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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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Abstract

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_2-VRSSNN-NH_2, NH_2-VRSSAA-NH_2, NH_2-VRAANN-NH_2, NH_2-VRSS-NH_2, NH_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2, 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_2,N^-,N^-) coordinated complexes was, however, enhanced by the asparaginyl moiety in the case of NH_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2. Among the hexapeptides the formation of dinuclear complexes was characteristic for NH_2-VRSSNN-NH_2 demonstrating the anchoring ability of the –SSNN– (SerSerAsnAsn) domain. The complexes of the heptapeptide NH_2-GGHSSNN-NH_2 were also studied and the data supported the above mentioned anchoring ability of the –SSNN– site.

Keywords
copper(II), stability constants, circular dichroism, ESR, amylin

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1. 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[1-3], while the impact of copper mediated ROS production was demonstrated in other works[4-6]. Moreover, it was also reported that copper(II) is able to modulate the proteolytic activity of IAPP degrading enzymes[7]. The biological significance of hIAPP promoted the investigations on the coordination chemistry of these peptides[8-9] 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[10,11]. 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[12]. 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 asparaginyl side chains. This observation was rather surprising in metallopeptide 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_2-VRSSNN-NH_2, its two mutants: NH_2-VRSSAA-NH_2 and NH_2-VRAANN-NH_2 and four tetrapeptides from the same domain: NH_2-VRSS-NH_2, NH_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2. Copper(II) complexes of tetraarginine (NH_2-RRRR-OH) were also studied for comparison and to obtain information on the possible impacts of guanidinium 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[13,14]) and it is linked to the previously suggested metal binding domain of rat amylin: NH_2-GGH-SSNN-NH_2

2. Experimental

2.1. Peptide synthesis and other materials

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-methyl-pyrrolidone (NMP), triisopropylsilane (TIS), 2,2'-(ethylenedioxy)diethanethiol, diethyl ether (Et_2O) 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.

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.

In the case of all other 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_2O/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_{18} 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.

2.2. Potentiometric measurements

The pH-potentiometric titrations were performed in 4 cm^3 samples at 2×10^{-3} - 3×10^{-3} M ligand concentration with the metal ion to ligand ratios 1:1 and 2:1 for binary systems. 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[15]. Protonation constants of the ligands and overall stability constants have been calculated by means of general computational programs, SUPERQUAD[16] and PSEQUAD[17] using equations (1) - (2).

$$pM + qH + rL = M_pH_qL_r \quad (1)$$

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

2.3. 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 $CuSO_4 \cdot 5H_2O$ enriched with ^{63}Cu to get better resolution of EPR spectra. Metallic copper (99.3% ^{63}Cu and 0.7% ^{65}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 [18,19]. The spectra of the individual species were determined by this method.

3. 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[10,14]. Therefore, the side chain residues of tetrapeptides cannot be independent metal binding sites, while specific sequences of a hexa- or hepta-peptide may provide 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.
Scheme 1.

3.1. 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. 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. This is best demonstrated by Figure 1, where the concentration distributions of three systems are plotted as a function of pH.
Table 1
Figure 1
Complex formation processes of oligopeptides generally starts in acidic pH range with the formation of a (NH_2,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_2-VRSS-NH_2. 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 form Table 1 that the stability constants of tetraarginine complexes are very similar to those of tetraglycine and tetraalanine 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 tetraalanine 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 $[CuH_{-2}L]$ complex of NH_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2 is very much favoured and exists as a major species in a relatively wide pH range. On the other hand, the species $[CuH_{-3}L]$ is favoured for NH_2-VRSS-NH_2 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_2-VRSS-NH_2 and NH_2-SSNN-NH_2. In the copper(II)–NH_2-VRSS-NH_2 system the species $[CuH_{-1}L]$ is the dominating complex below pH 7.0 and its ESR spectral parameters correspond well to those of the (NH_2,N^-,CO) coordination mode of peptides. For the other three tetrapeptides (NH_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2), however, $[CuH_{-2}L]$ is the major species in the same pH range and their CD and ESR spectral parameters can be best described by the simple (NH_2,2N^-) coordination mode. However, the positive Cotton effect around 480-500 nm in the CD spectra of the $[CuH_{-2}L]$ complexes of these peptides may suggest an interaction form 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 $[CuH_{-2}L]$ complexes of peptides containing asparaginyl residues.

The complex $[CuH_{-3}L]$ 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 (NH_2,3N^-) 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 NH_2-VRSS-NH_2 (see Figure 1 and 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. The lack of the C-terminal carboxylate residues in these peptides, however, may provide an explanation for this observation. In the case of tetraglycine, tetraalanine and tetraarginine 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 NH_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2 the asparaginyl side chains have an even more pronounced effect, but no chance for the extra stabilization for the complexes of NH_2-VRSS-NH_2 and it shifts the formation of the fully coordinated 4N complexes into a lower pH range. For the NH_2-SSNN-NH_2 the CD spectrum of $[CuH_{-3}L]$ is, however, different from that of NH_2-RRRR-OH or NH_2-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 $[CuH_{-4}L]$ of Ac-SSNN-NH_2[12].

Table 2
Figure 2
Figure 3

3.2. Complexes of hexapeptides

Our previous studies on the copper(II) complexes of the terminally blocked hexapeptide fragment 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 NH_2-VRSSNN-NH_2 and its two mutants NH_2-VRSSAA-NH_2 and NH_2-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.
Table 3
It is clear from Table 3 that formation of dinuclear complexes is characteristic only for the copper(II)–NH_2-VRSSNN-NH_2 system. A slight opalescence of the 2:1 = Cu(II):L samples was observed even with this ligand in the pH range 6-7 but it slowly 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_2-VRSSAA-NH_2 and NH_2-VRAANN-NH_2 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_2-VRSSNN-NH_2, 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 $[CuH_{-n}L]$ (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_2-VRSS-NH_2 in Table 1. This similarity in the thermodynamic data suggest the existence of the common $[NH_2,N^-]$, $[NH_2,N^-,N^-]$ and $[NH_2,N^-,N^-,N^-]$ coordination modes in the species $[CuH_{-1}L]$, $[CuH_{-2}L]$ and $[CuH_{-3}L]$, 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.
Table 4.
The formation of the 3N coordinated $[CuH_{-2}L]$ complexes overlaps significantly the 2N and 4N species and it is difficult to determine exact spectral parameters for this stoichiometry. The data obtained for $[CuH_{-1}L]$ and $[CuH_{-3}L]$ of NH_2-VRSSNN-NH_2, however, reveal a high similarity to those reported for NH_2-VRSS-NH_2 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 $[CuH_{-2}L]$ and $[CuH_{-3}L]$ 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_2-SSNN-NH_2, NH_2-SSNA-NH_2 and NH_2-AANN-NH_2 where a significant contribution of the aspargine moiety was suggested especially to the overall stability of the [$CuH_{-2}L$] 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_2-VRSSNN-NH_2 system, however, gives an unambiguous proof for the anchoring capacity of the free asparagine side chains (see Fig. 4).

Figure 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 [$CuH_{-1}L$] complex can be well extracted (see Table 3). Spectrum 5.b belongs to [$CuH_{-3}L$] 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 and it is due to a dimeric species with $g_z = 2.202$ and $A_z = 96 \times 10^{-4}$ cm^{-1}. The increasing baseline of this spectrum is also indicate some magnetic interaction between the copper(II) centers. 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 8.40 at 2:1 ratio with that calculated for the species [$Cu_2H_{-5}L$] is another indication for the existence of dinuclear complexes.

Figure 5

Figure 6

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 [$Cu_2H_{-5}L$]. 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_2-VRSSNN-NH_2 system obtained by increasing copper(II) concentration is plotted in Figure 7.

Figure 7.

It is evident from this Figure that at 1:1 ratio (or at 100% copper(II) concentration) and at pH 8.4 the characteristic negative Cotton effect of the dominating 4N [$CuH_{-3}L$] 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_2,N^-,N^-) and ($3N^-$) coordination modes in the species [$Cu_2H_{-5}L$]. 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 $3N^-$ species can be realized in several way, including the coordination of amide nitrogens of asparagine and peptide nitrogens together . (Scheme 2).

Scheme 2.

The structure of the supposed complexes, however, clearly shows the non-coordinated amide nitrogens in the molecule. The deprotonation and coordination of these groups result in the

formation of $[Cu_2H_{-6}L]$ and $[Cu_2H_{-7}L]$ 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).

3.3. Complexes of the heptapeptide NH_2-GGHSSNN-NH_2

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[13,14,22] . The heptapeptide NH_2-GGHSSNN-NH_2 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.

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_2-XXH.. sequence that the deprotonation of two amide nitrogens takes place in a cooperative process and the (NH_2,N^-,N^-,N_{im}) coordinated complexes are exclusively formed in a wide pH range[22]. The stability constants of the complexes [CuHL] and $[CuH_{-1}L]$ can also be calculated but these species are present in negligible concentration in the slightly acidic samples. As a consequence, $[CuH_{-2}L]$ 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[22]. In other words it means that in equimolar samples of copper(II) and NH_2-GGHSSNN-NH_2 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 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 $[Cu_2H_{-6}L]$ 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 $[CuH_{-2}L]$ complex of NH_2-GGH and $[CuH_{-4}L]$ of Ac-SSNN-NH_2 as it is demonstrated by Figure 8.

Figure 8.

These findings strongly support the participation of the deprotonated amide nitrogen of asparagine side chain in the metal binding this ligand and the former discussed ligands (NH_2-SSNN-NH_2, NH_2-AANN-NH_2, NH_2-VRSSNN-NH_2) as well.

4. 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[23,24]. 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.

References

1 B. Ward, K. Walker, C. Exley, J. Inorg. Biochem., 102 (2008), 371-375.
2 C. Exley, E. House, T. Patel, L. Wu, P.E. Fraser, J. Inorg. Biochem., 104 (2010) 1125-1129.
3 A. Sinopoli, A. Magri, D. Milardi, M. Pappalardo, P. Pucci, A. Flagiello, J.J. Titman, V.G. Nicoletti, G. Caruso, G. Pappalardo, Metallomics, 6 (2014) 1841-1852.
4 A. Masad, B.J. Tabner, J. Mayes, D. Allsop, Free Rad. Biol. Med., 51 (2011) 869-875.
5 E.C. Lee, E. Ha, S. Singh, L. Legesse, S. Ahmad, E. Karnaukhova, R.P. Donaldson, A.M. Jeremic, Phys. Chem. Chem. Phys., 15 (2013) 12558-12571.
6 L. Ma, X. Li, Y. Wang, W. Zheng, T. Chen., J. Inorg. Biochem., 140 (2014) 143-152.
7 F. Bellia, G. Grasso, J. Mass Spectr., 49 (2014) 274-279.
8 A. Amoresano, A. Flagiello, G. Impellizzeri, A. Magri, G. Pappalardo, P. Pucci, E. Rizzarelli, in Peptide Revolution: Genomics, Proteomics, Therapeutics, American Peptide Society (2003) 771-772.
9 M.J. Kim, H.T. Kim, Eur. J. Mass Spectr., 18 (2012) 51-58.
10 H. Sigel, R.B. Martin, Chem. Rev., 82 (1982) 385-426
11 I. Sóvágó, C. Kállay, K. Várnagy, Coord. Chem., Rev., 256 (2012) 2225-2233.
12 C. Kállay, Á. Dávid, S. Timári, E.M. Nagy, D. Sanna, E. Garribba, G. Micera, P. de Bona, G. Pappalardo, E. Rizzarelli, I. Sóvágó, Dalton Trans., 40 (2011) 9711-9721.
13 N. Camerman, A. Camerman, B. Sarkar, Can. J. Chem., 54 (1976) 1309-1316.
14 I. Sóvágó, K. Ősz, Dalton Trans., (2006) 3841-3854.
15 H. Irving, G. Miles, L.D. Pettit, Anal. Chim. Acta 38 (1967) 475-488.
16 L. Zékány and I. Nagypál, in Computational Methods for the Determination of Stability Constants (Eds. D.J. Leggett), Plenum Press, New York 1985.
17 P. Gans, A. Sabatini, A. Vacca, J. Chem. Soc., Dalton Trans., 1985, 1195.
18 A. Perczel, M. Hollosi, G. Tusnady, and G. D. Fasman, Croatica Chim. Acta., 1989, 62, 189-200.
19 A. Perczel, M. Hollosi, G. Tusnady and G. D. Fasman, Protein Engineering, 1991, 4, 669-679.
20 I. Sóvágó, D. Sanna, A. Dessi, K. Várnagy, G. Micera, J. Inorg. Biochem., 63 (1996) 99-117.
21 J.-F. Galey, B. Decock-Le Reverend, A. Lebkiri, L.D. Pettit, S.I. Pyburn, H. Kozlowski, J. Chem.Soc., Dalton Trans., (1991) 2281- 2287.
22 Á. Grenács, A. Kaluha, C. Kállay, V. Jószai, D. Sanna, I. Sóvágó, J. Inorg. Biochem., 128 (2013) 17-25.
23 W.J. Puspita, A. Odani, O. Yamauchi, J. Inorg. Biochem., 73 (1999) 203-213.
24 A. Janicka-Klos, E. Porciatti, D. Valensin, C. Conato, M. Remelli, S. Oldziej, G. Valensin, H. Kozlowski, Dalton Trans., 42 (2013) 448-458.

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

Species	NH_2-VRSS-NH_2	NH_2-SSNN-NH_2	NH_2-AANN-NH_2	NH_2-SSNA-NH_2	NH_2-RRRR-OH	NH_2-GGGG-OH (Ref. [20])	NH_2-AAAA-OH (Ref. [21])
pK(-COOH)	-	-	-	-	3.00(2)	3.24	3.52
pK($-NH_3^+$)	7.46(1)	6.99(1)	7.86(2)	7.01(1)	7.13(1)	7.94	8.13
$[CuL]^{2+/+}$	4.03(9)	-	-	-	-	5.06	4.77
$[CuH_{-1}L]^{+/0}$	-0.85(1)	-0.35(3)	-0.28(2)	-0.29(2)	-1.11(2)	-0.50	-0.45
$[CuH_{-2}L]^{0/-}$	-7.66(2)	-5.27(1)	-5.59(1)	-5.33(7)	-8.32(4)	-7.41	-8.09
$[CuH_{-3}L]^{-/2-}$	-15.02(1)	-13.99(3)	-14.36(2)	-13.97(2)	-17.22(5)	-16.59	-17.33
pK(H_{-1}/H_{-2})	6.81	4.92	5.31	5.04	7.21	6.91	7.64
pK(H_{-2}/H_{-3})	7.36	8.72	8.77	8.64	8.90	9.18	9.24

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

Peptide	Method	$[CuH_{-1}L]$	$[CuH_{-2}L]$	$[CuH_{-3}L]$
NH_2–AAAA–OH	UV–vis	652 / 93	563 / 138	515 / 165
	CD	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	–	–	–
NH_2–RRRR–OH	UV–vis	–	–	517 / 187
	CD	–	–	–
	ESR	175 / 2.252	179 / 2.216	206 / 2.173
NH_2–VRSS–NH_2	UV–vis	664 / 66	552 / 111	515 / 139
	CD	679 / –0.47 320 / +0.52	568 / –0.79 313 / +0.71	517 / –1.30 304 / +1.13
	ESR	177 / 2.254	–	203 / 2.182
NH_2–SSNN–NH_2	UV–vis	–	550 / 128	516 / 121
	CD	–	586 / –0.63 492 / +0.26 309 / +0.99	560 / –1.14 479 / +0.23 304 / +0.94
	ESR	–	205 / 2.209	200 / 2.180
NH_2–SSNA–NH_2	UV–vis		550 / 105	519 / 92
	CD	–	577 / –0.61 490 / +0.20 309 / +0.68	562 / –1.00 483 / +0.36 307 / +0.63
	ESR		208 / 2.203	200 / 2.181
NH_2–AANN–NH_2	UV–vis	–	547 / 109	510 / 103
	CD	–	580 / –0.46 492 / +0.16 306 / +0.68	554 / –0.84 470 / +0.05 303 / +0.55
	ESR	–	202 / 2.207	204 / 2.177

Table 3
pK values of hexapeptides and stability constants (log β_{pqr}) of their copper(II) complexes. (T = 298 K, I = 0.2 M)

Species	NH_2-VRSSNN-NH_2	NH_2-VRSSAA-NH_2	NH_2-VRAANN-NH_2
$[HL]^+$	7.50(3)	7.53(2)	7.55(2)
$[CuL]^{2+}$	3.76(18)	4.22(8)	4.11(9)
$[CuH_{-1}L]^+$	-0.61(1)	-0.69(1)	-0.65(1)
$[CuH_{-2}L]$	-7.13(5)	-7.47(2)	-7.80(2)
$[CuH_{-3}L]^-$	-14.20(3)	-14.71(2)	-15.45(2)
$[Cu_2H_{-4}L]$	-16.11(2)	-	-
$[Cu_2H_{-5}L]^-$	-23.74(4)	-	-
$[Cu_2H_{-6}L]^{2-}$	-33.37(7)	-	-
$[Cu_2H_{-7}L]^{3-}$	-42.25(4)	-	-
pK(H_{-1}/H_{-2})	6.52	6.78	7.15
pK(H_{-2}/H_{-3})	7.07	7.24	7.65

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

Peptide	Method	$[CuH_{-1}L]^+$	$[CuH_{-2}L]$	$[CuH_{-3}L]^-$
NH_2-VRSSNN-NH_2	UV-Vis	652 / 78	563 / 128	517 / 189
	CD	692 / –0.47 322 / +0.42	576 / –0.78 313 / +0.78	532 / –1.21 307 / +0.83
	ESR	176 / 2.256	–	206 / 2.183
NH_2-VRSSAA-NH_2	UV-Vis	673 / 70	564 / 138	520 / 194
	CD	675 / –0.23 318 / +0.29	565 / –0.50 314 / +0.39	531 / –0.97 307 / +0.45
NH_2-VRAANN-NH_2	UV-Vis	652 / 88	559 / 140	513 / 182
	CD	679 / –0.51 316 / +0.63	552 / –0.82 317 / +1.01	537 / –1.33 310 / +0.72

Table 5
Protonation and stability constants of the copper(II) complexes of the heptapeptide NH_2-GGHSSNN-NH_2 and the spectroscopic parameters obtained for the major species.
(T = 298 K, I = 0.2 M)

Species	log β_{pqr}	UV-Vis $\lambda_{max}/\varepsilon$	CD $\lambda_{max}/\Delta\varepsilon$
$[HL]^{+}$	7.91(1)	–	–
$[H_2L]^{2+}$	14.19(1)	–	–
$[CuHL]^{3+}$	11.43(31)	–	–
$[CuH_{-1}L]^{+}$	3.41(5)	–	–
$[CuH_{-2}L]$	–0.82(4)	530 / 93	590 / –0.24 500 / +0.85 306 / +0.30 276 / –0.25
$[Cu_2H_{-4}L]$	–11.14(19)	–	–
$[Cu_2H_{-5}L]^{-}$	–19.12(19)	–	–
$[Cu_2H_{-6}L]^{2-}$	–27.84(13)	534 / 103	574 / –0.55 491 / +0.42

Legends to Figures

Figure 1
Concentration distribution of the major species as a function of pH in the copper(II)-tetraalanine (a: ---), NH_2-SSNN-NH_2 (b:──) and NH_2-VRSS-NH_2 (c -○○○) systems. $c_{Cu(II)} = c_L = 2$ mM

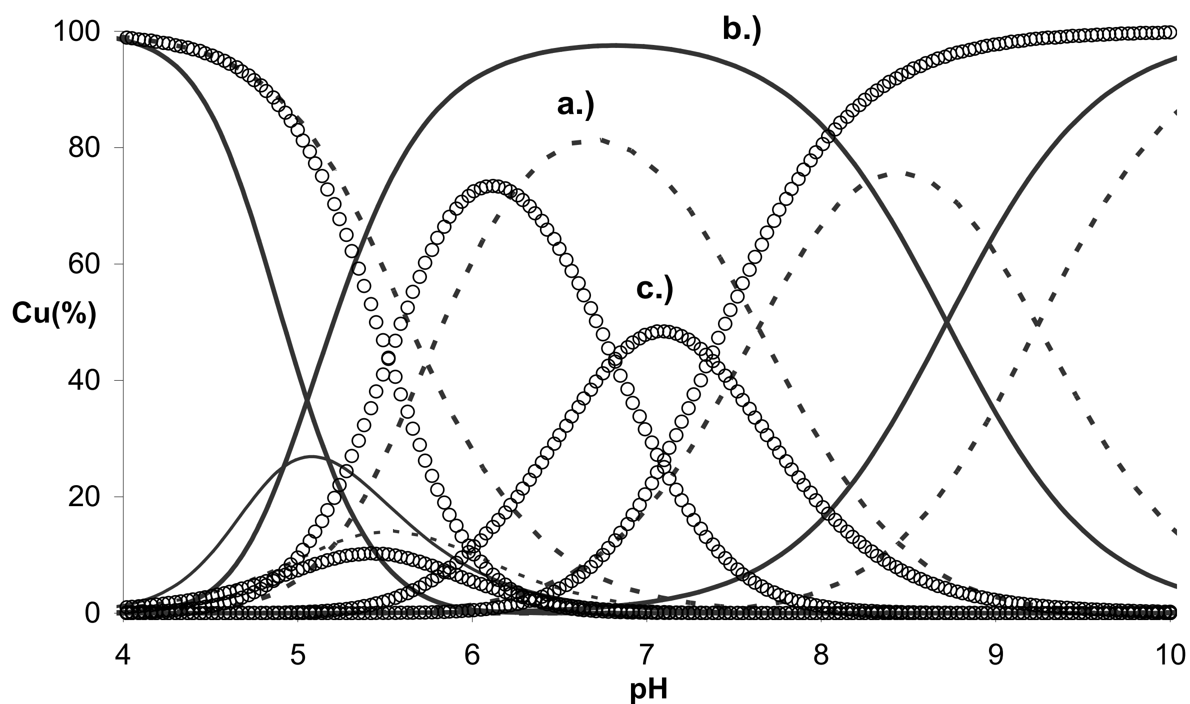


Figure 2
CD spectra of the copper(II) complexes of NH_2-VRSS-NH_2 measured in equimolar samples at pH 6.86 and 10.94 and for NH_2-SSNN-NH_2 at pH 7.03 and 10.94.

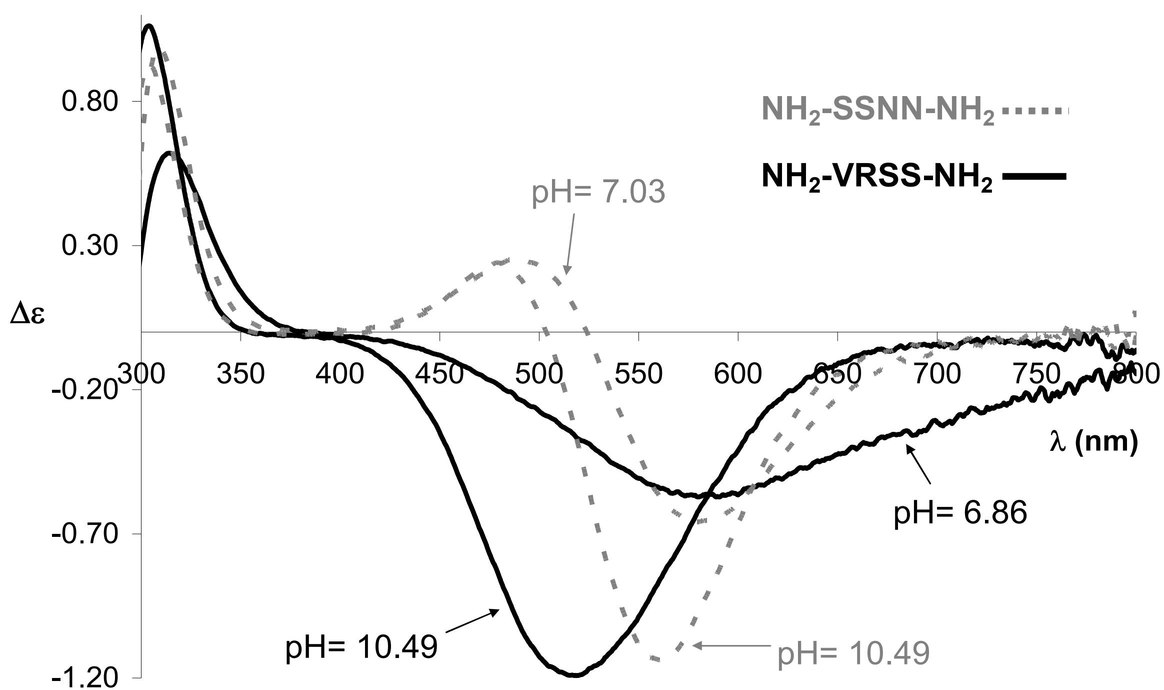


Figure 3
ESR spectra of the copper(II) complexes measured in equimolar samples of NH_2-VRSS-NH_2 at pH 7.11 (a) and 10.11 (b) and of NH_2-SSNN-NH_2 at pH 7.12 (c) and 9.96 (d).

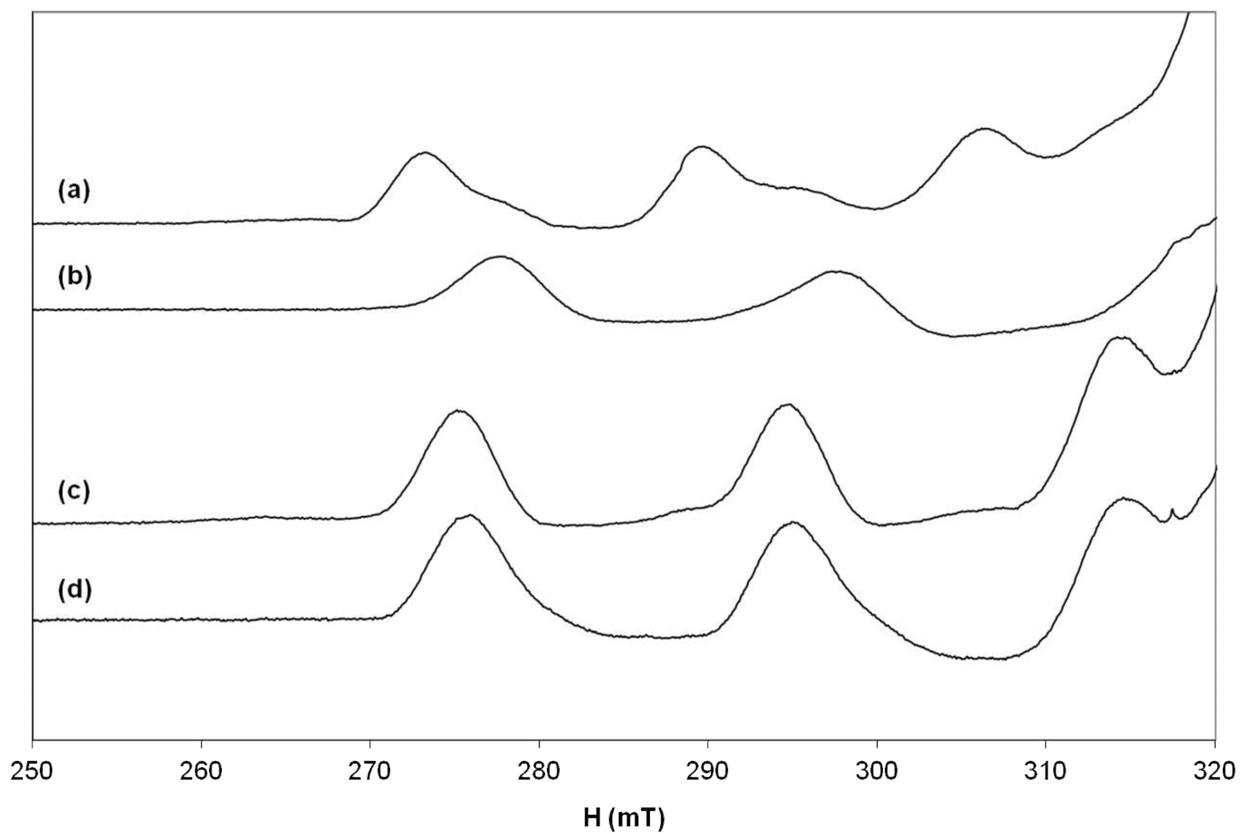


Figure 5

ESR spectra recorded in the copper(II)–NH_2-VRSSNN-NH_2 system at 1:1 ratio pH = 6.86 (a), 1:1 ratio pH = 10.08 (b) and 2:1 ratio pH = 10.01 (c).

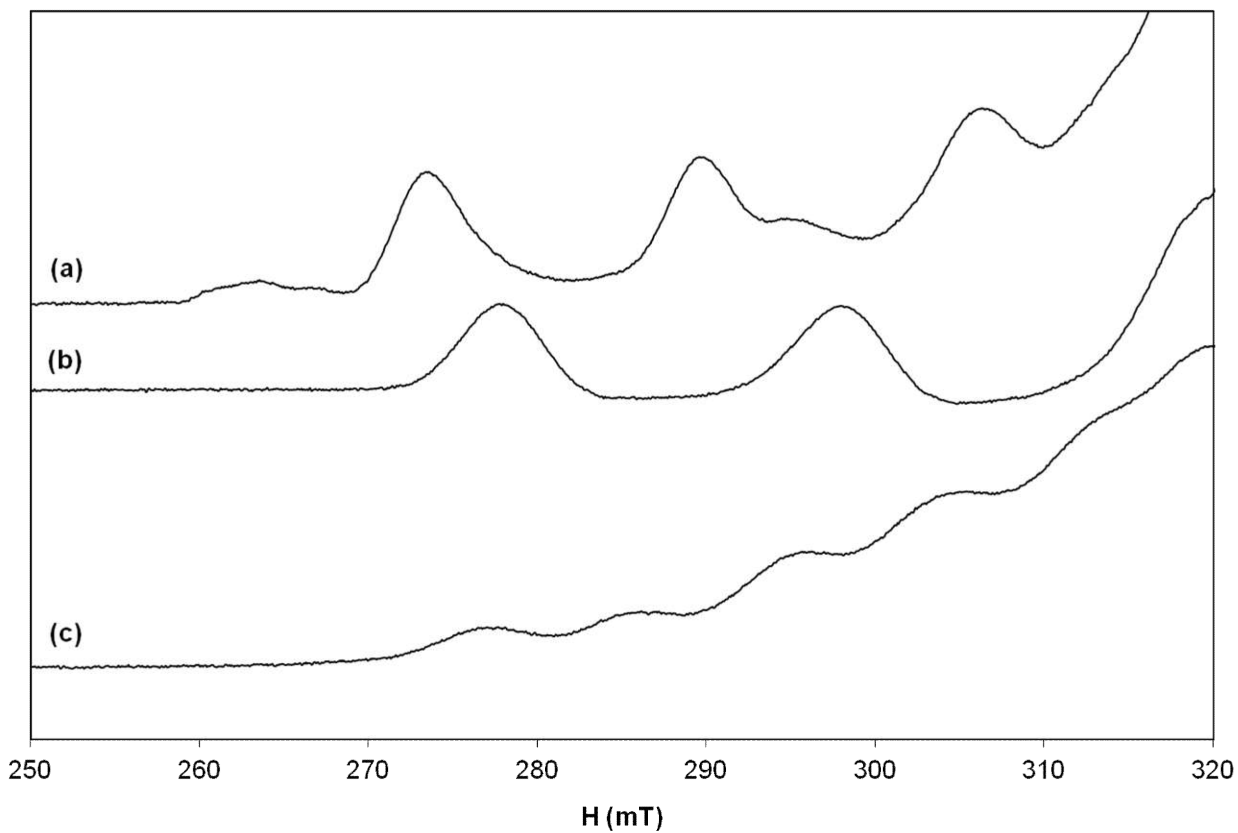


Figure 6

ESI-MS spectra recorded in the copper(II)-NH_2-VRSSNN-NH_2 system at 2:1 ratio pH = 8.4. experimental (a), calculated for $[Cu_2H_{-5}L]^-$ (b).

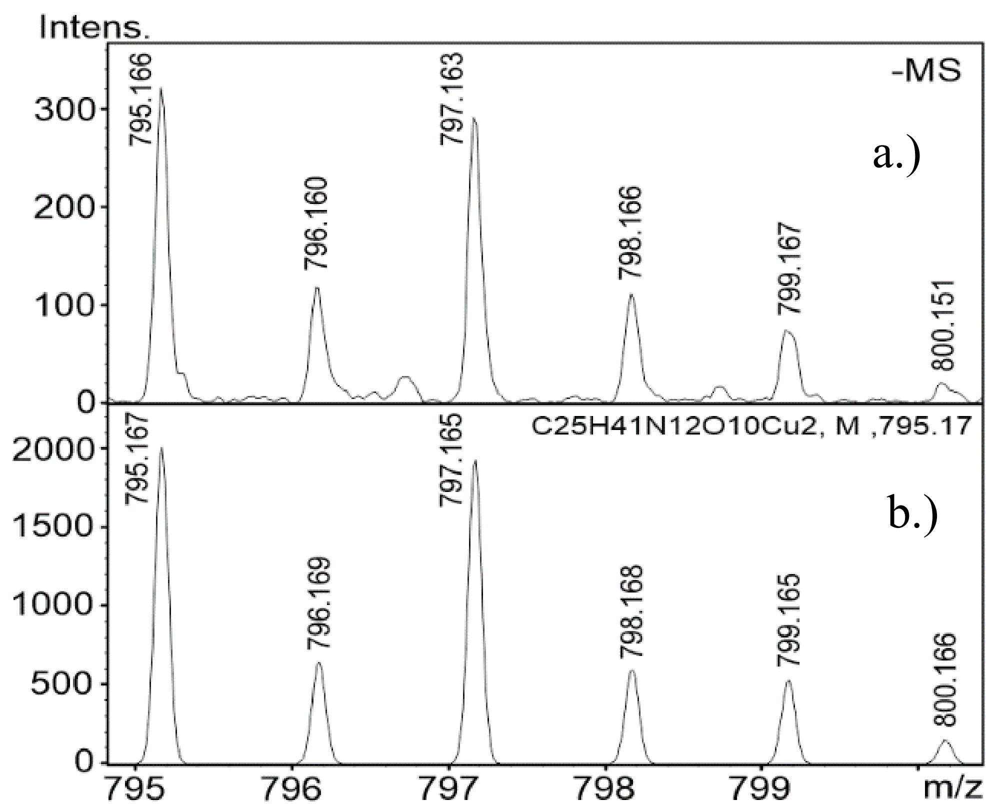
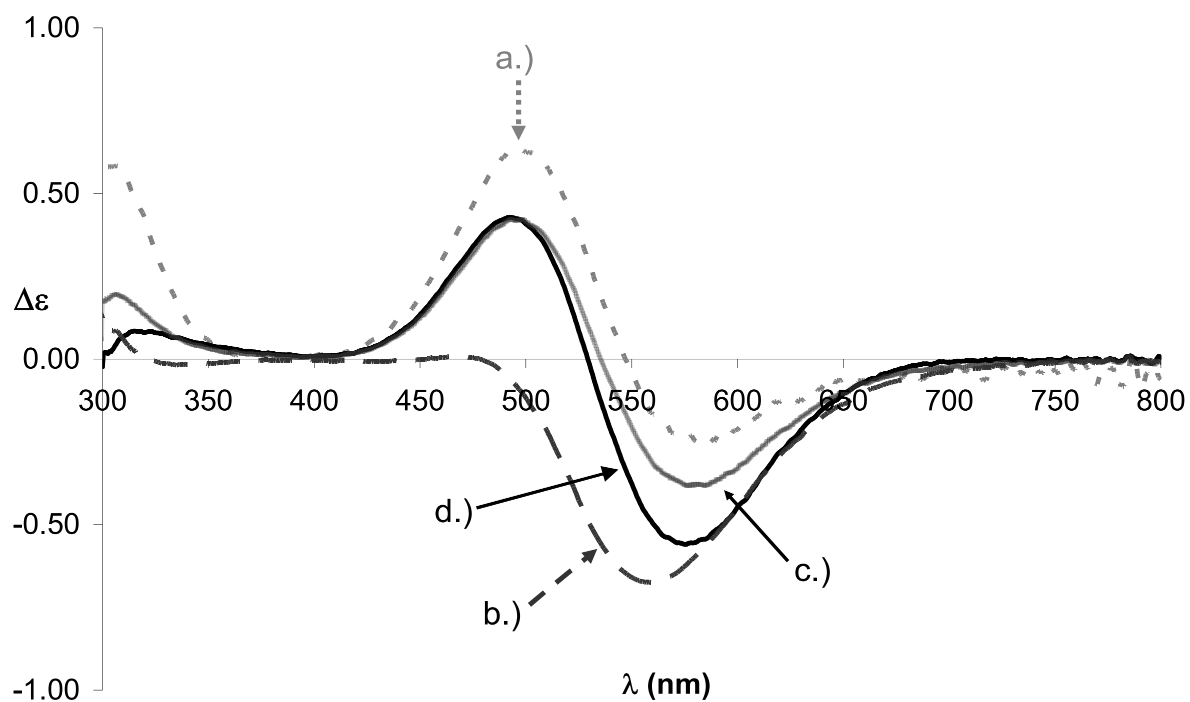


Figure 7

CD spectra of species [$CuH_{-2}L$] (a), species [$CuH_{-3}L$] (b) and the spectra recorded by increasing copper(II) concentration at pH 8.4, 120 % copper(II) (c), 140% (d), 160% (e), 180% (f) and 200% (g).

Figure 8

CD spectra of the $[CuH_{-2}L]$ complex of NH_2-GGH (a), $[CuH_{-4}L]$ of Ac-SSNN-NH_2 (b), calculated spectra of $[Cu_2H_{-6}L]$ of NH_2-GGHSSNN-NH_2 with the superposition of spectra "a" and "b" (c) and measured spectra of $[Cu_2H_{-6}L]$ of NH_2-GGHSSNN-NH_2.



Graphical abstract