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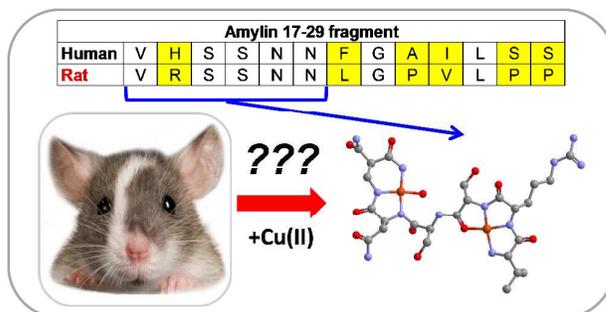
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The studies of rat amylin fragments and their mutants demonstrate the anchoring ability of -SerSerAsnAsn- domain.



Journal Name

ARTICLE

## Potentiometric and spectroscopic studies on the copper(II) complexes of rat amylin fragments. The anchoring ability of specific non-coordinating side chains

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Copper(II) complexes of peptides modelling the sequence of the 17-22 residues of rat amylin have been studied by potentiometric, UV-Vis, CD and ESR spectroscopic methods. The peptides were synthesized in N-terminally free forms, NH<sub>2</sub>-VRSSNN-NH<sub>2</sub>, NH<sub>2</sub>-VRSSAA-NH<sub>2</sub>, NH<sub>2</sub>-VRAANN-NH<sub>2</sub>, NH<sub>2</sub>-VRSS-NH<sub>2</sub>, NH<sub>2</sub>-SSNN-NH<sub>2</sub>, NH<sub>2</sub>-SSNA-NH<sub>2</sub> and NH<sub>2</sub>-AANN-NH<sub>2</sub>, providing a possibility for the comparison of the metal binding abilities of the amino terminus and the -SSNN-domain. The amino terminus was the primary ligating site in all cases and the formation of only mononuclear complexes was obtained for the tetrapeptides. The thermodynamic stability of the (NH<sub>2</sub>,N',N') coordinated complexes was, however, enhanced by the asparaginy moiety in the case of NH<sub>2</sub>-SSNN-NH<sub>2</sub>, NH<sub>2</sub>-SSNA-NH<sub>2</sub> and NH<sub>2</sub>-AANN-NH<sub>2</sub>. Among the hexapeptides the formation of dinuclear complexes was characteristic for NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> demonstrating the anchoring ability of the -SSNN- (SerSerAsnAsn) domain. The complexes of the heptapeptide NH<sub>2</sub>-GGHSSNN-NH<sub>2</sub> were also studied and the data supported the above mentioned anchoring ability of the -SSNN- site.

### Introduction

The deposition of islet amyloid polypeptide (IAPP or amylin) is a pathological feature of type 2 diabetes. The human form (hIAPP) is built up from 37 amino acid residues containing an internal histidine in positions 18. The presence of histidine in the peptide offers a potential and effective metal binding site in the ligand. In agreement with this expectation a series of recent studies supports that copper(II) and some other metal ions can mediate the aggregation processes of hIAPP. The inhibition of the aggregation was shown in several studies<sup>1-3</sup>, while the impact of copper mediated ROS production was demonstrated in other works<sup>4-6</sup>. Moreover, it was also reported that copper(II) is able to modulate the proteolytic activity of IAPP degrading enzymes<sup>7</sup>. The biological significance of hIAPP promoted the investigations on the coordination chemistry of these peptides<sup>8-9</sup> but the low solubility of the peptide fragments hindered these studies. The properties of human and rat amylin fragment were compared in some of these previous studies reflecting a significant difference in the metal binding capacity and pH dependence of aggregation processes. These differences can be easily explained by the

amino acid sequences of hIAPP and rIAPP. The most important difference is related to position 18, because the histidyl residue of hIAPP is replaced by arginine in rIAPP. There is no an effective donating group in the side chain of arginyl residue and, as a consequence, there is no any example in the literature on the anchoring ability of this residue in its metal complexes<sup>10,11</sup>. The copper(II) complexes of a tridecapeptide segment of rat amylin rIAPP(17-29) and some of its C-terminally shortened tetra- and hexa-peptide fragments and some mutants were investigated in our previous study<sup>12</sup>. It was found that the native fragments can bind one equivalent copper(II) ion at the physiological pH range, although there are no any strongly coordinating side chains in these molecules. The comparison of the data obtained for the various fragments suggested the specific role of the VRSSNN domain in copper(II) binding especially the polar seryl and asparaginy side chains. This observation was rather surprising in metalloprotein chemistry but the copper(II) binding by the VRSSNN and SSNN fragments were also supported by spectroscopic methods and DFT calculations. The exact binding mode of copper(II) in these complexes, however, remained partly unanswered promoting further studies in this subject.

Now in this paper we report the synthesis, potentiometric and spectroscopic studies on the copper(II) complexes of some N-terminally free fragments of rat amylin. These molecules include the hexapeptides modelling the 17-22 residues of rat amylin: NH<sub>2</sub>-VRSSNN-NH<sub>2</sub>, its two mutants: NH<sub>2</sub>-VRSSAA-NH<sub>2</sub> and NH<sub>2</sub>-VRAANN-NH<sub>2</sub> and four tetrapeptides from the same domain: NH<sub>2</sub>-VRSS-NH<sub>2</sub>, NH<sub>2</sub>-SSNN-NH<sub>2</sub>, NH<sub>2</sub>-SSNA-NH<sub>2</sub> and NH<sub>2</sub>-AANN-NH<sub>2</sub>. Copper(II) complexes of tetraarginine (NH<sub>2</sub>-RRRR-OH) and triserine (NH<sub>2</sub>-SSS-OH) were also studied for

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comparison and to obtain information on the possible impacts of guanidinium and seryl-OH side chains. Moreover, the studies on a heptapeptide are also included. Its N-terminus is reminiscent of that of albumin with outstanding Cu and Ni binding affinity (ATCUN motif<sup>13,14</sup>) and it is linked to the previously suggested metal binding domain of rat amylin: NH<sub>2</sub>-GGH-SSNN-NH<sub>2</sub>.

## Experimental

### Materials

The peptides NH<sub>2</sub>-SSS-OH (Ser<sub>3</sub>), NH<sub>2</sub>-AAAA-OH (Ala<sub>4</sub>) and NH<sub>2</sub>-RRRR-OH (Arg<sub>4</sub>) were purchased from Bachem and their concentration were checked by potentiometric titrations. The other tetra-, hexa- and heptapeptides (NH<sub>2</sub>-VRSS-NH<sub>2</sub>, NH<sub>2</sub>-SSNN-NH<sub>2</sub>, NH<sub>2</sub>-SSNA-NH<sub>2</sub>, NH<sub>2</sub>-AANN-NH<sub>2</sub>, NH<sub>2</sub>-VRSSNN-NH<sub>2</sub>, NH<sub>2</sub>-VRSSAA-NH<sub>2</sub>, NH<sub>2</sub>-VRAANN-NH<sub>2</sub> and NH<sub>2</sub>-GGH-SSNN-NH<sub>2</sub> were synthesized by means of a Liberty Peptide Synthesizer.

All solvents and chemicals used for synthesis were obtained from commercial sources in the highest available purity and used without further purification. All *N*-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids (Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Val-OH, 2-(1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and Rink Amide AM resin was purchased from Novabiochem. Peptide-synthesis grade *N,N*-dimethylformamide (DMF), *N,N*-diisopropyl-ethylamine (DIEA), 1,2-ethanedithiol and trifluoroacetic acid (TFA) were from Merck Ltd. *N*-hydroxybenzotriazole (HOBt), *N*-methylpyrrolidone (NMP), triisopropylsilane (TIS), 2,2'-(ethylenedioxy)diethanethiol, diethyl ether (Et<sub>2</sub>O) and 2-methyl-2-butanol were Sigma-Aldrich products. Piperidine, dichloromethane (DCM) and acetic acid (96%) were obtained from Molar Chemicals Ltd whereas acetonitrile (ACN) and acetic anhydride were from VWR International Ltd.

Stock solutions of copper(II) chlorides were prepared from analytical grade reagents and their concentration were measured gravimetrically via the precipitation of oxinates. The other stock solutions (KOH, HCl, KCl, potassium hydrogen phthalate) were also prepared from analytical grade reagents.

### Peptide synthesis

In the case of all synthesized peptides solid phase peptide synthesis was performed using a microwave-assisted Liberty Peptide Synthesizer (CEM, Matthews, NC), introducing the amino acid derivatives following the TBTU/HOBt/DIEA activation strategy on Rink Amide AM resin. Removal of the Fmoc protecting group was carried out at 80 °C with 30 Watts microwave power for 180 s by means of 20% piperidine in DMF. Coupling was achieved at 80 °C with 30 Watts microwave power, for 300 s, using 4 times amino acids excess, 0.5 M HOBt/0.5 M TBTU in DMF and 2 M DIPEA in NMP. Finally, the *N*-terminal Fmoc group was removed as mentioned before. Peptides were cleaved from their respective resins, with the simultaneous removal of the side chain protective groups, by treatment with a mixture of TFA/TIS/H<sub>2</sub>O/2,2'-

(ethylenedioxy)diethanethiol (94/2.5/2.5/1 v/v) for 1.5 h at room temperature. Each solution containing the free peptide was separated from the resin by filtration. The crude peptides were recovered from the pertinent solution by precipitation with cold diethyl ether. The precipitate was washed with cold diethyl ether and separated from it, then dried, re-dissolved in water, and finally lyophilized.

The purity of the peptides was checked by analytical *RP*-HPLC using a Jasco instrument, equipped with a Jasco MD-2010 plus multiwavelength detector. The analyses were performed by eluting solvent A (0.1% TFA in acetonitrile) and solvent B (0.1% TFA in water) on a Vydac C<sub>18</sub> chromatographic column (250 × 4.6 mm, 300 Å pore size, 5 μm particle size) at a flow rate of 1 mL/min, with a linear elution from 5% to 15% of B, in the case of tetrapeptides and from 3% to 40% of B in the other cases monitoring the absorbance at 222 nm. All the synthesized peptides showed the correct molecular mass, measured by mass spectrometry using the ESI-MS technique. Potentiometric measurements further confirmed the purity and the identity of the peptides.

### Potentiometric measurements

The pH-potentiometric titrations were performed in 4 cm<sup>3</sup> samples at 2 × 10<sup>-3</sup> - 3 × 10<sup>-3</sup> M ligand concentration with the metal ion to ligand ratios 1:1 for tetrapeptides and 1:1, 2:1 for hexa- and heptapeptides. During the titration, argon was bubbled through the samples to ensure the absence of oxygen and carbon dioxide. The samples were stirred by a VELP Scientific magnetic stirrer. All pH-potentiometric measurements were carried out at 298 K and at a constant ionic strength of 0.2 M KCl. pH measurements were made with a MOLSPIN pH-meter equipped with a 6.0234.100 combination glass electrode (Metrohm) and a MOL-ACS microburette controlled by a computer. The recorded pH readings were converted to hydrogen ion concentration as described by Irving et al<sup>15</sup>. Protonation constants of the ligands and overall stability constants have been calculated by means of general computational programs, SUPERQUAD<sup>16</sup> and PSEQUAD<sup>17</sup> using equations (1) - (2).

$$pM + qH + rL = M_p H_q L_r \quad (1)$$

$$\beta_{pqr} = \frac{[M_p H_q L_r]}{[M]^p \cdot [H]^q \cdot [L]^r} \quad (2)$$

### Spectroscopic measurements

UV-Vis spectra were recorded on a Perkin-Elmer Lambda 25 double beam spectrophotometer. In the case of the copper(II) complexes the same concentration range was used as for pH-potentiometry.

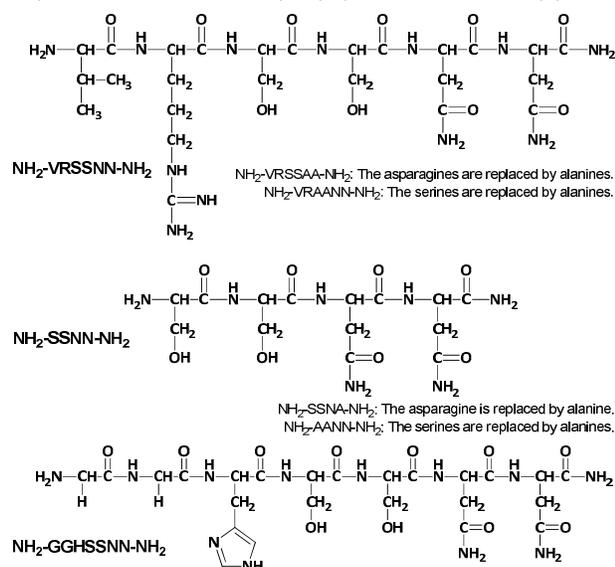
The ESR continuous wave spectra were recorded at 120 K, using a Bruker EMX X-band spectrometer (9.46 GHz) equipped with a HP53150A frequency counter. Copper(II) stock solution was prepared from CuSO<sub>4</sub>·5H<sub>2</sub>O enriched with <sup>63</sup>Cu to get better resolution of EPR spectra. Metallic copper (99.3% <sup>63</sup>Cu and 0.7% <sup>65</sup>Cu) was purchased from JV Isoflex, Moscow, Russia for this purpose and converted into the sulfate.

CD spectra of copper(II) complexes were recorded on a JASCO J-810 spectropolarimeter using 1 or 10 mm cells in the 200-800 nm range in the same concentration range as used for potentiometry.

The UV-Vis and CD spectra of the copper(II) ion containing systems were analyzed by the Convex Constraint Algorithm (CCA+) program<sup>18,19</sup>. The spectra of the individual species were determined by this method.

## Results and discussion

It is a common feature of all peptides that the terminal amino group is the primary metal binding site of the molecules, especially in the interaction with copper(II) ions<sup>10,14</sup>. Therefore, the side chain residues of tetrapeptides cannot be independent metal binding sites, so these tetrapeptides are not able to bind more than one copper(II) ion. The specific sequences of a hexa- or hepta-peptide, however, may provide



Tab.1. pK values of tetrapeptides and stability constants ( $\log \beta_{pqr}$ ) of their copper(II) complexes. (T = 298 K, I = 0.2 M)

Species	NH <sub>2</sub> -VRSS-NH <sub>2</sub>	NH <sub>2</sub> -SSNN-NH <sub>2</sub>	NH <sub>2</sub> -SSNA-NH <sub>2</sub>	NH <sub>2</sub> -AANN-NH <sub>2</sub>	NH <sub>2</sub> -SSS-OH	NH <sub>2</sub> -RRRR-OH	NH <sub>2</sub> -GGGG-OH <sup>20</sup>	NH <sub>2</sub> -AAAA-OH <sup>21</sup>
pK(-COOH)	—	—	—	—	3.08(1)	3.00(2)	3.24	3.52
pK(-NH <sub>3</sub> <sup>+</sup> )	7.46(1)	6.99(1)	7.01(1)	7.86(2)	7.13(1)	7.13(1)	7.94	8.13
[CuL]	4.03(9)	—	—	—	4.32(4)	—	5.06	4.77
[CuH <sub>-1</sub> L]	-0.85(1)	-0.35(3)	-0.29(2)	-0.28(2)	-0.01(1)	-1.11(2)	-0.50	-0.45
[CuH <sub>-2</sub> L]	-7.66(2)	-5.27(1)	-5.33(7)	-5.59(1)	-5.63(1)	-8.32(4)	-7.41	-8.09
[CuH <sub>-3</sub> L]	-15.02(1)	-13.99(3)	-13.97(2)	-14.36(2)	—	-17.22(5)	-16.59	-17.33
pK(H <sub>-1</sub> /H <sub>-2</sub> )	6.81	4.92	5.04	5.31	5.62	7.21	6.91	7.64
pK(H <sub>-2</sub> /H <sub>-3</sub> )	7.36	8.72	8.64	8.77	—	8.90	9.18	9.24
$\log \beta_{1-11} - pK(-NH_3^+)$	-8.31	-7.34	-7.30	-8.14	-7.14	-8.24	-8.44	-8.58

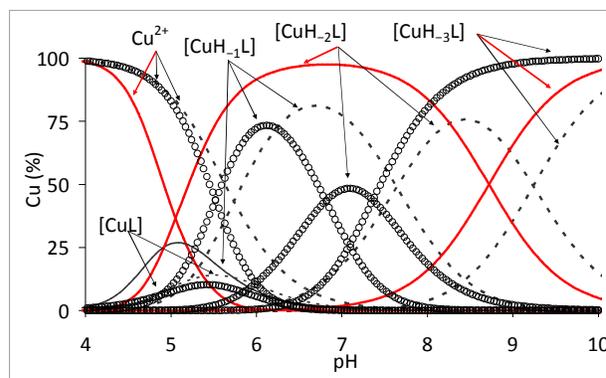


Fig.1. Concentration distribution of the major species as a function of pH in the copper(II)-tetraalanine (---), NH<sub>2</sub>-VRSS-NH<sub>2</sub> (—) and NH<sub>2</sub>-VRSS-NH<sub>2</sub> (ooo) systems.

$C_{Cu(II)} = C_L = 2 \text{ mM}$

a chance for the formation of dinuclear complexes.

The results of this study are in agreement with this expectation and justify the discussion of results in separate paragraphs for tetra- and hexa- or hepta-peptides. The structural formulae of studied ligands are depicted on Scheme 1.

### Complexes of tetrapeptides

Protonation constants of the tetrapeptides and the stability constants of their copper(II) complexes are included in Table 1. The corresponding data of tetra-Gly, tetra-Ala and tetra-Arg are also shown for comparison. The pK values of the ammonium group of tetrapeptides containing N-terminal serine are lower than those of other tetrapeptides, and similar trend can be observed in the case of NH<sub>2</sub>-SSS-OH (Ser<sub>3</sub>: pK(NH<sub>3</sub><sup>+</sup>)=7.13, Gly<sub>3</sub>: pK(NH<sub>3</sub><sup>+</sup>)=7.93<sup>20</sup>). This means that the formation of hydrogen bond between terminal amino group and N-terminal serine -OH group promotes the deprotonation of ammonium group. On the other hand it is clear from Table 1 that the complex formation processes of all tetrapeptides are basically the same, although there are significant differences in the pH ranges for the formation of amide coordinated species.

Tab.2. Spectroscopic parameters of the copper(II) complexes formed with tetrapeptides

Peptide	Method	[CuH <sub>1</sub> L]	[CuH <sub>2</sub> L]	[CuH <sub>3</sub> L]
NH <sub>2</sub> -AAAA-OH	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	652 / 93	563 / 138	515 / 165
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	680 / -0.30 300 / +0.76	564 / -0.88 304 / +0.90 280 / -0.60	527 / -1.19 304 / +0.56 280 / -2.0
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	—	—	—
NH <sub>2</sub> -RRRR-OH	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	—	517 / 187
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	—	—
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	175 / 2.252	179 / 2.216	206 / 2.173
NH <sub>2</sub> -VRSS-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	664 / 66	552 / 111	515 / 139
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	679 / -0.47 320 / +0.52	568 / -0.79 313 / +0.71	517 / -1.30 304 / +1.13
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	177 / 2.254	184 / 2.214	203 / 2.182
NH <sub>2</sub> -SSNN-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	550 / 128	516 / 121
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	586 / -0.63 492 / +0.26 309 / +0.99	560 / -1.14 479 / +0.23 304 / +0.94
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	—	205 / 2.209	200 / 2.180
NH <sub>2</sub> -SSNA-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	550 / 105	519 / 92
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	577 / -0.61 490 / +0.20 309 / +0.68	562 / -1.00 483 / +0.36 307 / +0.63
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	—	208 / 2.203	200 / 2.181
NH <sub>2</sub> -AANN-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	547 / 109	510 / 103
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	—	580 / -0.46 492 / +0.16 306 / +0.68	554 / -0.84 470 / +0.05 303 / +0.55
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	—	202 / 2.207	204 / 2.177

This is best demonstrated by Figure 1, where the concentration distributions of three systems are plotted as a function of pH.

Complex formation processes of oligopeptides generally starts in acidic pH range with the formation of an (NH<sub>2</sub>,CO)-coordinated species with [CuL] stoichiometry. The increase of the length of peptide chain and especially the presence of bulky side chains, however, significantly suppresses the thermodynamic stability of this species. [CuL] is probably present in all studied systems but its concentration is too low to calculate reliable stability constants, except for tetraglycine, tetraalanine and NH<sub>2</sub>-VRSS-NH<sub>2</sub>. As a consequence, the pK values for the deprotonation of the first amide nitrogens cannot be calculated but the speciation curves reveal the major differences in the stability of various species.

It is clear from Table 1 that the stability constants of Arg<sub>4</sub> complexes are very similar to those of Gly<sub>4</sub> and Ala<sub>4</sub> suggesting that even high numbers of arginyl side chains do not have a significant contribution to the overall stability of copper(II)-peptide complexes. The comparison of Ala<sub>4</sub> with the rat amylin

fragments, however, reveals a significant impact of specific side chains. It is obvious from both Table 1 and Figure 1 that the formation of [CuH<sub>2</sub>L] complex of NH<sub>2</sub>-SSNN-NH<sub>2</sub>, NH<sub>2</sub>-SSNA-NH<sub>2</sub> and NH<sub>2</sub>-AANN-NH<sub>2</sub> is very much favoured and exists as a major species in a relatively wide pH range. On the other hand, the species [CuH<sub>3</sub>L] is favoured for NH<sub>2</sub>-VRSS-NH<sub>2</sub> and predominates in almost the whole alkaline pH range. These observations suggest that the presence of two adjacent asparaginyl and/or seryl residues may enhance the stability of their copper(II) complexes.

The evaluation of UV-Vis, CD and ESR spectroscopic measurements helped to understand the above mentioned effects of side chain residues. Spectroscopic parameters of the major species formed in the copper(II)-tetrapeptide systems are collected in Table 2, while Figures 2 and 3 are used to compare the CD and ESR spectra of the copper(II) complexes of NH<sub>2</sub>-VRSS-NH<sub>2</sub> and NH<sub>2</sub>-SSNN-NH<sub>2</sub>. In the copper(II)-NH<sub>2</sub>-VRSS-NH<sub>2</sub> system the species [CuH<sub>1</sub>L] is the dominating complex below pH 7.0 and its ESR spectral parameters correspond well to those of the (NH<sub>2</sub>,N<sup>-</sup>,CO) coordination

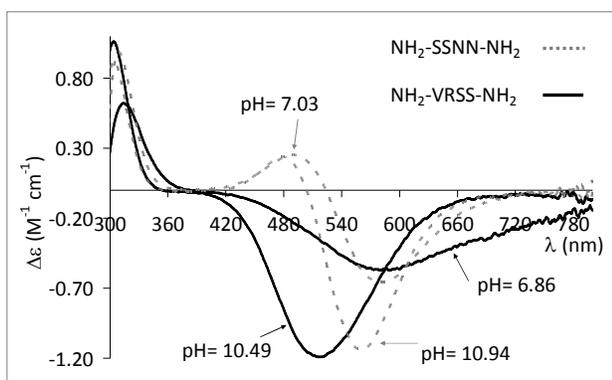


Fig. 2. CD spectra of the copper(II) complexes of  $\text{NH}_2\text{-VRSS-NH}_2$  measured in equimolar samples at pH 6.86 and 10.49 and for  $\text{NH}_2\text{-SSNN-NH}_2$  at pH 7.03 and 10.94.

mode of peptides. For the other three tetrapeptides ( $\text{NH}_2\text{-SSNN-NH}_2$ ,  $\text{NH}_2\text{-SSNA-NH}_2$  and  $\text{NH}_2\text{-AANN-NH}_2$ ), however,  $[\text{CuH}_2\text{-L}]$  is the major species in the same pH range and their CD and ESR spectral parameters can be best described by the simple  $(\text{NH}_2, \text{N}^-, \text{N}^-)$  coordination mode. However, the positive Cotton effect around 480-500 nm in the CD spectra of the  $[\text{CuH}_2\text{-L}]$  complexes of these peptides may suggest an interaction from the asparaginyl side chains. The comparison of EPR spectra of these peptide complexes reveals similar differences and supports the existence of a more saturated coordination environment in the  $[\text{CuH}_2\text{-L}]$  complexes of peptides containing asparaginyl residues.

The complex  $[\text{CuH}_3\text{-L}]$  is an exclusively formed species in all systems above pH 10.0 and systematic differences cannot be seen in their spectroscopic parameters. As a consequence, the  $(\text{NH}_2, \text{N}^-, \text{N}^-, \text{N}^-)$  coordination mode can be suggested for all tetrapeptides in the alkaline samples. pK values for the deprotonation of the third amide groups, however, reflect a high preference for the formation of this species with  $\text{NH}_2\text{-VRSS-NH}_2$  (see Figure 1 and next-to-the-last row in Table 1.). One may suggest a contribution from adjacent seryl side chains to copper(II) binding but neither CD and ESR spectra nor previous literature data do not support this assumption. But

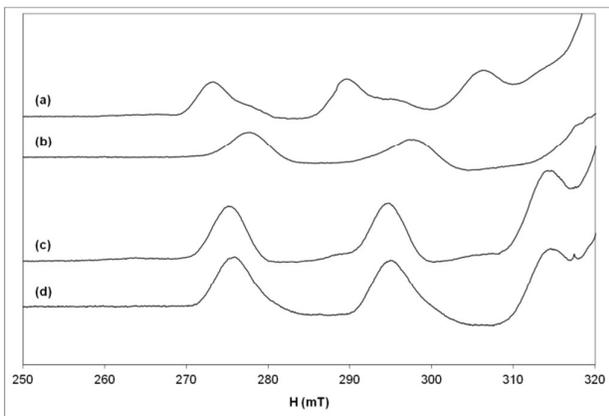


Fig. 3. ESR spectra of the copper(II) complexes measured in equimolar samples of  $\text{NH}_2\text{-VRSS-NH}_2$  at pH 7.11 (a) and 10.11 (b) and of  $\text{NH}_2\text{-SSNN-NH}_2$  at pH 7.12 (c) and 9.96 (d).

there are two facts, which should be taken into the account and may provide an explanation for this observation:

(i) the electron withdrawing character of seryl side chain  $-\text{OH}$  group

(ii) the lack of the C-terminal carboxylate residues.

The presence of electron withdrawing seryl- $\text{OH}$  group could promote indirectly the deprotonation of the adjacent amide nitrogen. A serine in the second position can enhance the stability of  $(\text{NH}_2, \text{N}^-)$  coordinated complexes, as it can be seen for  $\text{NH}_2\text{-SSNN-NH}_2$ ,  $\text{NH}_2\text{-SSNA-NH}_2$  and  $\text{Ser}_3$  peptides. (Because of the pK values of the first amide nitrogen cannot be calculated, only the comparison of the stability constants corrected by the protonation constants of ammonium group is possible. (Table 2, last row)) In the  $\text{Ser}_3$  and  $\text{NH}_2\text{-VRSS-NH}_2$  peptides seryl residues are in the third or/and fourth position, which results in the decreasing of  $\text{pK}(\text{H}_{-1}/\text{H}_{-2})$  and  $\text{pK}(\text{H}_{-2}/\text{H}_{-3})$  values compared to those of  $\text{Gly}_4$ ,  $\text{Ala}_4$  or  $\text{Arg}_4$ . On the other hand in the case of latter three tetrapeptides the presence of free carboxylates changes the charge of complexes. Moreover, it can contribute to the overall stability of the 2N and 3N coordinated complexes. In the case of  $\text{NH}_2\text{-SSNN-NH}_2$ ,  $\text{NH}_2\text{-SSNA-NH}_2$  and  $\text{NH}_2\text{-AANN-NH}_2$  the asparaginyl side chains have an even more pronounced effect, but no chance for the extra stabilization for the complexes of  $\text{NH}_2\text{-VRSS-NH}_2$ . So the above mentioned two effects shift the formation of the fully coordinated 4N complexes into a lower pH range. For the  $\text{NH}_2\text{-SSNN-NH}_2$  the CD spectrum of  $[\text{CuH}_3\text{-L}]$  is, however, different from that of  $\text{NH}_2\text{-RRRR-OH}$  or  $\text{NH}_2\text{-VRSS-NH}_2$  because of the presence of small positive Cotton effect at 470-480 nm. On the basis of this fact the deprotonation and coordination of asparagine amide nitrogen cannot be excluded. Similar coordination sphere around copper(II) ion was supported in the case of  $[\text{CuH}_4\text{-L}]$  of  $\text{Ac-SSNN-NH}_2$ .<sup>12</sup>

### Complexes of hexapeptides

Our previous studies on the copper(II) complexes of the terminally blocked hexapeptide fragment  $\text{Ac-VRSSNN-NH}_2$  revealed that this peptide can bind one equivalent copper(II) ion and this was explained by the anchoring role of the asparaginyl residues. On the other hand, the present results described in paragraph 3.1 for the N-terminally free tetrapeptides unambiguously demonstrated that the terminal amino groups are much more effective anchoring sites than the side chains of asparagine, although the latter also had some contribution to the overall stability of the complexes. Further understanding of these effects can be obtained from the studies on the corresponding N-terminally free hexapeptides including the natural (17-22) hexapeptide fragment of rat amylin  $\text{NH}_2\text{-VRSSNN-NH}_2$  and its two mutants  $\text{NH}_2\text{-VRSSAA-NH}_2$  and  $\text{NH}_2\text{-VRAANN-NH}_2$ . Protonation constants of these ligands and the stability constants of the corresponding copper(II) complexes have been determined by potentiometric titrations and the data are collected in Table 3. It is clear from Table 3 that formation of dinuclear complexes is characteristic only for the copper(II)- $\text{NH}_2\text{-VRSSNN-NH}_2$  system. A slight opalescence of the 2:1 =  $\text{Cu(II)}:\text{L}$  samples was observed even with this ligand in the pH range 6-7 but it slowly

Tab.3. pK values of hexapeptides and stability constants ( $\log \beta_{\text{pqr}}$ ) of their copper(II) complexes. (T = 298 K, I = 0.2 M)

Species	NH <sub>2</sub> -VRSSNN-NH <sub>2</sub>	NH <sub>2</sub> -VRSSAA-NH <sub>2</sub>	NH <sub>2</sub> -VRAANN-NH <sub>2</sub>
[HL]	7.50(3)	7.53(2)	7.55(2)
[CuL]	3.76(18)	4.22(8)	4.11(9)
[CuH <sub>-1</sub> L]	-0.61(1)	-0.69(1)	-0.65(1)
[CuH <sub>-2</sub> L]	-7.13(5)	-7.47(2)	-7.80(2)
[CuH <sub>-3</sub> L]	-14.20(3)	-14.71(2)	-15.45(2)
[Cu <sub>2</sub> H <sub>-4</sub> L]	-16.11(2)	—	—
[Cu <sub>2</sub> H <sub>-5</sub> L]	-23.74(4)	—	—
[Cu <sub>2</sub> H <sub>-6</sub> L]	-33.37(7)	—	—
[Cu <sub>2</sub> H <sub>-7</sub> L]	-42.25(4)	—	—
pK(H <sub>-1</sub> /H <sub>-2</sub> )	6.52	6.78	7.15
pK(H <sub>-2</sub> /H <sub>-3</sub> )	7.07	7.24	7.65

dissolved by increasing pH and the experimental data only out of this range were used for calculations. In the case of the other two hexapeptides NH<sub>2</sub>-VRSSAA-NH<sub>2</sub> and NH<sub>2</sub>-VRAANN-NH<sub>2</sub> high amount of copper(II)-hydroxide precipitate was formed above pH 6.0 ruling out the computer evaluation of the titration curves recorded in the presence of metal ion excess. However, it is important to note that the dissolution of the precipitates of these samples was also observed above pH 11.0 suggesting that all three peptides can form dinuclear complexes with copper(II) but it occurs in the physiological pH range for NH<sub>2</sub>-VRSSNN-NH<sub>2</sub>, while only in strongly alkaline samples for the other two ligands.

The stability constants obtained for the mononuclear complexes, however, reveal a high similarity in the copper(II) complexes of the three hexapeptides. The species [CuL] can be detected in all cases but in very low concentration, while the [CuH<sub>-n</sub>L] ( $n = 1-3$ ) complexes are present in comparable concentration for all systems. Moreover, it can be stated that the stability constants and stepwise deprotonation constants of the amide nitrogens in Table 3 can be best compared to those of NH<sub>2</sub>-VRSS-NH<sub>2</sub> in Table 1. This similarity in the thermodynamic data suggest the existence of the common [NH<sub>2</sub>,N<sup>-</sup>], [NH<sub>2</sub>,N<sup>-</sup>,N<sup>-</sup>] and [NH<sub>2</sub>,N<sup>-</sup>,N<sup>-</sup>,N<sup>-</sup>] coordination modes in the species [CuH<sub>-1</sub>L], [CuH<sub>-2</sub>L] and [CuH<sub>-3</sub>L], respectively. In other words it means that the side chain residues have only a minor contribution to the thermodynamic stability of the mononuclear complexes and the spectroscopic data (see Table 4) provide further support for this assumption. Although the participation of the side chain residues can be excluded in the metal binding, the indirect effect of seryl-OH in the third and fourth position of NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> and NH<sub>2</sub>-VRSSAA-NH<sub>2</sub> peptides is reflected in the lower pK(H<sub>-1</sub>/H<sub>-2</sub>) and pK(H<sub>-2</sub>/H<sub>-3</sub>) values compared to those of NH<sub>2</sub>-VRAANN-NH<sub>2</sub>. The deprotonation processes of amide nitrogens of latter peptide take place around same pH range as that of common larger oligopeptide (e.g. for Gly<sub>5</sub> pK<sub>2</sub>(amide)=6,81 and pK<sub>3</sub>(amide)=7,89<sup>22</sup>).

The formation of the 3N coordinated [CuH<sub>-2</sub>L] complexes overlaps significantly the 2N and 4N species and it is difficult to determine exact spectral parameters for this stoichiometry. The data obtained for [CuH<sub>-1</sub>L] and [CuH<sub>-3</sub>L] of NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> and NH<sub>2</sub>-VRSSAA-NH<sub>2</sub>, however, reveal a high similarity to those reported for NH<sub>2</sub>-VRSS-NH<sub>2</sub> supporting the negligible contribution of side chain residues in metal ion coordination.

Moreover, the lack of any positive Cotton effect around 480 nm in the CD spectra of [CuH<sub>-2</sub>L] and [CuH<sub>-3</sub>L] complexes rules out the involvement amide side chains of asparaginyl residues in copper(II) binding. At the first sight this observation seems to be in contradiction with the results obtained for the tetrapeptides NH<sub>2</sub>-SSNN-NH<sub>2</sub>, NH<sub>2</sub>-SSNA-NH<sub>2</sub> and NH<sub>2</sub>-AANN-NH<sub>2</sub> where a significant contribution of the asparagine moiety was suggested especially to the overall stability of the [CuH<sub>-2</sub>L] complexes. The increase in the length of peptide chain can be responsible for this observation. In the case of the hexapeptide the asparagine moieties are out of the fused chelate rings created by the nitrogen donors of the first 3 or 4 amino acid residues. This binding mode provides a chance for the free rotation of these residues. On the contrary, the amide groups of asparagine is also involved in metal binding in the tetrapeptide complexes hindering the above mentioned free rotation of the side chain residues.

The formation of dinuclear complexes in the copper(II)-NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> system, however, gives an unambiguous proof for the anchoring capacity of the free asparagine side chains (see Fig. 4).

Both ESR spectra and ESI MS measurements provide a clearcut evidence for the existence of dinuclear complexes in the 2:1 copper(II):peptide systems. Figure 5 is used to demonstrate the ESR spectra recorded under three different conditions. Spectrum 5.a was recorded in equimolar samples at pH 6.86. The formation of all mononuclear species overlap under this condition but the parameters for the 2N coordinated [CuH<sub>-1</sub>L] complex can be well extracted (see Table 3). Spectrum 5.b

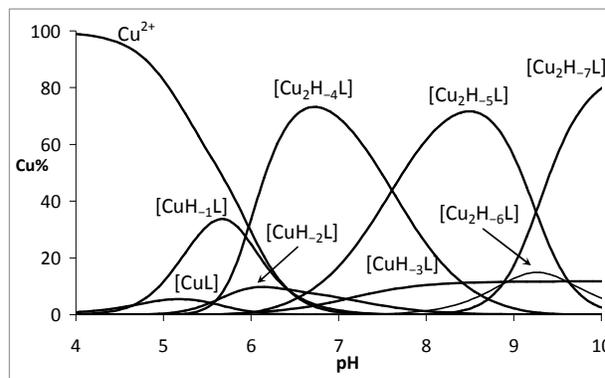


Fig.4. Distribution curves in the copper(II)-NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> system at 2:1 ratio  $c_L = 2$  mM

Tab.4. Spectroscopic parameters of the copper(II) complexes formed with hexapeptides

Peptide	Method	[CuH <sub>1</sub> L]	[CuH <sub>2</sub> L]	[CuH <sub>3</sub> L]
NH <sub>2</sub> -VRSSNN-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	652 / 78	563 / 128	517 / 189
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	692 / -0.47 322 / +0.42	576 / -0.78 313 / +0.78	532 / -1.21 307 / +0.83
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	176 / 2.256	—	206 / 2.183
NH <sub>2</sub> -VRSSAA-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	673 / 70	564 / 138	520 / 194
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	675 / -0.23 318 / +0.29	565 / -0.50 314 / +0.39	531 / -0.97 307 / +0.45
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	—	—	—
NH <sub>2</sub> -VRAANN-NH <sub>2</sub>	UV-vis (nm / M <sup>-1</sup> cm <sup>-1</sup> )	652 / 88	559 / 140	513 / 182
	CD (nm / M <sup>-1</sup> cm <sup>-1</sup> )	679 / -0.51 316 / +0.63	552 / -0.82 317 / +1.01	537 / -1.33 310 / +0.72
	ESR A <sub>  </sub> (x 10 <sup>-4</sup> cm <sup>-1</sup> ) / g <sub>  </sub>	—	—	—

belongs to [CuH<sub>3</sub>L] which is a single species above pH 9.0. Spectrum 5.c was taken at 2:1 metal to ligand ratio at pH 9.17; it cannot be due to two different copper(II) species because otherwise an anomalously low g value (2.134) would be obtained for the second one. So we have to suppose that this spectrum corresponds to a dimeric species where the interaction with the two copper(II) nuclei originates 2nI + 1, that is 7 lines (being n = number of copper(II) nuclei and I = nuclear spin of copper = 3/2) with g<sub>||</sub> 2.202 and A<sub>||</sub> ~96x10<sup>-4</sup> cm<sup>-1</sup>. Unfortunately there is no detectable signal at half field. ESI-MS measurements were also performed in this system at 2:1 ratio and the spectra are plotted in Figure 6. The good agreement of the isotopic distribution of the spectra recorded at pH 8.4 at 2:1 ratio with that calculated for the species [Cu<sub>2</sub>H<sub>5</sub>L] is another indication for the existence of dinuclear complexes

The ESR and ESI-MS measurements provide sufficient proof for the existence of dinuclear species but the characterization of the binding modes of copper(II) ions in these complexes is a more complicated task. Careful evaluation of the ESR spectra recorded for the dinuclear species (e.g. Figure 5.c) seems to support the presence of two metal ions in a similar 3N coordination mode in the species [Cu<sub>2</sub>H<sub>5</sub>L], in fact the A<sub>||</sub>

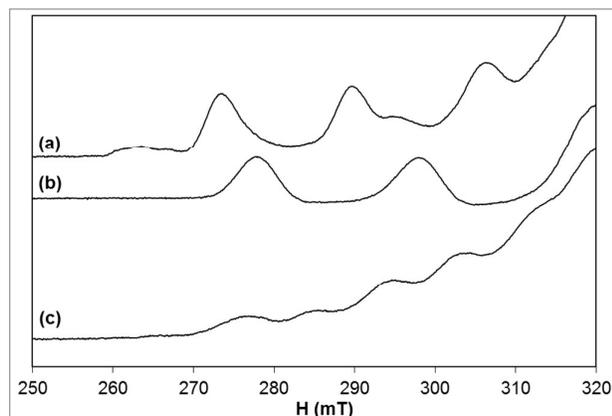


Fig.5. ESR spectra recorded in the copper(II)-NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> system at 1:1 ratio pH = 6.86 (a), 1:1 ratio pH = 10.08 (b) and 2:1 ratio pH = 9.17 (c).

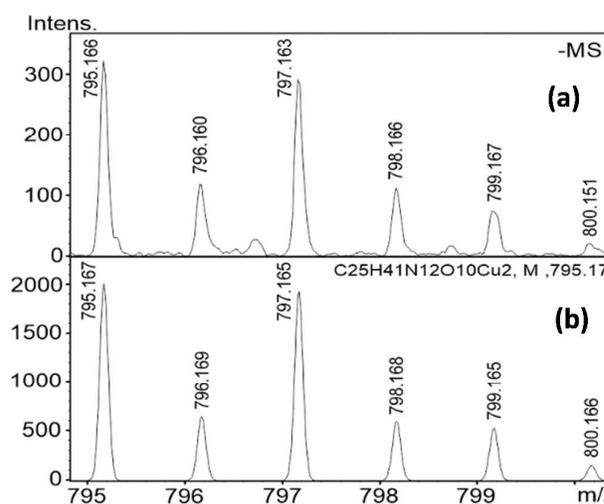


Fig.6. ESI-MS spectra recorded in the copper(II)-NH<sub>2</sub>-VRSSNN-NH<sub>2</sub> system at 2:1 ratio pH = 8.4. Experimental (a), calculated for [Cu<sub>2</sub>H<sub>5</sub>L] (b)

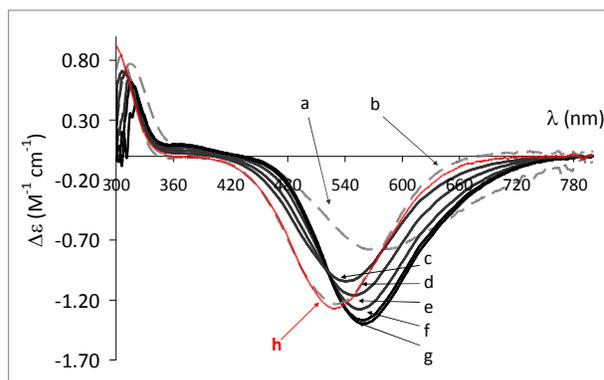
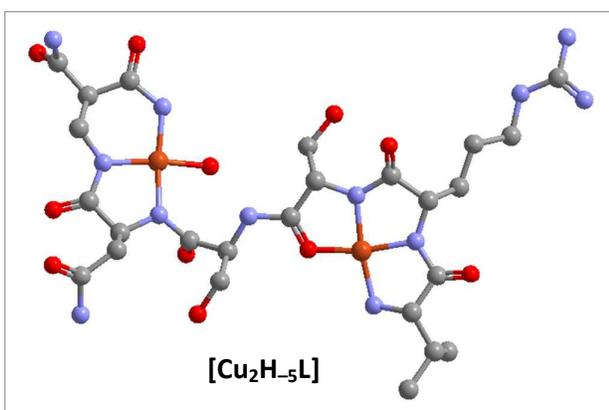


Fig.7. NH<sub>2</sub>-VRSSNN: CD spectra of species [CuH<sub>2</sub>L] (a), species [CuH<sub>3</sub>L] (b) and the spectra recorded by increasing copper(II) concentration at pH 8.4, 120% copper(II) (c), 140% (d), 160% (e), 180% (f), 200% (g) and at pH=11.5, 200% (h).

value obtained for the dimeric species is one half of that expected for 3N coordination. Metal ion dependent changes of CD spectra provide similar conclusion for the coordination modes of copper(II) ions. CD spectra of the copper(II)–NH<sub>2</sub>–VRSSNN–NH<sub>2</sub> system obtained by increasing copper(II) concentration is plotted in Figure 7. It is evident from this Figure that in equimolar solution (100% copper(II) concentration) at pH 8.4 the characteristic negative Cotton effect of the dominating 4N [CuH<sub>3</sub>L] species is measured at 530 nm (spectrum 7.b), while it is 550 nm for the 3N complex (see spectrum 7.a). Figure 7 clearly demonstrates that the increase of copper(II) concentration at a constant pH shifts the absorption maxima towards the higher wavelength values. This observation supports the co-existence of the (NH<sub>2</sub>,N<sup>−</sup>,N<sup>−</sup>) and (N<sup>−</sup>,N<sup>−</sup>,N<sup>−</sup>) coordination modes in the species [Cu<sub>2</sub>H<sub>5</sub>L]. The former belongs to the amino terminus, while the latter is created around the asparaginyl sites. The metal binding on the C-termini of the molecule resulting in 3N<sup>−</sup> species can be realized in several ways, including the coordination of amide nitrogens of asparagine and peptide nitrogens together. (Scheme 2).

The structure of the supposed complexes, however, clearly shows non-coordinated amide nitrogens in the molecule. The deprotonation and coordination of these groups result in the formation of [Cu<sub>2</sub>H<sub>6</sub>L] and [Cu<sub>2</sub>H<sub>7</sub>L] complexes above pH 10. These processes are accompanied by small shift of the absorption maximum and Cotton effect of UV-Vis and CD-spectra, respectively (spectrum 7.h).



Scheme 2.

### Complexes of the heptapeptide NH<sub>2</sub>–GGHSSNN–NH<sub>2</sub>

Huge number of literature studies supports the outstanding copper(II) binding affinity of the peptides containing histidine as the third amino acid from the N-terminus in the sequence<sup>13,14,23</sup>. The heptapeptide NH<sub>2</sub>–GGHSSNN–NH<sub>2</sub> provides a good chance to compare the binding affinities of the GGH and SSNN sequences. Stability constants of the copper(II) complexes of this ligand and the spectroscopic parameters of the major species are collected in Table 5.

In agreement with the expectations the peptide can bind two equivalents of copper(II) ions. It is a common feature of the peptides with NH<sub>2</sub>–XXH sequence that the deprotonation of two amide nitrogens takes place in a cooperative process and the (NH<sub>2</sub>,N<sup>−</sup>,N<sup>−</sup>,N<sub>im</sub>) coordinated complexes are exclusively formed in a wide pH range<sup>23</sup>. The stability constants of the complexes [CuHL] and [CuH<sub>1</sub>L] can also be calculated but these species are present in negligible concentration in the slightly acidic samples. As a consequence, [CuH<sub>2</sub>L] is a single species from pH 5.0 to 10.0 and its spectral parameters are in good agreement with those reported for similar sequences<sup>23</sup>. In other words it means that in equimolar samples of copper(II) and NH<sub>2</sub>–GGHSSNN–NH<sub>2</sub> the amino terminus is the exclusive copper(II) binding site and the polar side chains of Ser and Asn residues do not have any contribution to the stability of copper(II) complexes. On the other hand, stable dinuclear complexes are formed in the presence of excess of

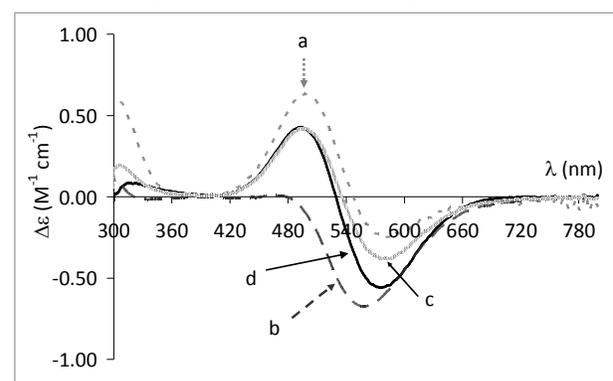


Fig.8. CD spectra of the [CuH<sub>2</sub>L] complex of NH<sub>2</sub>–GGH (a), [CuH<sub>4</sub>L] of Ac–SSNN–NH<sub>2</sub> (b), calculated spectra of [Cu<sub>2</sub>H<sub>6</sub>L] of NH<sub>2</sub>–GGHSSNN–NH<sub>2</sub> with the superposition of spectra “a” and “b” (c) and measured spectra of [Cu<sub>2</sub>H<sub>6</sub>L] of NH<sub>2</sub>–GGHSSNN–NH<sub>2</sub> (d).

Tab.5. Protonation and stability constants of the copper(II) complexes of the heptapeptide NH<sub>2</sub>–GGHSSNN–NH<sub>2</sub> and the spectroscopic parameters obtained for the major species. (T = 298 K, I = 0.2 M)

Species	log β <sub>pqr</sub>		UV-vis λ <sub>max</sub> /ε	CD λ <sub>max</sub> /Δε	ESR A <sub>  </sub> (x 10 <sup>−4</sup> cm <sup>−1</sup> ) / g <sub>  </sub>
[HL]	7.91(1)	–	–	–	–
[H <sub>2</sub> L]	14.19(1)	–	–	–	–
[CuHL]	11.43(31)	–	–	–	–
[CuH <sub>1</sub> L]	3.41(5)	–	–	–	–
[CuH <sub>2</sub> L]	−0.82(4)	530 / 93	590 / −0.24 500 / +0.85 306 / +0.30 276 / −0.25	198 / 2.192	
[Cu <sub>2</sub> H <sub>4</sub> L]	−11.14(19)	–	–	–	–
[Cu <sub>2</sub> H <sub>5</sub> L]	−19.12(19)	–	–	–	–
[Cu <sub>2</sub> H <sub>6</sub> L]	−27.84(13)	534 / 103	574 / −0.55 491 / +0.42		

copper(II) ions indicating that the SSNN domain can be an independent binding site. Unfortunately, the formation of dinuclear complexes is accompanied with rather small spectral changes therefore reliable parameters were obtained only for the final species  $[\text{Cu}_2\text{H}_6\text{L}]$  predominating above pH 10.0. The CD spectra of this species, however, can be well fitted with a simple superposition of the CD spectra of the  $[\text{CuH}_2\text{L}]$  complex of  $\text{NH}_2\text{-GGH}$  and  $[\text{CuH}_4\text{L}]$  of  $\text{Ac-SSNN-NH}_2$  as it is demonstrated by Figure 8.

These findings strongly support the participation of the deprotonated amide nitrogen of asparagine side chain in the metal binding of this ligand and the former discussed ligands ( $\text{NH}_2\text{-SSNN-NH}_2$ ,  $\text{NH}_2\text{-AANN-NH}_2$ ,  $\text{NH}_2\text{-VRSSNN-NH}_2$ ) as well.

## Conclusions

The results of combined potentiometric and spectroscopic studies on the copper(II) complexes of rat amylin fragments and their mutants provide sufficient information for the understanding of the possible role of Arg, Ser and Asn side chains in copper(II) binding. At first, all data rule out the involvement of arginyl residues in copper(II) binding, the coordination chemistry of the model peptide tetrarginine is quite similar to that of tetraalanine. The effect of the 19-22 domain SSNN sequence is, however, more complicated and largely depends on the length of the peptides and on the presence of other strongly coordinating groups. The comparison of the data obtained for the N-terminally free and acylated peptides unambiguously demonstrates the dominating role of the terminal amino group over the asparaginyl side chains in copper(II) binding. The coordination behaviors of the tetra- and hexa-peptides are, however, slightly different. In the case of tetrapeptides, when the side chains of asparagine are in the close vicinity of the coordinated peptide backbone a significant enhancement of thermodynamic stability can be observed, although the major binding modes are basically the same. Similar stabilizing role of non-coordinating side chain residues has already been observed in other peptide complexes, too<sup>24,25</sup>. On the contrary, this stability enhancement was not observed for the corresponding hexapeptides when the free rotation of the possibly coordinating side chains can occur. At the same time, these side chains can be independent anchoring sites for the binding of another copper(II) ion in dinuclear complexes. The primary ligating sites of these molecules are, however, always the amino terminus and/or a histidyl residue and the SSNN sequence can provide a secondary metal binding site.

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