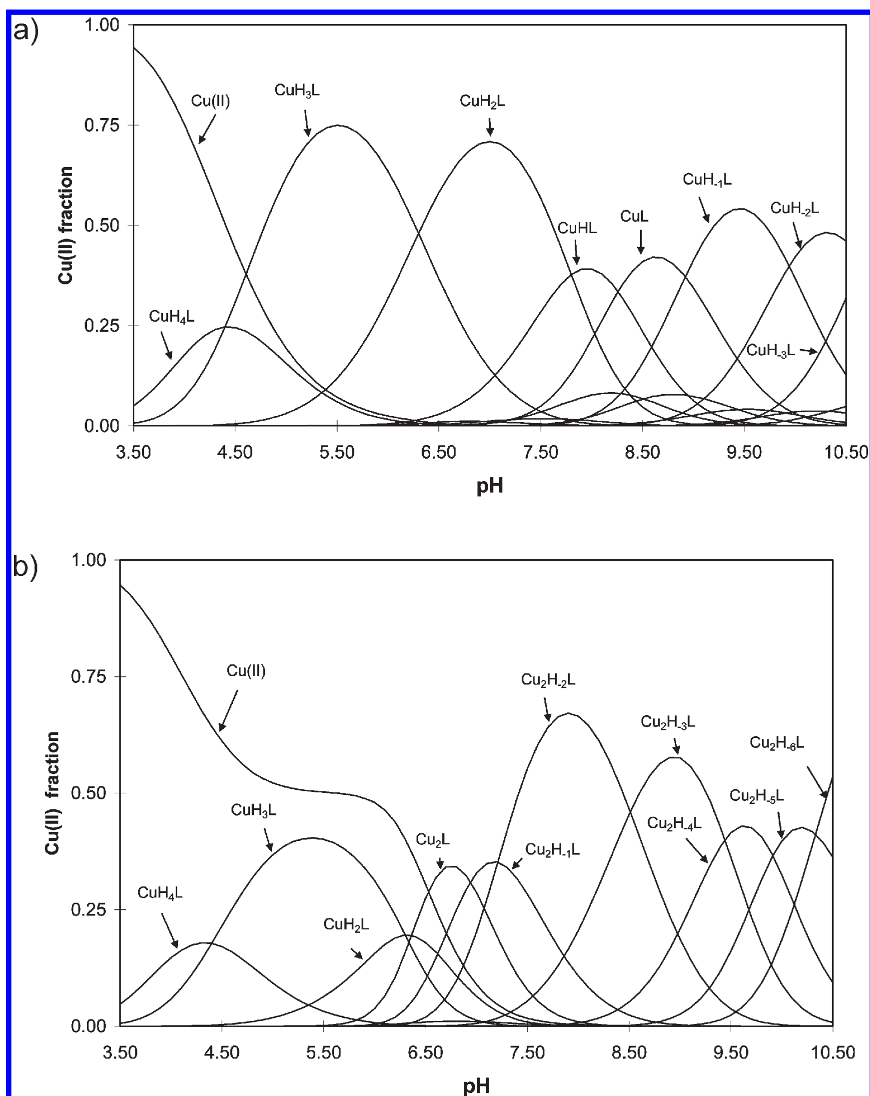

Figure 2. Species distribution of the complexes formed in the Cu(II)−(1−2,10−21)NPγ system at (a) 1:1 and (b) 2:1 Cu(II)-to-ligand ratios. [Cu(II)] = 0.001 M for the 1:1 molar ratio and [Cu(II)] = 0.002 M for the 2:1 molar ratio.

value may correspond to the deprotonation and the coordination of the imidazole nitrogen to the Cu(II) ion.[50,53] The d−d transition energy at 678 nm, the presence in CD spectra of a $N_{Im} \rightarrow$ Cu(II) charge transfer transition at 384 nm, and the EPR parameters A_{II} = 133 G and g_{II} = 2.301 (Figure 3a) suggst the 2N {NH_2,β-COO^-, N_{Im}} coordination mode (Table 5). The low value of A_{II} (133 G) for the 2N copper(II) complex may reflect distortion of the complex plane expected when a macrochelate ring is formed (Scheme 1).[50,51,54] The log K^* value for the CuH_3L complex is higher by about 1 order of magnitude in comparison to that of G_5H, but it is comparable to that of 1−10H, suggesting the coordination of the β-carboxylate group of Asp[1] in this complex (Table 3). With an increasing of the pH, the CuH_2L complex is formed after deprotonation of the first amide nitrogen. The pK_1 value (deprotonation constant of the first amide nitrogen) for the ligand studied is comparable to those of 1−10H and DAAA, but it is about 1 order of magnitude higher in comparison to that of G_5H, supporting the coordination of the β-carboxylate group of the Asp[1] residue in the CuH_3L complex (Table 4). The spectroscopic data for the CuH_2L species, the d−d transition energy at 610 nm, the EPR parameters A_{II} = 150 G and g_{II} =2.239 (Figure 3b), and the presence in CD spectra of the $N_{am}^- \rightarrow$ Cu(II) charge transfer transition at 314 nm suggest the 3N {NH_2, N_{am}^-, CO, N_{Im}} complex with a macrochelate ring (Table 5, Scheme 1, Figure 1Sb, Supporting Information). It should be mentioned that for a 1:1 metal-to-ligand molar ratio when two anchoring sites in the peptide exist (N-terminal amine group and imidazole of His residue), the coordination isomers cannot be excluded, especially at high pH. The next CuHL and CuL complexes are formed in the subsequent deprotonation and coordination to copper(II) ions of second and third amide nitrogens with pK_2 = 7.80 and pK_3 = 8.26 values (Table 4). At a pH above 9, the UV−vis, CD, and EPR spectra are similar to each other, indicating the same coordination mode of the ligand in the CuL, $CuH_{-1}L$, $CuH_{-2}L$, and $CuH_{-3}L$ complexes. The EPR parameters A_{II} = 212 G and g_{II} =2.178 (Figure 3c), the

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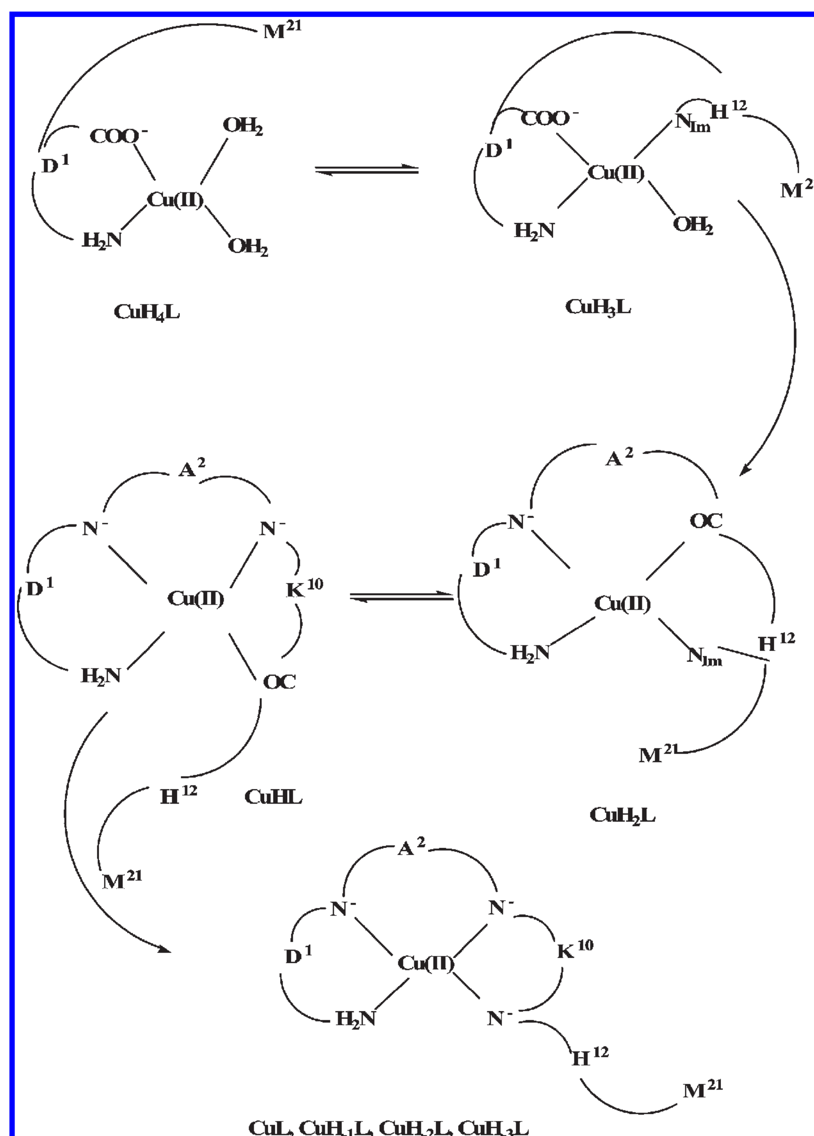
Scheme 1. Schematic Representation of the Complexes Formed in a Cu(II)−(1-2,10-21)NPγ 1:1 System with Increasing pH



d−d transition energy of 507 nm, and in CD spectra the presence of the $N_{am}^- \rightarrow$ Cu(II) charge transfer transition at 309 nm correspond to a four nitrogen $\{NH_2, 3N_{am}^-\}$ bonding mode (Scheme 1, Figure 1Sb, Supporting Information). The log K^* values for the 3N $\{NH_2, N_{am}^-, CO, N_{Im}\}$ and 4N $\{NH_2, 2N_{am}^-, N_{Im}\}$ species are similar to those of 1−10H and G_5H, supporting the same coordination modes in the complexes formed (Table 3). The (1−2,10−21)NPγ peptide forms the 4N $\{NH_2, 3N_{am}^-\}$ complex, which is more stable in comparison to those of the 1−10H and G_5H by about 1 and 1.7 orders of magnitude (Table 3). This stabilization may result from highly organized structures of peptide in copper(II) complexes.[41,55,56]

The polynuclear complexes can be obtained if anchoring sites (NH_2, amine and histidyl residues) are well separated binding sites of a large peptide fragment of native proteins. In these systems, the copper(II) ions can be coordinated independently from each other by three or four nitrogen atoms of the peptide backbone.[37,40,57]

Potentiometric titration curves reveal that (1−2, 10−21)NPγ can keep 2 equiv of copper(II) ions in solution, and precipitation was not observed at any pH values at the metal-to-ligand molar ratios of 1:1 and 2:1. This is indirect proof of the formation of polynuclear species in solution, and both computer calculation of potentiometric data and spectroscopic measurements support this assumption. It is clear from Figure 2b that the formation of the dinuclear complexes starts above pH 6 in parallel with the involvement of amide nitrogen atoms in metal binding as it was observed for similar systems.[37,58−61] For the studied solutions up to pH 6.5,

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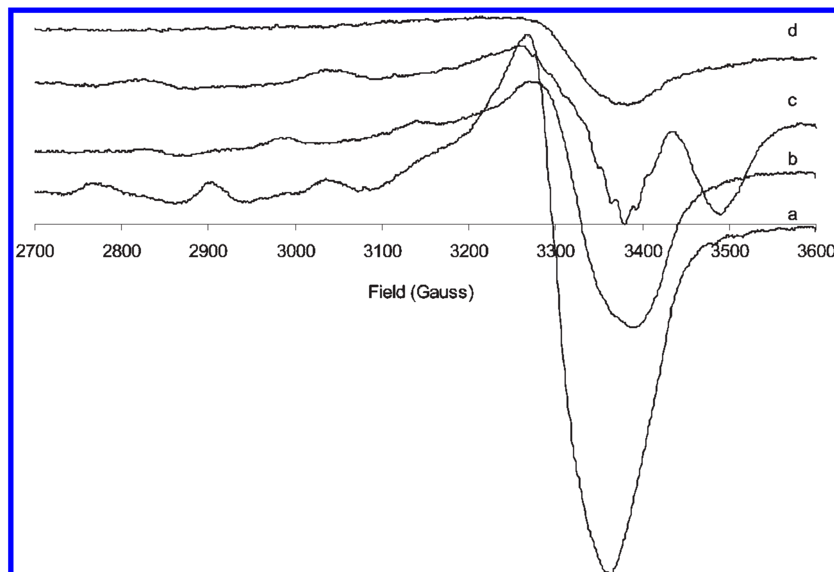
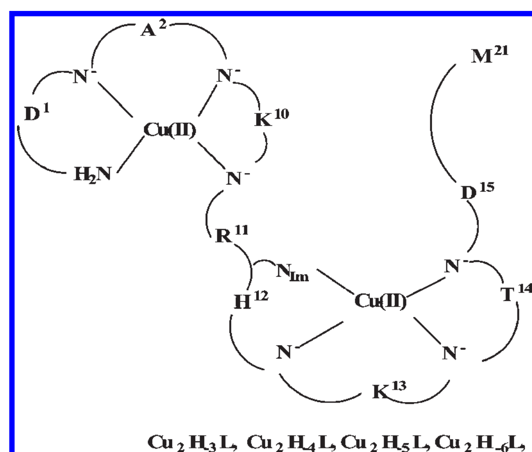


Figure 3. The X-band anisotropic EPR spectra of the Cu(II)−(1−2,10−21)NPγ system with a 1:1.1 metal-to-ligand molar ratio at different pH's: 5.5 (a), 6.5 (b), and 10.0 (c) and a 2:1 metal-to-ligand molar ratio at a pH range of 7−10.5 (d).

Scheme 2. The Binding Sites of the $Cu_2H_{-3}L$ to $Cu_2H_{-6}L$ Species Formed in the 2:1 Copper(II)−(1-2,10-21)NPγ System



the EPR parameters A_{II} 122 G and g_{II} =2.415 clearly support the formation of the aqua complex of copper(II) ions with a major 50% concentration (Figure 2b). The stoichiometry of the first measurable dinuclear complex is $Cu_2H_{-2}L$, suggesting that the binding of two copper(II) ions requires the involvement of amide functionalities for both metal ions. The real stoichiometry of $Cu_2H_{-2}L$ is $Cu_2H_{-5}LH_3$ containing $\{NH_2, 2N^-\}\{N_{Im}, 3N^-\}$ or $\{NH_2, 3N^-\}\{N_{Im}, 2N^-\}$ coordination modes of the ligand around the each copper(II) ion. The next dinuclear complexes $Cu_2H_{-3}L$, $Cu_2H_{-4}L$, $Cu_2H_{-5}L$, and $Cu_2H_{-6}L$ are formed with an increasing of pH, and the metal binding sites for these species are similar and can be easily obtained from superposition of the $\{NH_2, 3N^-\}$ and $\{N_{Im}, 3N^-\}$ coordination modes starting from the NH_2-terminal and N_{Im} of His[12] to the C-termini of the peptide, respectively (Scheme 2). It should be mentioned that measurable CD activity of the samples of d−d transitions can be recorded only above pH ~ 6 (Figure 1Sc, Supporting Information). This implicates the involvement of peptide amide groups in the metal binding. In UV−vis spectra, the absorption maximum of the species $Cu_2H_{-3}L$ to $Cu_2H_{-6}L$ appears at about 510−550 nm with a molar absorptivity of 150 M^{-1} cm^{-1}. The bandwidth of this band is higher in comparison to those of the mononuclear species. It strongly suggests that these stoichiometries are superimpositions of 4N $\{NH_2, 3N^-\}$ and 4N $\{N_{Im}, 3N^-\}$ coordinated copper(II) ions for which the absorption maxima were recorded in the ranges 507 and 540 nm, respectively (Table 5). The EPR parameters for the dinuclear species (pH 6−10.5) cannot be calculated because a significant line broadening is observed, suggesting some magnetic interactions between the coordinated metal ions of the species $Cu_2H_{-2}L$ to $Cu_2H_{-6}L$.

3.3. Oxidation of the Fragments of Neuropeptide γ. 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 antioxidant defenses.[62,63] In general, the ROS are formed ubiquitously in biological systems by both enzymatic and metal-catalyzed oxidation (MCO) reactions.[63−65] 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.[66−69] 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

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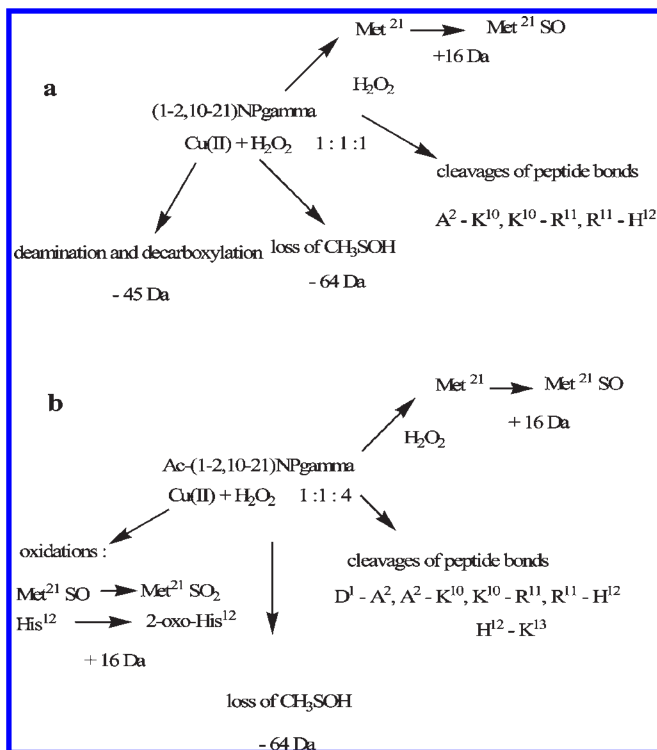
to generate ·OH[70,71] which immediately oxidizes neighboring amino acid residues. The free radical generating Cu(II)−ascorbate and mercaptans−H_2O_2 systems can be replaced by a Cu(II)−H_2O_2 system.[72] For Cu(II) complexes with peptides, it is assumed that hydrogen peroxide reduces peptide−Cu(II) to peptide−Cu(I); this is followed by the reaction of Cu(I) with hydrogen peroxide to give ·OH.[73] The individual amino acids involved in metal binding to a protein can be conveniently identified through site specific metal catalyzed oxidation.[74,75] The spectroscopic data for the copper(II) complexes of the (1−2,10−21)NPγ and Ac-(1−2,10−21)NPγ fragments of neuropeptide γ in a 0.1 M phosphate buffer at pH 7.4 are similar to those obtained in aqueous solutions at pH 7.4 (data not shown, Figures 1 and 2a, Table 5). For the Ac-(1−2,10−21)NPγ fragment, the copper(II) ions are coordinated by the imidazole nitrogen of the histidine residue (H^{12}) and two amide nitrogens of H^{12} and R^{11} residues, while for the (1−2,10−21)NPγ fragment at pH 7.4, the CuH_2L species dominates with a $\{NH_2, N^-, CO, N_{Im}\}$ coordination mode (Scheme 1). The chromatograms of both fragments of neuropeptide γ after 24 h incubation at 37 °C for the peptides alone, with Cu(II) only, with hydrogen peroxide, and with Cu(II)−H_2O_2 indicate that the solutions containing peptides alone and the copper(II) ions with a 1:1 peptide to copper(II) molar ratio were not changed in comparison to the peptide alone before incubation (data not shown).

Methionine is one of the most reactive amino acid residues subjected to oxidation. Methionine sulfoxide is the principal oxidation product in most cases and can be further oxidized to methionine sulfone.[76−78] LC-MS analysis of the systems containing (1−2,10−21)NPγ or Ac-(1−2,10−21)NPγ and hydrogen peroxide revealed the presence of monooxidized fragments of neuropeptide γ. For the 1:1 (1−2,10−21)NPγ−H_2O_2 system in a chromatographic fraction eluting at 9.4 min, a triply charged molecular ion $[M+3H]^{3+}$ with m/z 540.5 is present (Table 1S, Supporting Information), while for the 1:4 Ac-(1−2,10−21)NPγ−H_2O_2 system, doubly $[M+2H]^{2+}$ and triply $[M+3H]^{3+}$ charged molecular ions with m/z 831.9 and 555.0, respectively, are present in a fraction eluting at 10.3 min (Table 2S, Supporting Information). Each of these fragments displayed a molecular mass +16 Da higher than the respective native sequence (Tables 1S and 2S, Supporting Information). This change in the molecular mass is indicative of the addition of a single oxygen atom and may correspond to formation of the methionine sulfoxide. When the copper(II) ions are added to the (1−2,10−21)NPγ−H_2O_2 system with 1:1:1 molar ratio, then further modifications of the peptide are observed. Peptides with an N-terminal Asp residue under-

Scheme 3. Schematic Representation of the Products of Copper(II)-Catalyzed Oxidation for the (1−2,10−21)NPγ (a) and Ac-(1−2,10−21)NPγ (b).



went oxidative deamination and decarboxylation to pyruvate (−45 Da)[79] or oxidative decarboxylation in aqueous buffers with metal ions.[80,81] In a chromatographic peak eluting at 11.5 min (Table 1S, Figure 2S, Supporting Information), peaks in the MS spectra occurring at m/z 534.7 may correspond to the triply charged molecular ion of the peptide after deamination, and those at 780.3 and 520.2 may indicate the presence of doubly and triply charged, respectively, molecular ions of the peptide modified by deamination and decarboxylation of the D^1 residue (Figure 2S, Supporting Information). The cleavage of the A^2−K^{10} peptide bond was observed, supporting the involvement of the N-terminal part of the peptide in the coordination of metal ions (Table 1S, Supporting Information). Mass spectrometry for the chromatographic fraction eluted at 14.1 min yielded a doubly $[M+2H]^{2+}$ charged molecular ion of 565.3 Da (Table 1S, Supporting Information) which may be assigned to the H^{12}−M^{21} fragment, supporting the presence of the histidine residue around the copper(II) ions.[82] The molecular ions for the (1−2,10−21)NPγ fragment of neuropeptide γ after Cu(II)-catalyzed oxidation of the 1:1 complex−hydrogen peroxide system in Table 1S (Supporting Information) are proposed and presented in Scheme 3a.

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Histidine is a very important amino acid residue critical for the function of many enzymes. His is susceptible to radical oxidation, leading to very complex products. The conversion of His to aspartic acid results in a −22 Da product, while a +16 Da species corresponds to 2-oxo-histidine, consistent with MCO studies of histidine oxidation.[83] In oxidation of the proteins, the −22, −10, +5, and +16 Da oxidation products of His were also identidied.[76] It should be mentioned that the reaction of histidine with hydroxyl radicals is complicated, and all the oxidation products have not been fully characterized.[84] For oxidation of the Cu(II)−Ac-(1−2,10−21)NPγ system in the presence of hydrogen peroxide, the 1:4 complex−H_2O_2 molar ratio was used, and further oxidations of the methionine and histidine residues were observed. Mass spectrometry for the chromatographic fraction eluting at 10.8 min (Table 2S, Supporting Information) contains the doubly charged molecular ions with *m*/*z* 839.3 and 847.4 Da (Figure 3Sa, Supporting Information) and the triply charged molecular ions with *m*/*z* 559.5 and 565.7 Da (Figure 3Sb, Supporting Information). The masses of these oxidation products are +16 and +32 Da higher than the parent oxidized with methionine to a sulfoxide peptide ion, suggesting further oxidation of the methionine sulfoxide to sulfone and histidine to 2-oxohistidine (Table 2S). For this peptide, the coordination of the His[12] residue to copper(II) ions is suggested; therefore, fragmentation by the cleavage of the peptide bonds near the His residue should be observed. Mass spectrometry for the chromatographic fraction eluted at 13.0 min yielded masses of 497.2 and 1011.4 Da (Table 2S, Supporting Information). These masses may be assigned to the doubly charged molecular ion of the K^{13}−M^{21} fragment and to the molecular $[M+H]^+$ ion of the K^{13}−M^{21} fragment with an oxidized Met residue to sulfoxide, respectively. Other molecular ions of copper(II)-catalyzed oxidation products for the Ac-(1−2,10−21)-NPγ fragment of neuropeptide γ were also detected, and the modifications of this peptide in Table 2S are proposed and in Scheme 3b are presented. It should be also mentioned that, for both peptides (Tables 1S and 2S) in MS spectra, molecular ions with a loss of 64 Da (CH_3SOH) were observed, and it is diagnostic for the formation and presence of methionine sulfoxide.[74]

Conclusions

The tachykinins are a family of undecapeptides that are widely distributed throughout the body, including the central nervous system (CNS). There is some evidence that they function as neuromodulators rather than neurotransmitters. The role of tachykinins (neurokinins) in a variety of CNS developmental or disease processes was suggested.[16,85] For (1−2,10−21)NPγ, the coordination of Cu(II) ions starts from the N-terminal amino group of the Asp[1] residue, which stabilizes the 1N {NH_2, β-COO^-} and 2N {NH_2, β-COO^-, N_{Im}} complexes by the coordination of the β-carboxylate oxygen to metal ion. In the pH range 4−9, the imidazole nitrogen of His[12] is coordinated, and the macrochelate in the molecule with the 2N {NH_2, β-COO^-, N_{Im}} to 4N {NH_2, $2N^-$, N_{Im}} coordination mode is formed. At a pH above 9, the (1−2,10−21)NPγ forms 4N complexes with a classical peptide type coordination starting from the N-terminal amino group up to the third deprotonated amide groups, as in the case of peptides with noncoordinating side chains (Scheme 1). The introduction of the acethyl group at the N-terminal amine group changes the coordination mode of the peptide. The histidyl imidazole−N (H^{12}) donor atom was found to be the major copper(II) binding, site and the 3N complex containing two additional deprotonated amide-N donors is the major species in the physiological pH range. (1−2,10−21)NPγ contains two binding sites: histidyl residue (H^{12}) and the N-terminal amine group. The high water solubility of the resulting metal complexes allowed us to obtain a complete complex speciation at different, 1:1 and 2:1, metal-to-ligand ratios. Dinuclear complexes are only formed above pH 6 in parallel with the involvement of amide functions in metal binding. The N-terminus and the histidyl side chain work as independent anchoring sites for metal binding with the subsequent deprotonation and coordination of amide functions.

The individual amino acids involved in metal binding to a protein can be conveniently identified through site-specific metal catalyzed oxidation.[74,75] For both fragments of neuropeptide γ, the methionine residue is converted to methionine sulfoxide in the reaction with hydrogen peroxide, while for the 1:1:4 Cu(II)−Ac-(1−2,10−21)NPγ−hydrogen peroxide system, oxidations of histidine residue to 2-oxo-histidine and methionine sulfoxide to sulfone were observed. For the Cu(II)−peptide−H_2O_2 system, a loss of sulfunic acid (CH_3SOH) from the oxidized methionine (M^{21}) residue was detected. Under experimental conditions, the (1−2,10−21)-NPγ and Ac-(1−2,10−21)NPγ fragments of neuropeptide γ also undergo fragmentations by cleavage of the R^{11}−H^{12} and H^{12}−K^{13} peptide bonds indicating the involvement of the histidine residue in the copper(II) binding sites. The presented paper may be utilized in the interpretation of the results obtained for the neuropeptide γ and similar systems containing peptides with free amine groups and the histidine residue in a position higher than the third of the peptide sequence.[41,50,57]

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Supporting Information Available: CD spectra, mass spectra, and additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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