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Structural analysis of copper(I) interaction with amyloid β peptide

Giuseppe De Gregorio[a,1], Francesco Biasotto[a,1], Aleksandra Hecel[b], Marek Luczkowski[c], Henryk Kozlowski[d], Daniela Valensin[a,*]

[a] Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Via A. Moro 2, Siena, Italy
[b] Faculty of Chemistry, University of Wroclaw, F. Joliot-Curie 14, 50383 Wroclaw, Poland
[c] Department of Biotechnology, University of Wroclaw, F. Joliot-Curie 14a, Wroclaw, Poland
[d] Opole Medical School in Opole, Katowicka 68, 45060 Opole, Poland

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ABSTRACT

The N-terminal fragment of Aβ (β = beta) peptide is able to bind essential transition metal ions like, copper, zinc and iron. Metal binding usually occurs *via* the imidazole nitrogens of the three His residues which play a key role in the coordination chemistry. Among all the investigated systems, the interaction between copper and Amyloid β assume a biological relevance because of the interplay between the two copper oxidation states, Cu(II) and Cu (I), and their involvement in redox reactions. Both copper ions share the ability to bind Amyloid β. A huge number of investigations have demonstrated that Cu(II) anchors to the N-terminal amino and His6, His13/14 imidazole groups, while Cu(I) forms a linear complex by coordinating to the His13 and His14 dyad. In this study we have analyzed Cu(I) interaction with the Amyloid β fragment encompassing the first 16 amino-acids. Our data were obtained by means of NMR spectroscopy which provided relevant structural details of the metal complexes. Our findings are consistent with the involvement of two or three His in the Cu(I) coordination sphere and indicate that His6 effectively participates to the metal binding.

1. Introduction

Alzheimer's Disease (AD) is a severe neurological disorder characterized by brain atrophy and impairment, which ultimately leads to death [1,2]. The number of people affected by AD has been exponentially increased in the last ten years and it is believed that it will be bigger and bigger because of the growing of life expectancy [3]. The scientific community has been hardly working to understand the cause of AD and to identify valid therapeutic approaches able to contrast the disease progression [see for example reviews [4–7]]. Unfortunately, no therapy is available so far.

It is well accepted that AD is associated with the accumulation of toxic species consisting of oligomeric forms of amyloid β peptide (Aβ) [8–11]. Aβ is derived by the amyloidogenic proteolytic cleavage of Amyloid Precursor Protein (APP) and it consist of 40/42 amino acids [12,13]. The N-terminal part (Aβ16) is soluble in water being rich in His, Asp and Glu residues, while the C-terminus is highly hydrophobic and insoluble.

Aβ is a copper binding peptide and interacts with either Cu(II) or Cu (I) ions with relatively high affinity [14–23]. The affinity constant for Cu(II) interaction with Aβ16 ranges between subfemtomolar ($K_a = 10^{10}\,M^{-1}$), for the first site, to subnanomolar for the second site ($K_a = 10^{7}\,M^{-1}$) [24,25] There is a discrepancy on affinity of Aβ16 towards Cu(I) that lies between subnanomolar ($K_a = 10^{7}\,M^{-1}$) [26] and subfemtomolar ($K_a = 10^{10}\,M^{-1}$) [27]. The metal binding regions for both copper oxidation states, are located at the N-terminus.

The characterization of Cu(II) coordination sphere has been widely investigated and the main metal binding mode comprises a 3N1O donor set. According to the pH value, copper is bound to specific nitrogen atoms [14,15,28]. Two His imidazole (His6 and His13/His14) and NH_2 amino groups constitute the metal coordination sphere at pH lower than 7.8. On the other hand, deprotonated amide nitrogen of Ala2 substitutes one His imidazole when the pH is raised.

Compared to the huge amount of investigations on copper(II) interaction, relatively few studies have been directed to characterize the structural features of Cu^{+}-Aβ association. In fact, Cu(I) is EPR, CD and UV-VIS inactive and NMR and XAS spectroscopies only can be applied. There is the consensus that HisHis motif is predominantly involved in Cu(I) binding in Aβ peptides [14,29–33]. This bis-His motif has a key role in the redox activity of the copper Aβ complexes [34,35].

In addition, NMR investigations on the Cu(I)-Aβ16 system point out the occurrence of dynamical processes, where equivalent ligands

* Corresponding author.
E-mail address: daniela.valensin@unisi.it (D. Valensin).
[1] Authors equally contributed to this work.

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exchange for one coordination position [31]. In particular, the chemical shift experienced by all the three His's signals support copper coordination by all the three His side chains (His 6, 13 and 14) [31]. However, the data are consistent with a dynamical equilibrium between different His-Cu-His arrangements as well, with His 6 involved with the His dyads. It has been hypothesized that the transient 3-His coordination of Cu(I) may be important in the further stabilization of Cu(II) forms, thus affecting the kinetics of the Cu redox couple.

The importance played by His residue in Cu(I) binding is also evident from studies on Cu(I) interaction with Aβ peptides containing His/Ala substitution. In particular, previous investigations on Cu(I) affinity indicate that single His/Ala substitution reduced the affinities in a similar fashion independently on which His is substituted [26,27,36].

The effects of His/Ala substitution on Cu(I) binding affinity was also evaluated by investigating peptides derived from the copper transporter, Ctr1, indicating that His residues constitute a strong affinity binding site and they may contribute to Cu(I) acquisition. Replacement and substitution of Met residues has no effect on the picomolar affinity of the peptide for Cu(I), strongly indicating that His residues, rather than Mets motifs, are important for high affinity Cu(I) binding to Ctr1 model peptides at the physiological pH of 7.4 [37].

As for Aβ, Ctr1 model peptides contain three His, one at position 3 and the other two as dyad at position 5 and 6. Single replacement of histidine at the fifth or sixth position (H5A and H6A peptides) has little effect, on the other hand, the binding constant is reduced by about one order of magnitude when both His are replaced. Moreover, Cu(I) binding affinity is similarly decreased when the histidine at the third position is substituted (H3A). These results indicate that all three His have a specific role in the Cu(I) coordination sphere: (i) H3 and H5 or H6 are fundamental for the stability of the metal complex and H5 and H6 are exchangeable and/or have a cumulative contribution to Cu(I) complex formation.

In order to better understand the role played by the three His, in copper binding to amyloid β, we decided to investigate Cu(I) interactions by 2D NMR spectroscopy with the final aim to obtain the structural details of the metal complexes. Beside Cu(I), we also analyzed Ag(I)-Aβ interaction. Ag(I) is in fact used as metal binding probe for Cu(I) [19,38,39].

2. Experimental

2.1. Peptides and reagents

Aβ16 was synthesized on an Activotec Activo-P11 automated peptide synthesizer, with Fmoc-protected amino acids using Fmoc chemistry. Rink-amide resin was used as the solid support so that the resulting peptides would be amidated at the C-terminus. Cleavage from the resin was performed for 90 min in a 90% trifluoroacetic acid (TFA) solution containing 5% thioanisole, 2% anisole, and 3% ethanedithiol as free radical scavengers. After precipitation with cold ether, the peptides were redissolved in water and lyophilized to obtain a fluffy off-white powder. The solid was redissolved in 10% acetic acid and purified by reversed phase HPLC on a Varian Prostar HPLC with a preparative C18 column (Varian Pursuit XRs C 18) with a semi-linear gradient of 0.1% TFA in water to 0.1% TFA in 9:1 CH_3CN/H_2O over 45 min. The identity of the peptides was verified by ESI (electrospray ionization) mass spectrometry. The purified peptide was lyophilized and stored at −20 °C until use.

2.2. Mass spectrometry

High-resolution mass spectra were obtained on a Bruker micrOTOF-Q spectrometer equipped with an Apollo II electrospray ionization source with an ion funnel. The mass spectrometer was operated in the positive ion mode with the following parameters: scan range *m/z* 400–2000, dry gas-nitrogen, temperature 170 °C, ion energy 5 eV, transfer time 120 ps. Capillary voltage was optimized to 4500 V to obtain the highest S/N ratio. The small changes of voltage (± 500 V) did not significantly affect the optimized spectra. The samples (metal/ligand in a 1:1.2 stoichiometry, $[Ag(I)] = 1 \times 10^{-4}$ M) were prepared in a (1:1 $MeOH/H_2O$) mixture 1 mM carbonate buffer pH 6.5. Variation of the solvent composition down to 5% of MeOH did not change the speciation. The sample was infused at a flow-rate of 3 ml min^{-1}. The instrument was calibrated externally with the Tunemix_mixture (Bruker Daltonik, Germany) in quadratic regression mode. Data were processed by using the Bruker Compass Data Analysis 4.0 program.

2.3. Potentiometry

Potentiometric measurements were performed at constant temperature of 298 K in 0.1 mol dm^{-3} $NaClO_4$ under argon atmosphere on a MOLSPIN pH-meter system using a Mettler Toledo InLab semi micro combined electrode calibrated in hydrogen concentrations using 4 mM $HClO_4$ [40]. Stability constants both for protons and Ag(I) complexes were calculated from titrations carried out over the range pH 3–11. A total volume of 1.5 ml. NaOH was added by using a 0.5 ml micrometer syringe. The Ag(I) concentrations were 0.5 mM, the Ag(I): ligand/molar ratios were 1:1.2. The SUPERQUAD program was used for stability constant calculations [41]. Reported log β values refer to the overall equilibria:

$$pCu + qH + rL = Cu_pH_qL_r \quad (1)$$

$$\beta = \frac{[Cu_pH_qL_r]}{[Cu]^p[H]^q[L]^r} \quad (2)$$

charges are omitted for clarity; $\log K_{step}$ values refer to the protonation process:

$$Cu_pH_{q-1}L_r + H = Cu_pH_qL_r \quad (3)$$

(charges omitted; p might also be 0).

Standard deviations were computed by SUPERQUAD and refer to random errors only. They are, however, a good indication of the importance of a particular species in the equilibrium. The speciation diagrams were plotted with the HYSS 2006 program [42].

2.4. UV–vis measurements

Absorption spectra were recorded on a Cary 300 Bio spectrophotometer (Varian Inc., Palo Alto, CA) in the 360–220 nm range. Measurements were performed for 3 ml sample in quartz cell with 1 cm path length. The final peptide concentration was 0.1 mM, the metal to ligand molar ratio was 1:1.2. The solutions were prepared in 4 mM $HClO_4$ solution at 0.1 M $NaClO_4$ ionic strength. Data were processed using Origin 7.0.

2.5. NMR measurements

Aβ16 was dissolved in 20 mM or 2 mM phosphate buffer in H_2O at pH 7.3, obtaining a final concentration ranging from 400 to 500 μM for NMR experiments, 100 μM for CD experiments. The desired concentration of Ag(I) ions was achieved by using a stock solution of 20 mM $AgNO_3$ (Sigma Chemical Co.) in D_2O. For Cu(I) complexes 10 mM $[Cu(CH_3CN)4]BF_4$ stock solution was prepared in 20 mM phosphate buffer in D_2O at pH 7.3 containing 5% v/v CH_3CN. Ascorbic acid was added to the peptides (ratio 2:1) just before use in order to avoid any possible oxidation of Cu(I) to Cu(II). All the solutions were degassed with water-saturated N_2 just before use and kept under inert N_2 atmosphere. TMSP-2,2,3,3-d_4, 3-(trimethylsilyl)-[2,2,3,3-d_4] propansulfonate, sodium salt, was used as internal reference standard for NMR measurements.

NMR spectra were acquired at 278 and 288 K using Bruker Advance spectrometer operating at proton frequency of 600 MHz. NMR spectra

were processed with XwinNMR 3.6 and Top-Spin 2.0 software and analyzed with the program Cara [43]. Suppression of residual water signal was achieved either by presaturation or by excitation sculpting [44], using a selective 2 ms long square pulse on water. Proton resonance assignment of the peptides was obtained by 2D ^{1}H-^{1}H COSY (COrrelated SpectroscopY), TOCSY (Total Correlated SpectroscopY) and NOESY (Nuclear Overhauser Effect SpectroscopY) and ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Coherence) experiments were performed.

2.6. Structure determination

NOE cross peaks in 2D ^{1}H-^{1}H NOESY spectra acquired on Cu(I)-Aβ16 complexes at 278 K were integrated with Cara program and were converted into upper internuclear distances with the routine CALIBA of the program package DYANA [45]. The intra- and inter-residue constraints obtained were used to generate an ensemble of 30 structures by the standard protocol of simulated annealing in torsion angle space implemented in DYANA (using 10,000 steps). In order to take into account, the copper coordination mode, distance constraints between metal and the histidine nitrogen atoms were imposed. No dihedral angle restraints and no hydrogen bond restraints were applied. The final structures were analyzed using the program MOLMOL [46].

3. Results and discussion

The interaction between Aβ16 and Cu(I) was investigated by 2D NMR spectroscopy with the aim to obtain the structural characterization of the metal binding sites. In addition to Cu(I), the Ag(I)-Aβ16 complexes were also analyzed in order to understand if silver can be used as metal probe for copper(I) binding in Aβ16.

Cu(I) coordination to Aβ16 yields to local electronic perturbations which, in turn, generate chemical shift variations of the nuclei close to the metal biding site. Both up field and down field chemical shift variations may occur depending on the shielding or deshielding effects of the magnetic field. By comparing the NMR spectra of the metal bound and the apo Aβ16 it is easy to identify the peptide's regions perturbed by the metal ion. As shown in Fig. 1, the most affected signals are those of the three His (H6, H13 and H14) and residues nearby.

By analyzing the chemical shift variations of His aromatic protons we could also determine the imidazole nitrogens bound to Cu(I). Metal binding to Nδ usually causes larger changes on Hε, on the other hand similar chemical shift variations on Hε and Hδ resonances are observed when Cu(I) coordinates to Nε. Our findings indicate that His13 and His14 bind Cu(I) by Nε, while His6 interacts through Nδ, as clearly shown by the data reported in Table 1.

Table 1
Effects induced by Cu(I) binding on His aromatic nuclei.

Chemical shift variation (ppm) of aromatic protons of His residues			His aromatic nitrogen atom bound to Cu(I)
His-6	Hδ	0.03	Nδ
	Hε	0.08	
His-13	Hδ	0.12	Nε
	Hε	0.09	
His-14	Hδ	0.05	Nε
	Hε	0.06	

The chemical shift variations of all the detected NMR signals are reported in Fig. 2. Besides His signals, significant deviations are observed for Glu3, Phe4, Arg5, Asp7, Ser8, Gly9, Tyr10, Glu11, Val12 and Gln15. These effects can be easily explained if we consider additional copper binding to His6. Fig. 2 shows that the signals perturbed by the metal ion belong to residues linking His6 to the HisHis dyad and this is behavior is consistent with the structural rearrangements induced by the His6 and His13/His14 approaching.

In order to get more insights on the structural details of the Cu(I) complexes we collected ^{1}H-^{1}H TOCSY and NOESY NMR spectra at lower temperature (T = 278 K). In fact, at room temperature amide resonances are very weak and broad such to limit the acquisition and the positive outcome of the NOESY experiments. The analysis of effects induced by the cupreous ions, on ^{1}H chemical shift of Aβ16 at T 278 K, reveal that temperature does not influence Cu(I) coordination. Similarly, to what we observed at T 298 K, all the three His interact with Cu(I) at lower temperature (278 K).

NOEs correlations observed either for the free peptide or for the Cu (I) complexes were then converted into proton-proton distance constraints to be used in the calculation of the structures with the program DYANA. The obtained results are shown in Fig. 3.

The family structures of the apo Aβ16 reveal the presence of a turn in the region encompassing Ser8 and Glu11, in agreement with what

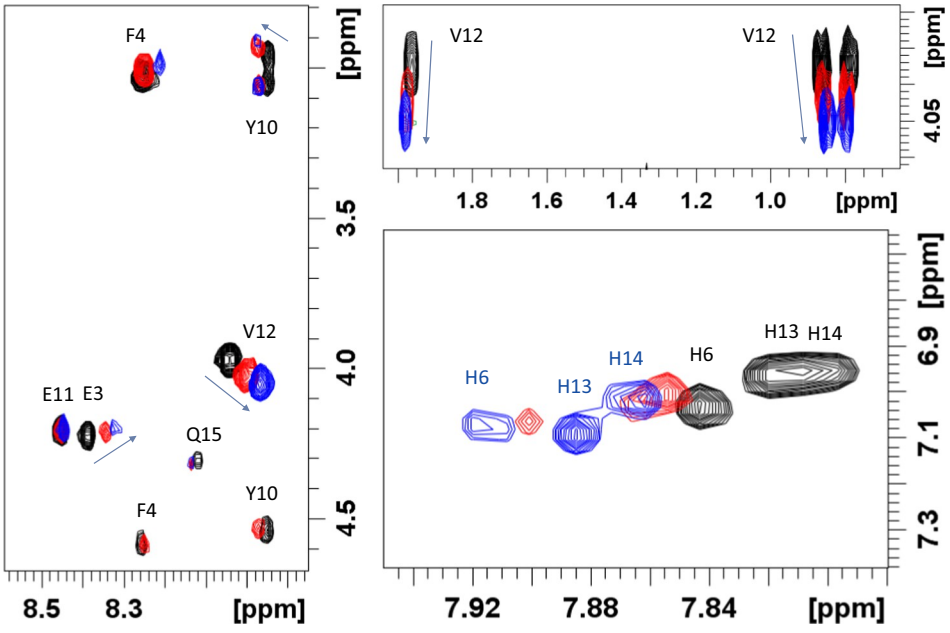

Fig. 1. ^{1}H-^{1}H 2D TOCSY experiments of Aβ16 0.5 mM in absence (black) and in presence of 0.5 (red) and 0.75 (blue) Cu(I) eqs. T = 298, pH 7.

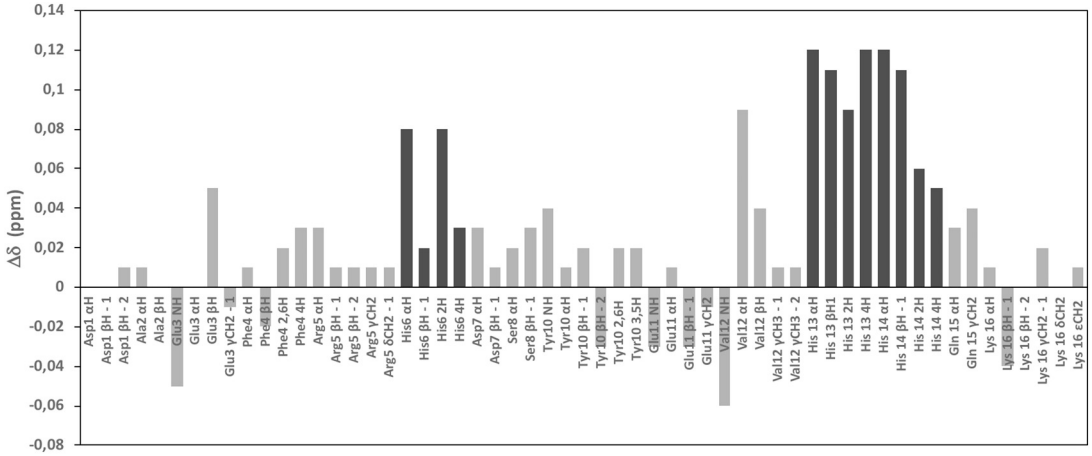

Fig. 2. Chemical shift variations of Aβ16 signals calculated by comparing the NMR spectra in absence and in presence of 0.75 Cu(I) eqs. T = 298 K, pH 7.

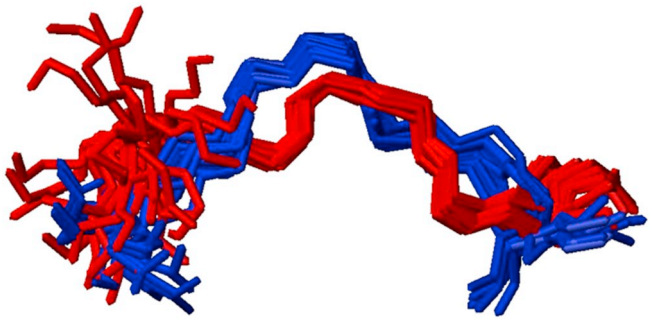

Fig. 3. Superimposition of the best 30 structures of Aβ16 (red) and Cu(I)-Aβ16 complex (blue). The obtained RMSD value (taken for residues 6–14) is 1.08 ± 0.50 Å for backbone atoms.

previously found by other research groups [47]. Such turn is destroyed when the metal complex is formed. In fact, for the Cu(I) complex we observe a different backbone bending which brings His13 and His14 pair close to the His6 residue. These findings agree with the observed chemical shift variations showed in Fig. 2.

These results agree with the previous NMR findings [31], suggesting the occurrence of a dynamic exchange between different Cu(I) binding modes containing just two His imidazole or all the three His. In order to take into account all the proposed sites we run four different simulated annealing calculations by including copper – nitrogen distances of the donor set, to the proton – proton distances previously calculated. The models are classified as the following:

1. H6-H13-H14-Cu(I): all the three His are bound to the metal ion.
2. H6-H13-Cu(I): His6 and His13 only, are bound to Cu(I).
3. H6-H14-Cu(I): His6 and His14 only, are bound to Cu(I).
4. H13-H14-Cu(I): His13 and His14 only, are bound to Cu(I).

The obtained results are shown in Fig. 4 and they indicate that H6-H13-H14-Cu(I) and H6-H13-Cu(I) models are the most significant ones. The structures are better defined and the RMDS values smaller, compared to H6-H14-Cu(I) and H13-H14-Cu(I) models.

The involvement of His6 in copper coordination sphere is also in agreement with the reaction schemes proposed for the preorganization electron transfer (POET) mechanism between Cu(II)/Cu(I) bound to Aβ [34]. In fact, His6 is a binding donor atom for Cu(II) as well and its interaction with both metal ions might impact the redox chemistry of Aβ. In addition, it has been recently shown that copper catalyzed ROS production is strictly correlated to specific copper-Aβ coordination mode. In particular, this binding, denoted as a “in-between” state, involves the N-terminus of Aβ (Asp-1) and one His residues only [48–50].

In order to better understand the Cu(I) coordination sphere we also investigated the Ag(I)-Aβ16 complex by using NMR spectroscopy. Our first aim is to verify if Ag(I) ions behave as Cu(I). In fact, it is well accepted that Ag(I) forms stable linear complexes and it can be used as probe for Cu(I). Similarly, for Cu(I) data, we collected 2D NMR spectra in absence and in presence of Ag(I) (Fig. 5). The general trend of the chemical shift variations is very similar both for Cu(I) and Ag(I), strongly supporting the occurrence of similar rearrangement of Aβ16 backbone upon metal binding (Fig. 5A). However, the two metal ions produce different electronic environments around the His moieties as indicated by the chemical shift perturbations of His Hε protons which are down-field or up-field shifted in presence of copper and silver ions, respectively (Fig. 5B).

As previously found for Cu(I), our findings point out that Ag(I) binding brings the HisHis pair close to His6, by canceling the turn present in apo Aβ16 form. As for copper, silver may adopt either trigonal or linear geometry by coordinating three or two His respectively, even if the latter is the preferred one. Contrary to Cu(I), Ag(I) induces homogenous shift of imidazole protons (ca ± 0.04 ppm) of all the His, strongly suggesting Nε only as the donor atoms. This finding is in contrast to what we observed for Cu(I), which coordinates His6 *via* Nδ and not *via* Nε. Such diversity is explained by considering that silver-Aβ complexes usually contain two His rather than three, as for Cu(I). Moreover, the well-fixed His6 orientation (Fig. 6) limits the mobility of its side-chain in the tri-coordinated Cu(I)-Aβ system. Finally, the identification of the nitrogen atoms by NMR relies on the evaluation of the entity of the chemical shift variations. These values, in fact, are strictly dependent on the change of the electronic chemical environment upon metal binding, and they represent a weighted average between all the possible coordination spheres.

We have investigated silver binding to Aβ16 by means of mass spectrometry (MS) as well. The results indicate that at pH 6.5 apo Aβ16 (m/z = 489.48, 652.31 and 977.97) and its silver bound complex (m/z = 516.21, 687.94 and 1031.42) exist as monomers (Fig. 7a). The MS data can be modeled with doubly deprotonated Aβ16 and one Ag(I) ion bound and presumably reflecting silver coordination by two imidazoles (Fig. 7b). No species of higher ligand content with respect to metal have been detected, but this may be because it is difficult to detect polymeric forms of ligand or its complexes. Low intensity signal corresponding to trace amounts (< 1%) of dinuclear $Ag(I)_2$-Aβ16 species (m/z = 723.57) has been identified as well (data not shown). This effect may result from weak interaction between a second Ag(I) ion and the peptide in the gas phase. Silver complexation is used as ionization efficiencies enhancer in electrospray ionization applications [51]. It is noticeable that frequently solution-phase and gas-phase structures of silver-peptide

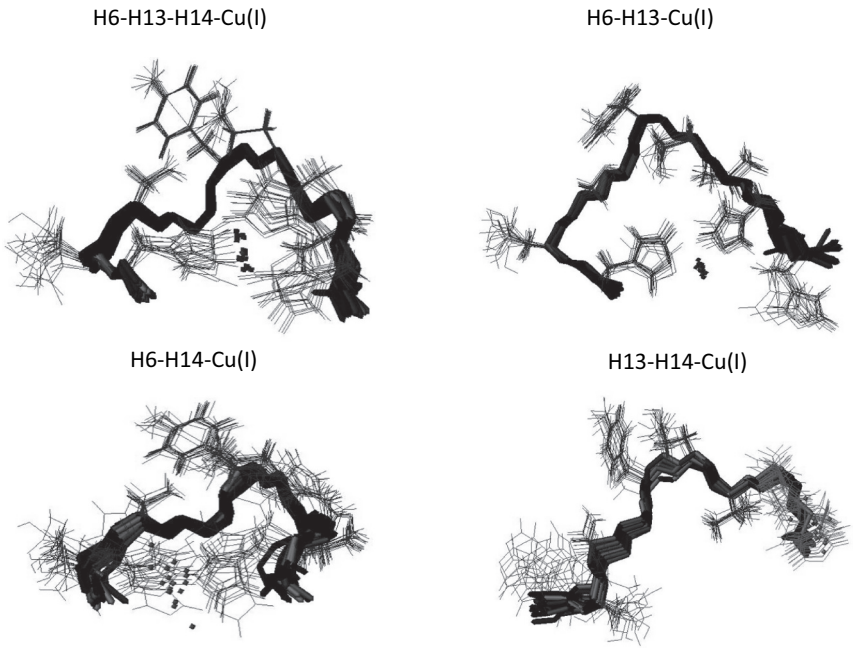

Fig. 4. Superimposition of the best 30 structures of the Cu (I)-Aβ16 complexes. The obtained RMSD values (taken for residues 6–14) are (i) 0.20 ± 0.12 Å (H6-H13-H14-Cu(I)), (II) 0.39 ± 0.21 Å (H6-H13-Cu(I)), (III) 0.44 ± 0.27 Å (H6-H14-Cu(I)) and (iv) 0.57 ± 0.36 Å (H13-H14-Cu(I)) for backbone atoms.

complexes are not identical. Water is an effective competitor with the peptide backbone for silver ion solvation in solution. In a gas phase, after the removal of water, the only effective "solvent" is a peptide backbone that wraps around the silver ions and chelates it [52].

To evaluate the acid-base properties of Aβ16 and its binding affinity towards Ag(I) ion potentiometric titrations were performed. In the measured pH range Aβ16 (DAEFRHDSGYEVHHQK-NH_2) behaves like $H_{10}L$ acid (Table 2). The ten protonation constants correspond to consecutive proton binding to the ε-amino group of Lys, phenyl group of Tyr, the terminal α-amino group, imidazole nitrogen atoms of three His residues, γ-carboxylate of two glutamate residues and β-carboxylate of two aspartate residues. The protonation of the guanidinyl function of the Arg residue cannot be followed. Subsequently, potentiometric titration at a 1:1.2 Ag(I) to Aβ16 ratio was carried out. Comprehensive

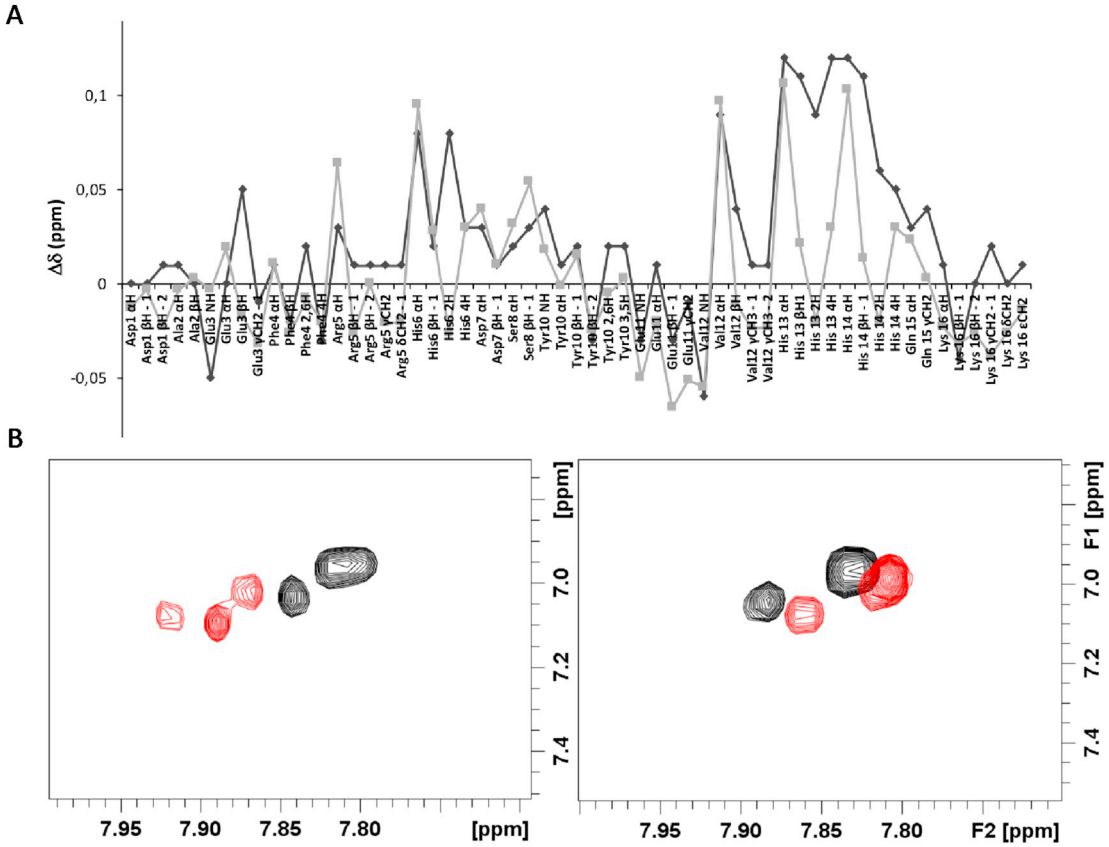

Fig. 5. A. Comparison of the chemical shift variations of Cu(I)-Aβ16 and Ag(I)-Aβ16 systems. T = 298 K, pH 7. B. ^{1}H-^{1}H 2D TOCSY experiments of Aβ16 0.5 mM in absence (black) and in presence (red) of: Left. 0.75 Cu(I) eqs.; Right 0.8 Ag(I) eqs. T = 298, pH 7.

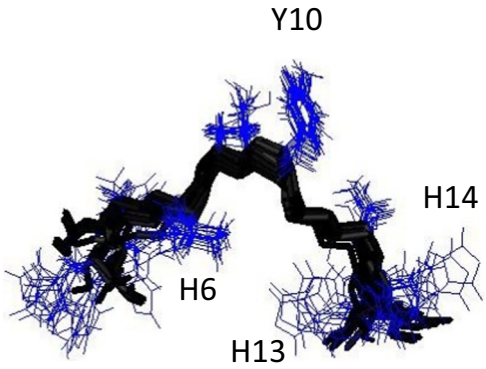

Fig. 6. Superimposition of the best 30 structures of Aβ16 showing the side chain of aromatic residues. The obtained RMSD value (taken for residues 6–14) is 1.08 ± 0.50 Å for backbone atoms.

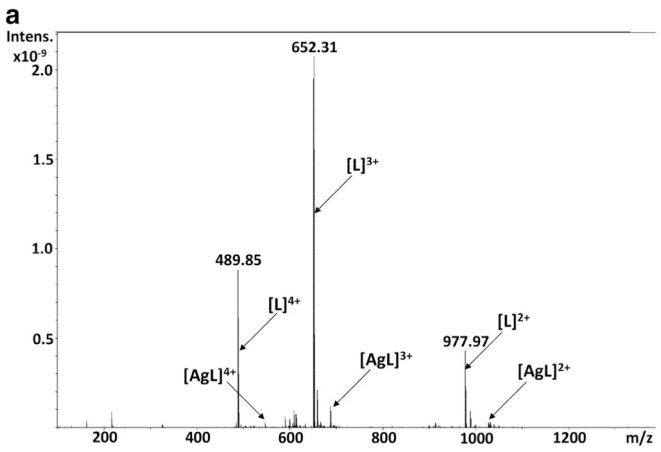

Fig. 7a. ESI-MS spectra of the Ag(I) complex of the Aβ16 peptide at pH 6.5 in carbonate buffer (1 mM). [Aβ16] = 1×10^{-4} M; Ag(I)/Aβ16 ratio 1:1; MeOH/H_2O = 1:1.

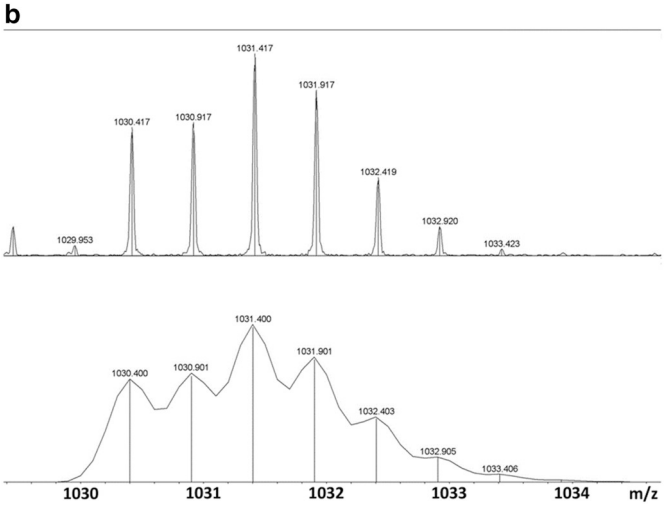

Fig. 7b. ESI-MS spectra of the Ag(I) complex of the Aβ16 peptide at pH 6.5 in 1 mM carbonate buffer (top) and its simulated spectra (bottom).

analysis of potentiometric data reveals the presence of 6 complex species, AgH_5L, AgH_4L, AgH_3L, AgH_2L, AgHL and AgL (Table 3, Fig. 8). As a result of silver instability in alkaline pH range, predefined stability constant for Ag(I) hydrolysis product was used in all calculations [53]. AgH_5L complex, fully formed at pH 5.5, results from the deprotonation

Table 2
Protonation constants for the Aβ16 at T = 298.2 K and I = 0.1 M in $NaClO_4$; $c_{Aβ16} = 6 \times 10^{-4}$ M.

Species	logβ	logK	Residue
HL	10.47(1)	10.47	Lys
H_2L	20.28(1)	9.81	Tyr
H_3L	28.12(2)	7.83	α-NH_2
H_4L	35.03(2)	6.91	His
H_5L	41.47(3)	6.44	His
H_6L	47.23(3)	5.76	His
H_7L	51.65(4)	4.42	Glu
H_8L	55.56(4)	3.91	Glu
H_9L	58.78(5)	3.22	Asp
$H_{10}L$	61.34(5)	2.56	Asp

Table 3
Stability constants for the Ag(I) complexes of Aβ16 at T = 298.2 K and I = 0.1 M in $NaClO_4$; $c_{Aβ16} = 6 \times 10^{-4}$ M, $c_{Ag(I)} = 5 \times 10^{-4}$ M.

Species	logβ	pK	logK*[a]
AgH_5L	44.92(3)	–	3.45
AgH_4L	38.93(6)	5.99	3.90
AgH_3L	32.60(4)	6.33	4.48
AgH_2L	25.23(2)	7.36	4.95
AgHL	15.99(3)	9.24	5.52
AgL	5.78(4)	10.21	
AgOH	−8.56[b]	–	

[a] log K* = log b(CuH_jL) − log b(H_nL) (where the index j corresponds to the number of the protons in the coordinated ligand to the metal ion and n corresponds to the number of protons coordinated to the ligand).
[b] Taken from the reference [44].

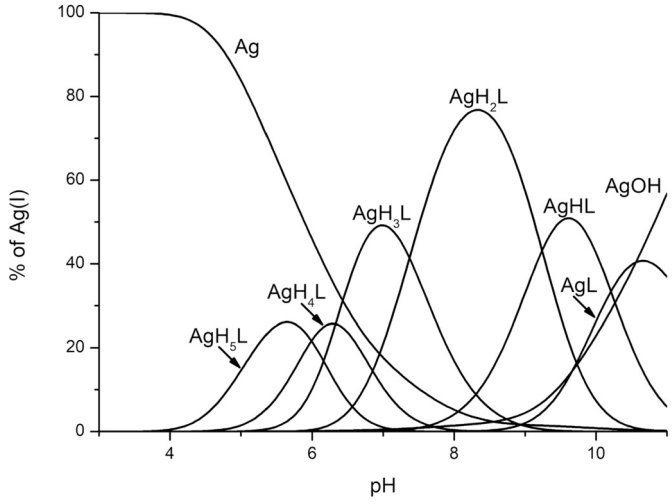

Fig. 8. Species distribution diagram for Ag(I) complexes of the Aβ16 peptide; T = 298.2 K and I = 0.1 M in $NaClO_4$; $c_{Aβ16} = 6 \times 10^{-4}$, $c_{Ag(I)} = 5 \times 10^{-4}$ M.

and metal ion binding to one His imidazole. In this pH range carboxylic groups of the Asp and Glu are deprotonated. The protonation corrected stability constant logβ* [54,55] (logβ* = 3.45) fully supports the model of Ag(I) ion bound to single His residue. Further deprotonations result into the formation of AgH_4L and AgH_3L species in which two and three histidyl residues are deprotonated, respectively. The evaluated logβ* values (3.90 and 4.48) do not change significantly indicating the weak interaction between Ag(I) and second and third imidazole moieties in consequence allowing the system to switch between different binding modes (*vide supra*). The three species forming at alkaline pH range, AgH_2L, AgHL and AgL stem from the proton dissociation processes of the α-amino group, phenyl of Tyr and ε-amino group of Lys residue. These residues are not involved in the metal ion sequestration. While

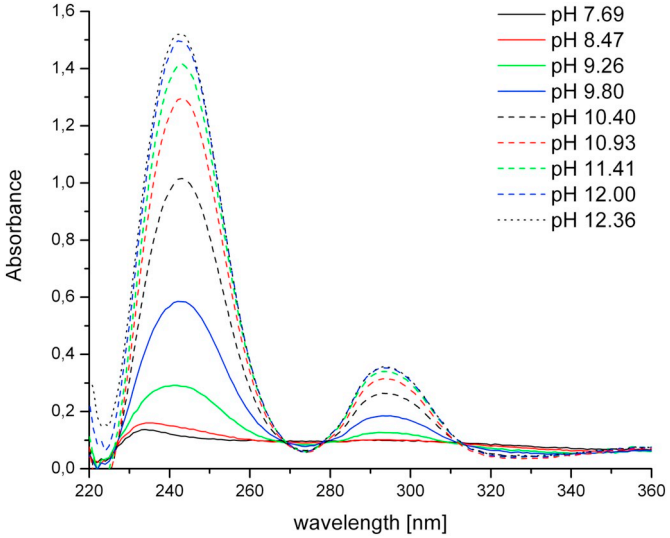

Fig. 9. Difference absorption spectra for Ag(I) complexes of the Aβ16 peptide; T = 298.2 K and I = 0.1 M in $NaClO_4$; $c_{Aβ16} = 1.3 \times 10^{-4}$ M, $c_{Ag(I)} = 1.1 \times 10^{-4}$ M.

Table 4
K_d values at pH 7.4 of Ag(I) complexes of Aβ16, computed from stability of model systems [48].

Peptide	K_d from/$[M]_{50}$ (mol l^{-1})	Total substrate conc. (P_0) (mol l^{-1})	Total Ag(I) conc. (M_0) (mol l^{-1})
Aβ16	2.64×10^{-5}	6.0×10^{-4}	1.0×10^{-4}–1.0×10^{-3}

logK value describing the phenolate protonation decrease in the presence of metal, proton dissociation yields difference spectra characteristic Tyr-O^-, thus proving that aromatic residue is not involved in Ag(I) binding (Fig. 9) [56].

The application of potentiometric techniques is necessary to determine the speciation of the different metal complex species according to pH and molar ratios between metal and ligand. Unfortunately, it gives sparse information on biological significance of the interaction. The protonation corrected stability constants (logβ*) do not easily translate to affinity. The broad picture tangles even more when mixture of species is observed. At pH 7.4, commonly considered physiological for most living systems in normal condition, the AgH_3L and AgH_2L species are in the equilibrium. However, application of the dissociation constants approach [57] overwhelms these difficulties by allowing evaluation of the conditional affinity constants for physiologically relevant pH values reflected as apparent dissociation constants (K_d),

$$K_d = \frac{[M][L]}{[ML]}$$

defined to by the equilibrium:

$$ML = M + L$$

The calculated dissociation constants accompanied by experimental conditions are summarized in Table 4. The calculated value ($K_d = 2.64 \times 10^{-5}$) is two orders of magnitude lower than affinity constant estimated for Cu(I)-Aβ16 interaction ($K_d = 1.33 \times 10^{-7}$) [26], though it is still reasonable, since the average copper concentration in the brain may reach 100 μM [58]. Our previous work on Ag(I) interaction with prion neurotoxic domain fragment indicates that Cu(I) has definitely higher affinity to imidazole nitrogens than Ag(I) [39]. Cu(I) affinity to imidazole nitrogen atoms, due to ion's smaller size and different electron density, is definitely higher then found for analogical Ag(I) systems. This may explain the difference in complexes' stability for investigated metal ions.

4. Conclusions

Our NMR findings on Cu(I) interaction with the N-terminal Aβ16 fragment indicate the involvement of all the three His residues in metal ion binding in agreement with previous NMR investigations [31]. The structural studies obtained by the analysis of NOEs correlations of the metal complex, show that the N-terminal histidine (His6) is close the His13-His14 dyad and it strongly supports the presence of a dynamic equilibrium between two molecular species engaging either three (Nδ-His6, Nε-His13 and Nε-His14) or two (Nδ-His6, Nε-His13) imidazole nitrogen atoms. The probability of Cu(I) interaction with the His13-His14 pair is lower, though possible. Parallel NMR investigations on Ag(I)-Aβ16 interaction suggest similar structural rearrangements to that observed for Cu(I), indicating that Ag(I) has strong potential in probing Cu(I) interaction with Aβ16. The only observed differences are in the nitrogen donor atoms, which correspond to (i) Nε for all the His residues coordinated to silver, (ii) while are Nδ, Nε and Nε for His6, His13 and His14 coordinated to Cu(I). The binding of different nitrogen atoms for His6 might be dependent on the different size of the two metal ions, being the Cu(I) ionic radius much smaller than the Ag(I) one.

NMR findings for Ag(I)-Aβ16 system are supported by comprehensive analysis performed with the application of mass spectrometry and potentiometric studies. Silver(I) ion interaction with metal binding domain of Aβ yields monomeric species that excludes existence of two independent binding motives in Aβ16 and supports His6, His13 or/and His14 involvement in metal ion sequestration. Furthermore, analysis of potentiometric data suggests weak interaction of Ag(I) with second and third His residues and strengthens the hypothesis of dynamic equilibrium between species in solution. We also calculated the K_d values of the Ag(I) complexes, which were one/two order of magnitude different from the Cu(I). Such diversity depends on the fact that Cu(I) has higher affinity towards His nitrogen atoms than Ag(I), and suggest that silver (I) is not a good metal probe for investigating copper(I) interactions with multi-His containing peptides.

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