

Time- and pH-dependent copper binding to A β (1-16) peptide: an electrospray ionization-mass spectrometric approach

Marilena Manea,^a Gitta Schlosser,^b and Manuela Murariu^{c,*}

^aUniversity of Konstanz, Department of Chemistry and Zukunftskolleg,
Universitaetsstrasse 10, 78457 Konstanz, Germany

^bEötvös Loránd University, Research Group of Peptide Chemistry, 1/A Pázmány Péter
Sétány, 1117 Budapest, Hungary

^cPetru Poni Institute of Macromolecular Chemistry, 41A Grigore Ghica Voda Alee,
700487 Iasi, Romania, manuelam@icmpp.ro

Abstract

An elevated concentration of copper ions in the brain of Alzheimer's disease (AD) patients has been reported in many studies and might be associated with an increased aggregation of β -amyloid (A β) peptides. In the present work, the interaction with copper ions of a model β -amyloid peptide, A β (1-16), was investigated by electrospray ionization-mass spectrometry (ESI-MS) at two pH values, 7.4 and 6.6, as well as at various peptide: copper ion ratios in the first minutes after components mixing and time intervals. Our results indicated that copper ions specifically bound to A β (1-16) peptide in solution and that the complex formation increased with time. Once formed in solution, Cu²⁺-A β (1-16) complexes could easily be detected in the gas phase by ESI-MS. The pH shift from 7.4 to 6.6 only slightly influenced the Cu²⁺ binding to A β (1-16). No oligomerization of A β (1-16) peptide was noticed in the first minutes of copper-peptide interaction.

Keywords: ESI-MS; β -amyloid peptide; Copper-peptide complex; pH, Alzheimer's disease.

Introduction

There is increasing evidence that heavy metal ions are involved in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD).¹⁻⁵ The aggregation of β -amyloid peptides (A β) and subsequent neurotoxicity may be triggered by abnormal

interactions with neocortical metal ions, especially copper, zinc, iron and aluminum.^{2,6} Recent data suggest that the intracellular pool of A β is regulated by the activity of endothelin-converting enzymes at the sites of production.⁷ However, the deregulation of copper seems to be intimately involved in the pathogenesis of AD.^{8,9} Copper is known to accelerate the aggregation of A β (1-40) and A β (1-42) peptides, the major components of A β deposits.¹⁰ Nevertheless, short and easy-to-handle peptides have been used to study the structures and mechanisms that are also relevant for the native and longer peptides involved in neurodegenerative diseases.^{11,12} Moreover, some previous ESI-MS studies have reported on the binding of Cu²⁺ to A β (1-16) at physiological pH and its effect on peptide conformation and oligomerisation.¹² The copper binding sites are located in the N-terminal region of A β peptides.^{2,13-15} Furthermore, the mode of copper binding to peptides seems to be highly pH dependent.¹⁶⁻¹⁹ The most common methods used to investigate the interaction of metal ions with amyloidogenic peptides have already been reviewed.^{20,21} Yet, it is less known if copper-peptide complexes are formed in the gas phase as non-specific adducts or in solution.

We explore here the hypothesis that under in-solution conditions (pH, time of reaction, metal and peptide concentrations) various species of copper-peptide complexes may result, which are mostly conserved in the gas phase and can be detected by ESI-MS. Thus, the present work aims at investigating the formation of complexes between A β (1-16) and copper ions in solution, both at pH 6.6 and pH 7.4.

Experimental

Materials. All reagents were of analytical grade or highest available purity. The solutions were prepared using MilliQ grade water (18 M Ω ·cm). The A β (1-16) peptide (H-DAEFRHDSGYEVHHQK-OH) was purchased from Bachem AG (Bubendorf, Switzerland).

ESI-ion trap mass spectrometry. Electrospray ionization mass spectrometric analyses were carried out on a Bruker Daltonics Esquire 3000plus ion trap mass spectrometer (Bremen, Germany). Spectra were acquired in the 50-2000 m/z range. Binding of metal ions to A β (1-16) peptide was investigated by ESI-ion trap MS using 5 mM ammonium acetate, pH 7.4 or 6.6 as a solvent. Peptide concentration was 10 μ M and the peptide: copper ion ratios were 1:1, 1:2 and 1:10. A β (1-16) and CuSO₄ were first dissolved in 5 mM ammonium acetate and mixed prior to MS analysis. Copper binding to A β (1-16) peptide was followed over time, in the first minutes after mixing, while recording the mass spectra (no preincubation of the samples was performed). The experiments were carried out either under nearly physiological

conditions (5 mM salt concentration, pH 7.4) or at pH 6.6, representing the pH value determined in the case of inflammatory processes.

Circular dichroism (CD) spectroscopy. CD spectra were recorded on a Jasco J-715 spectropolarimeter at room temperature, in quartz cells of 0.05 cm path length, under constant nitrogen flush. The A β (1-16) peptide was dissolved in 5 mM ammonium acetate, at a concentration of 0.2 mM, while the molar ratio Cu²⁺: A β (1-16) was 2:1. The spectra were recorded after preincubating the samples for 1 min and 7 min, respectively; the spectra were averages of six scans in the wavelength range 180 - 260 nm. Molar ellipticity was expressed as deg cm² dmol⁻¹.

Results

Copper binding to A β (1-16) peptide. Using ESI-MS, we found that the binding of Cu²⁺ to A β (1-16) peptide was significantly influenced by the molar ratio of peptide: copper ion and the time of peptide-metal ion contact, being only slightly influenced by the pH. However, more copper was bound to A β (1-16) at pH 6.6 than at pH 7.4, although the complex formation was faster at pH 7.4. A β (1-16) peptide, at a concentration of 10 μ M in 5 mM ammonium acetate, pH 7.4, displayed a peak in the ESI-mass spectrum at m/z 652.6 and another one at m/z 978.5 that were assigned to [M+3H]³⁺ and [M+2H]²⁺ ions (Fig. 1a). Under the same experimental conditions and upon adding Cu²⁺, the intensity of the peptide peak decreased to 26.8% in the first 0.1-0.5 min after mixing the peptide with the copper ions. The average mass spectrum between 7.3 and 7.6 min showed a further decrease in the intensity of the peptide peak (15.3%), whereas that of [M+Cu+H]³⁺ ion was found to be 220.7% higher than that of the peptide (Fig. 1c). Copper ions demonstrated a high affinity toward A β (1-16), since [M+Cu+H]³⁺ ion appeared immediately after mixing the two components and its intensity was high enough (96.1% of peptide intensity). The formation of copper-peptide complex was also observed at m/z 1009.4, the peak being assigned to [M+Cu]²⁺ ion.

A β (1-16) peptide bound only one Cu²⁺ at both pH 7.4 and 6.6, at a molar ratio of 1:1 peptide: Cu²⁺. At pH 6.6, for the time interval 0.2-0.7 min from mixing the components (Fig. 2a), the [M+Cu+H]³⁺: [M+3H]³⁺ intensity ratio was found to be lower than that at pH 7.4 (57.1% vs 96.1%). However, while the intensity of the peak corresponding to [M+Cu+H]³⁺ ion was rather the same ($6 \cdot 10^4$ at pH 6.6 vs $4.9 \cdot 10^4$ at pH 7.4), that of the peptide ion was much higher ($10.5 \cdot 10^4$ at pH 6.6 vs $5.1 \cdot 10^4$ at pH 7.4), most probably due to the higher proton concentration of the solution and better ionization of the peptide under slightly acidic

conditions. Over time (7.9-8.1 min, pH 6.6), the intensity of $[M+Cu+H]^{3+}$ ion doubled, while that of the peptide ion decreased three times (Fig. 2b). When compared with the results obtained at pH 7.4, both the $[M+Cu+H]^{3+}$ intensity and the $[M+Cu+H]^{3+} : [M+3H]^{3+}$ intensity ratio were found to be higher ($12.0 \cdot 10^4$ vs $6.4 \cdot 10^4$; 3.43 vs 2.66).

When copper: A β (1-16) ratio increased up to 10, the intensity of the peaks corresponding to the triply charged ions, $[M+Cu+H]^{3+}$, suddenly increased as well; no trace of peptide ion was found at pH 7.4, while a low intensity peak was observed at m/z 652.7, being assigned to $[M+3H]^{3+}$ molecular ion. At pH 6.6 and about 0.5 min reaction time, the $[M+3H]^{3+} : [M+Cu+H]^{3+} : [M+2Cu-H]^{3+}$ intensity ratio was 1: 8.9: 2.8. In only eight minutes, $[M+3H]^{3+}$ ion completely disappeared, $[M+Cu+H]^{3+}$ decreased dramatically, while $[M+2Cu-H]^{3+}$ became the main peak in the mass spectrum (Fig. 2d). Both at pH 7.4 and pH 6.6, the $[M+2Cu-H]^{3+}$ ion peak had the same intensity. Interestingly, $[M+Cu+H]^{3+}$ ion displayed at m/z 673.3 and pH 7.4 a peak as intense as that characteristic for the $[M+3Cu-3H]^{3+}$ ion at pH 6.6. Hence, more copper was bound to A β (1-16) at pH 6.6 than at pH 7.4, although the complex formation was faster at pH 7.4.

Copper binding stoichiometry. ESI-MS showed binding stoichiometries of up to 1:3 (pH 7.4) and 1:4 (pH 6.6) for the peptide-Cu²⁺ complexes, when Cu²⁺: peptide molar ratio was 10:1 (Fig. 3). We assumed that in spite of the elevation of both peptide and copper ion concentrations during the ionization process than in the incubation buffer, the ratio between the copper ion and A β (1-16) would be fixed even after evaporation of the solvent as shown by other studies.^{12,22} Moreover, since there is an increase in the copper content of Cu²⁺-A β (1-16) complexes from fractions with higher copper to peptide ratios, this would suggest that the metal was attached to the peptide prior to ESI-MS analysis. We demonstrate here that A β (1-16) is able to bind up to three Cu²⁺ ions at pH 7.4, and up to four Cu²⁺ ions when the pH value decreased to 6.6.

CD spectroscopy. The CD spectra presented in Fig. 4 indicated that the binding of copper ions to A β (1-16) could significantly influence the peptide conformation. The far UV CD spectrum of an aqueous solution of A β (1-16) (0.2 mM concentration) was characterized by two negative maxima at 191 nm and 198 nm, and other two positive ones at 183 nm and 220 nm, indicating predominant random coil conformation. In the first minute of copper interaction with A β (1-16), when a relatively small proportion of metal ions was bound to the peptide, a slight conformational change of the peptide was observed. The spectral features were comparable to those of the peptide in the absence of copper ions; however, the bands at 183 and 191 nm had increased intensity, while the negative maximum at 198 nm shifted to

196.5 nm. Moreover, the positive band at 200 nm decreased to negative values and shifted to 203 nm. Over time (7-8 min), more copper bound to A β (1-16), resulting in a completely different CD spectrum of the peptide, in which only its ellipticity in the range from 200 nm to 260 nm was similar to that for the spectrum recorded at about 1 min from mixing. Two negative maxima at 199.5 nm (69.1% of the negative ellipticity of peptide at 198 nm) and 194.5 nm (73.7%) were the main changes in the spectrum, while the positive maximum at 183 nm shifted to 186 nm and had decreased ellipticity. The CD spectrum after 7 min may indicate the presence of a mixture of conformers.

These data clearly indicate that copper ions bind to A β (1-16) over time in solution and not in the gas phase. Nevertheless, the possibility of forming non specific adducts in the gas phase was not excluded.

Time- and pH dependences of copper binding. The time-dependent formation of species of A β (1-16):Cu²⁺ complex was followed by mass spectrometry at pH 6.6 and 1:10 peptide: copper molar ratio (Fig. 5), and at pH 7.4 and 1:1 peptide: copper molar ratio (Fig. 6). At high copper: peptide molar ratio, the free peptide almost disappeared in the first 2 minutes, while the intensity of 1:1 Cu²⁺:A β (1-16) species increased dramatically in the first minute after mixing to decrease quickly in the second one. Once formed, the 1:1 Cu²⁺:A β (1-16) complex did bind the second copper ion to form the 2:1 Cu²⁺:A β (1-16) complex, whose intensity also increased for the time interval from minute 1 to minute 2, and remained almost constant up to minute 6. The 3:1 Cu²⁺:A β (1-16) complex showed an increasing intensity for 1.5 min after mixing the solutions, followed by a rather unchanged intensity of MS peak for the time interval of 0.5 min, between minute 1.5 and minute 2, the time when the intensity of 2:1 Cu²⁺:A β (1-16) complex increased based on copper binding to 1:1 Cu²⁺:A β (1-16) complex (Fig. 5). After minute 6, an increase in the intensity of 3:1 Cu²⁺:A β (1-16) complex on the expense of 2:1 Cu²⁺:A β (1-16) complex intensity was observed, while the 3:1 Cu²⁺:A β (1-16) complex formation for 2-4.5 min interval was dependent on 1:1 Cu²⁺:A β (1-16) complex. Taken together, the sum of the intensities of complexes with one, two, and three copper ions increased quickly in the first two minutes and remained almost constant for the next minutes of experiments.

At 1:1 copper: A β (1-16) peptide molar ratio, the intensity of free peptide peak decreased slowly and continuously over the experimental time (Fig. 6). The 1:1 Cu²⁺:A β (1-16) complex formed quickly in the first two minutes from mixing the two solutions, but the process continued up to minute 4, when a maximum of intensity was attained. Both intensities of 2:1 Cu²⁺:A β (1-16) and 3:1 Cu²⁺:A β (1-16) complexes increased slowly.

Discussion

Our results revealed the time-dependent formation of peptide-metal ion complexes in solution, namely A β (1–16): Cu²⁺ complexes that could be detected by ESI-MS in the gas phase. The complex formation was mainly dependent on the metal concentration and less on pH; however, it seemed to be faster at higher pH, whereas the lower pH stimulated the appearance of complexes containing more than two copper ions.

In contrast to other studies demonstrating the formation of oligomeric species of A β (1–16) peptide in the presence of Cu²⁺ ions,^{2,11-12} we noticed no oligomerization in the first minutes of copper-peptide interaction during the ESI-MS analysis. Working under different experimental conditions (preincubated samples, pH 5.5, higher concentrations of copper ions and peptide), copper was found to accelerate the aggregation of A β (1-16).¹² However, such complexes did not appear under the experimental conditions described here, probably due to the short time of copper-peptide interaction.

One can expect to find more copper ions bound to A β monomer at pH 7.4 than at pH 6.6, since higher pH may favor copper binding regardless of peptide sequence.²³ Nevertheless, more copper was found to be bound at lower pH, suggesting that brain pH changes could also play a role in various neurodegenerative pathologies.

These results are in agreement with other findings related to pH-dependent metal binding to peptides.^{22,24-26} Our data could be highly important to understand metal binding to peptides *in vivo*. For example, if transient concentrations of metals may appear in the body, peptides and proteins do not immediately bind such metals. The binding process takes minutes as we showed in this work and depends on the metal and peptide concentrations, as well as on pH variations. These findings may explain why short stress conditions followed by a period of rest do not damage the cell, while long acting, but low intensity stressors may result in neurodegeneration.

Concluding remarks

Taken together, our results indicate that copper ions specifically bind to A β (1-16) peptide in solution and that this process is time-dependent. Changing the pH value from 7.4 to 6.6, only slightly influences the Cu²⁺ binding to the peptide. Nevertheless, more copper ions bind to A β (1-16) at pH 6.6 than at pH 7.4, although the complex formation is faster at pH 7.4. These

results could be useful to understand the long lasting, pH-dependent interaction of copper ions with β -amyloid peptides involved in the pathogenesis of AD.

Declaration of interest

Marilena Manea, Gitta Schlosser, and Manuela Murariu declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Acknowledgments

The authors gratefully acknowledge the financial support provided by the Romanian Government (Contract CNCSIS IDEI 313/2011).

References

1. Zecca L, Youdim MBH, Riederer P, Connor JR, Crichton RR. Iron, brain ageing and neurodegenerative disorders. *Nat. Rev. Neurosci.* 2004; **5**: 863-873.
2. Bush AI. The metallobiology of Alzheimer's disease. *Trends Neurosci.* 2003; **26**: 207-214.
3. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. *Nat. Rev. Drug Discovery* 2004; **3**: 205-214.
4. Gaggelli E, Kozłowski H, Valensin D, Valensin G. Copper homeostasis and neurodegenerative disorders (Alzheimer's, prion, and Parkinson's diseases and amyotrophic lateral sclerosis). *Chem. Rev.* 2006, **106**, 1995-2044.
5. Gradinaru R, Ionas A, Pui A, Zbancioc G, Drochioiu G. Interaction of inorganic mercury with CoA-SH and acyl-CoAs. *Biometals* 2011; **24**: 1115-1121.
6. Bush AI. The metal theory of Alzheimer's disease. *J. Alzheimers Dis.* 2013; **33**: S277-S281.
7. Pacheco-Quinto J, Eckman EA. Endothelin-converting enzymes degrade intracellular β -amyloid produced within the endosomal/lysosomal pathway and autophagosomes. *J. Biol. Chem.* 2013; **288**: 5606-5615.
8. Eskici G, Axelsen PH. Copper and oxidative stress in the pathogenesis of Alzheimer's disease. *Biochemistry* 2012; **51**: 6289-6311.
9. Damante CA, Ösz K, Nagy Z, Pappalardo G, Grasso G, Impellizzeri G, Rizzarelli E, Sóvógó I. Metal loading capacity of A β N-terminus: a combined potentiometric and spectroscopic study of zinc(II) complexes with A β (1-16), its short or mutated peptide fragments and its polyethylene glycol-ylated analogue. *Inorg. Chem.* 2009; **48**: 10405-10415.
10. Bush AI, Tanzi RE. Therapeutics for Alzheimer's disease based on the metal hypothesis. *Neurotherapeutics* 2008; **5**: 421-432.
11. Alies B, Hureau C, Faller P. The role of metal ions in amyloid formation: general principles from model peptides. *Metallomics* 2013; **5**: 183-192.

12. Ali FE, Separovic F, Barrow CJ, Yao S, Barnham KJ. Copper and zinc mediated oligomerisation of A β peptides. *Int. J. Pept. Res. Ther.* 2006; **12**: 153-164.
13. Syme CD, Nadal RC, Rigby SEJ, Viles JH. Copper binding to the amyloid- β (A β) peptide associated with Alzheimer's disease. *J. Biol. Chem.* 2004; **279**: 18169-18177.
14. Minicozzi V, Stellato F, Comai M, Serra MD, Potrich C, Meyer-Klaucke W, Morante S. Identifying the minimal copper- and zinc-binding site sequence in amyloid- β peptides. *J. Biol. Chem.* 2008; **283**: 10784-10792.
15. Smith DP, Smith DG, Curtain CC, Boas JF, Pilbrow JR, Ciccotosto GD, Lau T-L, Tew DJ, Perez K, Wade JD, Bush AI, Drew SC, Separovic F, Masters CL, Cappai R, Barnham KJ. Copper-mediated amyloid-beta toxicity is associated with an intermolecular histidine bridge. *J. Biol. Chem.* 2006; **281**: 15145-15154.
16. Schlosser G, Stefanescu R, Przybylski M, Murariu M, Hudecz F, Drochioiu G. Copper-induced oligomerization of peptides: a model study. *Eur. J. Mass Spectrom.* 2007; **13**: 331-337.
17. Drochioiu G, Manea M, Dragusanu M, Murariu M, Dragan ES, Petre BA, Mezo G, Przybylski M. Interaction of β -amyloid(1-40) peptide with pairs of metal ions: an electrospray ion trap mass spectrometric model study. *Biophys. Chem.* 2009; **144**, 9-20.
18. Murariu M, Dragan ES, Drochioiu G. Synthesis and mass spectrometric characterization of a metal-affinity decapeptide: copper-induced conformational alterations. *Biomacromolecules* 2007; **8**: 3836-3841.
19. Murariu M, Dragan ES, Drochioiu G. Model peptide-based system used for the investigation of metal ions binding to histidine-containing polypeptides. *Biopolymers* 2010; **93**: 497-508.
20. Faller P, Hureau C, Dorlet P, Hellwig P, Coppel Y, Collin F, Alies B. Methods and techniques to study the bioinorganic chemistry of metal-peptide complexes linked to neurodegenerative diseases. *Coord. Chem. Rev.* 2012; **256**: 2381-2396.
21. Grasso G. The use of mass spectrometry to study amyloid- β peptides. *Mass Spectrom. Rev.* 2011; **30**: 347-365.
22. Murariu M. Characterization of pH-dependent non-covalent copper-tetraglycine complexes by ESI-MS. *Int. J. Mass Spectrom.* 2013; **351**: 12-22.
23. Drochioiu G, Damoc EN, Przybylski M. Novel UV assay for protein determination and the characterization of copper-protein complexes by mass spectrometry. *Talanta* 2006; **69**: 556-564.
24. Murariu M, Dragan ES, Drochioiu G. Electrospray ionization mass spectrometric approach of conformationally-induced metal binding to oligopeptides. *Eur. J. Mass Spectrom.* 2010; **16**: 511-521.
25. Murariu M, Dragan ES, Drochioiu G. IR, MS and CD investigations on several conformationally-different peptides. *Int. J. Pept. Res. Ther.* 2009; **15**: 303-311.
26. Drochioiu G, Ion L, Ciobanu C, Habasescu L, Mangalagiu I. Letter: Mass spectrometric approach of high pH- and copper-induced glutathione oxidation. *Eur. J. Mass Spectrom.* 2013; **19**: 71-75.

Figure captions

Figure 1. ESI-ion trap mass spectra of A β (1-16) peptide (10 μ M peptide concentration), which show time-dependent formation of complexes with copper ions at pH 7.4 in 5 mM ammonium acetate solution.

Figure 2. ESI-ion trap mass spectra of A β (1-16) peptide (10 μ M peptide concentration in 5 mM ammonium acetate solution, pH 6.6) in the presence of copper ions (1:1 and 1:10 peptide: copper molar ratios).

Figure 3. ESI-ion trap mass spectra of A β (1-16) peptide in the presence of highly concentrated copper ion solutions, at two pH values (10 μ M peptide concentration in 5 mM ammonium acetate; pH 7.4 and 6.6, respectively; [Cu²⁺]: [A β (1-16)] 1:10). The spectra show the formation of Cu²⁺-A β (1-16) complexes in which the monomer peptide binds up to 3 or even 4 copper ions.

Figure 4. Circular dichroism spectra of A β (1-16) and its copper complexes formed in the first minutes after mixing the two solutions.

Figure 5. Time dependent copper binding to A β (1-16) at pH 6.6 and 1:10 peptide: copper molar ratio.

Figure 6. Time dependent copper binding to A β (1-16) at pH 7.4 and 1:1 peptide: copper molar ratio.