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Characterization of Mn(II) ion Binding to the Amyloid- β Peptide in Alzheimer's Disease

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Short title: Mn(II) ion binding to the amyloid- β peptide

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Abstract

Growing evidence links neurodegenerative diseases to metal exposure. Aberrant metal ion concentrations have been noted in Alzheimer's disease (AD) brains, yet the role of metals in AD pathogenesis remains unresolved. A major factor in AD pathogenesis is considered to be aggregation and amyloid formation by amyloid- β (A β) peptides. Previous studies have shown that A β displays specific binding to Cu(II) and Zn(II) ions, and such binding has been shown to modulate A β aggregation. Here, we use nuclear magnetic resonance (NMR) spectroscopy to show that Mn(II) ions also bind to the N-terminal part of the A β (1-40) peptide, with a weak binding affinity in the milli- to micromolar range. Circular dichroism (CD) spectroscopy, solid state atomic force microscopy (AFM), fluorescence spectroscopy, and molecular modeling suggest that the weak binding of Mn(II) to A β may not have a large effect on the peptide's aggregation into amyloid fibrils. However, identification of an additional metal ion displaying A β binding reveals more complex AD metal chemistry than has been previously considered in the literature.

Key words: manganese, neurodegeneration, metal-protein binding, spectroscopy, molecular dynamics

Introduction

The molecular events underlying neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyloid lateral sclerosis (ALS) are still not known. In the case of AD, increasing evidence links disturbed metal homeostasis and deposition of stable amyloid aggregates of misfolded proteins to the disease, as self-assembly, aggregation and misfolding of the amyloid- β (A β) peptide have been suggested to be either the primary cause or a secondary consequence of disease development. The amyloid hypothesis is supported by the genetic link between inherited risk alleles in familial AD and senile plaques found post-mortem in AD brains [1,2]. The *in vivo* aggregation of A β follows a distinct pathway with diffuse plaques formed in the basal temporal cortex and progression to hippocampal departments and eventually the complete neocortex [3,4]. Imbalance in A β misfolding, clearance and deposition is not limited to exclusively brain parenchyma [5], indicating that systemic misfolding events could be a consequence of external influences [6].

The A β monomer is a soluble unstructured peptide, 39-43 amino acid residues long and generated from the transmembrane amyloid precursor protein (APP) by β - and γ -secretase enzymatic cleavages [7-9]. Two hydrophobic segments are present in respectively the central and C-terminal parts of the peptide, and one hydrophilic part is located in the N-terminus. The mechanisms of A β aggregation are not yet completely elucidated, and intermediate structures along the aggregation pathway are not well-defined [10,11]. The starting point with a monomeric peptide in random coil conformation, and the end point with stable fibrils, are however well characterized. Fibrils studied with cryo-electron microscopy (cryo-EM) and solid state nuclear magnetic resonance (ssNMR) reveal a hydrophobic core and the N-terminus is considered to be flexible, possibly wrapped around the core [9,12]. *In vitro* aggregation kinetic studies reveal perturbed aggregation and structural propensities, in the presence of small molecules or metal ions [13,14].

Mounting evidence derived from clinical and biochemical studies indicates a putative role for metals in AD pathogenesis [15-18]. The interactions of A β with copper and zinc ions have received much attention [19-22]; the monomeric A β peptide is known to display moderate affinity for Cu(II) and Zn(II) ions with binding constants in the micro- to nanomolar range, and with a higher affinity to more ordered structures [19,20]. The A β peptide has a net charge of minus three at physiological pH, and cationic metal ions and small molecules are therefore of interest for possible interactions [23]. A well-defined

specific binding site for both Cu(II) and Zn(II) ions to the monomeric peptide has been proposed, involving metal ion coordination by the three histidines (H6, H13, H14) and the N-terminal aspartic acid (D1) [24–28]. The N-terminal alanine and E11 residues have also been suggested as alternative ligands, and thus multiple metal binding conformations seem possible [29].

Metals with neurotoxic properties such as Mn ions [30] have been assigned a pathogenic role in various neurodegenerative disorders [31,32]. Nerve cells of the brain are in equilibrium with the extracellular space and with the cerebrospinal fluid (CSF). Manganese is a selective choroid plexus toxicant [33] accumulating in the choroid plexus, and some data suggest that Mn(II) is the most blood brain barrier (BBB) permeable species among the several metal ions studied [34]. Mn transport mechanisms across the BBB are complex and seem to involve several proteins [35] regulating Mn concentration in the CSF. Manganese is found in several chemical forms and oxidation states; in human tissue Mn(II) and Mn(III) are of special interest [36]. Mn(II) is the most stable state among the oxidation states for manganese, and it has paramagnetic properties with a high spin electron state with one single electron in every 3d-orbital.

Despite extensive studies of the role of metal ions in AD in general [15,16,37–40], A β binding to Mn ions in particular has not been studied in any detail. Studies *in vitro* have focused on A β interactions with Cu(II) and Zn(II) ions [18,20,41,42], due to their physiological presence in the human brain. However, Atwood et al. found *in vitro* aggregated A β peptides at pH 7.4 in similar concentrations from both Mn and Cu incubation at physiological metal concentrations [43]. At lower pH, Mn(II) did not contribute to aggregation to the same extent as Cu(II) and Zn(II) ions did. Additionally, Bush *et al.* found in a Zn(II) competitive assay with APP that Mn(II) competed off Zn(II) to approximately the same extent as Cd(II), Pb(II), Al(III), Hg(II) and Cu(II) did [44]. One study using Mn(II) ions as paramagnetic probes in A β /micelle studies found evidence for an N-terminal Mn(II) binding specificity [45]. Despite these studies, the effects of Mn/A β interactions on A β aggregation and the molecular details of the interactions involved are so far unknown. Here, we used NMR and fluorescence spectroscopy, circular dichroism (CD), molecular modeling, and solid state atomic force microscopy (AFM) to study the interactions between Mn(II) ions and the A β peptide. A β (1-16) and A β (1-40) peptides were used for the computational and experimental studies, respectively, with a focus on characterizing the binding properties and the effects of Mn(II) binding on A β amyloid aggregation.

Materials and Methods

Lyophilized A β (1-40) peptides, with the primary sequence DAEFR₅HDSGY₁₀EVHHQ₁₅KL VFF₂₀AEDVG₂₅SNKGA₃₀IIGLM₃₅VGGVV₄₀, were bought either unlabeled or isotope ¹³C, ¹⁵N-labeled from AlexoTech AB (Umeå, Sweden). The A β peptides were dissolved to monomeric form as previously described [46], and peptide concentration determined by weight. Mn(II) acetate was purchased from Sigma-Aldrich Co.

Nuclear magnetic resonance spectroscopy

A Bruker Avance 700 MHz spectrometer with a cryogenic probe was used to record 2D ¹H, ¹⁵N-HSQC, and 2D ¹H, ¹³C-HSQC spectra at +5 °C of 77 or 84 μ M ¹³C, ¹⁵N-labeled A β (1-40) peptides in 20 mM HEPES buffer at pH 7.2 or sodium phosphate buffer at pH 7.35 (90/10 H₂O/D₂O), both before and after titration with Mn(II) ions. The spectra were referenced to trimethylsilyl propanoic acid (TSP). The assignment of the amide crosspeaks and of the aromatic and C α -H crosspeaks for the A β (1-40) peptide is based on previous work [47–50]. The data was processed and analyzed with the Topspin version 3.2 software. A non-quantitative apparent dissociation constant (K_D^{app*}) was determined from 2D ¹H, ¹³C-HSQC crosspeak signal intensity changes. A global fit analysis, using the resonances from residues with

significant signal attenuation upon Mn(II) ion titration, was performed with a binding model assuming a single binding site described by Eq. 1:

$$I = I_0 + \frac{I_\infty - I_0}{2 * [A\beta]} * \left((K_D + [Me] + [A\beta]) - \sqrt{(K_D + [Me] + [A\beta])^2 - 4 * [Me] * [A\beta]} \right) \quad (\text{Eq. 1})$$

Where I_0 is the intensity in the absence of metal ions, I_∞ the intensity when saturated, K_D is the dissociation constant for the A β /Me complex, and $[Me]$ and $[A\beta]$ are the metal concentration and A β peptide concentration.

Fluorescence spectroscopy

For intrinsic Y10 fluorescence measurements a sensitive spectrofluorometer (Jobin Yvon Horiba Fluorolog 3 Longjumeau, France) was used to monitor the fluorescence of Y10, the only A β residue with relatively high fluorescence. The excitation wavelength was set to 276 nm and emission spectra were recorded between 290 nm and 350 nm at room temperature. A quartz cell with a 4 mm path length with an experimental volume of 1 ml, and a slit opening of 5 nm was used. Quenching of the Y10 fluorescence at an A β concentration of 10 μ M was investigated in a metal titration series with increasing Cu(II) ion concentration of 1, 2, 3, 4, 5, 6, 7, 9, 11, 16, 21, 36, 66, 81, 181, 281, 481, 681, and 881 μ M. The maximum intensity around 307 nm was used for analysis. Competitive measurements for Mn(II) ions (60 and 90 μ M) with Cu(II) were performed. The fluorescence data were fitted to a binding model assuming a single binding site with no correction for buffer conditions [27,51] using Eq 1.

A β peptide (15-20 μ M) in 20 mM HEPES buffer pH 7.2 and 40 μ M Thioflavin T (ThT) were used for aggregation kinetics measurements [52]. Several concentrations of Mn(II) ions were used in different measurements series. Thioflavin T was excited at 440 nm and emission was measured every three minutes at 480 nm in a 96-well plate reader from FLUOstar Omega (BMG LABTECH, Germany) under quiescent conditions, with eight replicates per condition. The kinetic parameters characterizing the amyloid aggregates were determined from a sigmoidal curve-fitting using Eq.2:

$$F(t) = F_0 + \frac{A}{1 + \exp[r_{\max}(T_{1/2} - t)]} \quad (\text{Eq. 2})$$

Here F_0 is the fluorescence intensity baseline, A is the amplitude, $r_{\max}$ is the maximum growth rate and $T_{1/2}$ is defined as the time when 50% of the monomer concentration is depleted.

Molecular dynamics calculations

The starting point for the molecular dynamics (MD) simulations performed in this work was the suggested NMR structure of A β (1-16) bound to a Zn(II) ion, with H6, H13, H14 and E11 as binding ligands (PDB ID: 1ZE9) [53]. The truncated A β (1-16) version was selected for modeling to reduce the size of the system, thereby allowing much longer simulation timescales. The Zn(II) atom in the original structure was manually replaced with octahedral Mn(II), Zn(II), and Cu(II) dummy models, as previously described [54,55]. The use of dummy models allowed us to capture both key structural and electrostatic properties of the different divalent metals using a classical force field, without the need for artificial bonds or restraints, thus allowing for extensive conformational sampling of the peptide. Three different NMR conformers - 1, 3 and 8, referred to as Struct1, Struct2 and Struct3 in this article - were considered for the simulations with each metal, in order to have multiple starting structures and achieve more extensive conformational sampling.

All MD simulations were performed using the GROMACS simulation package v. 5.0.4 [56,57], and the OPLS-AA force field [58,59]. Each peptide monomer was capped with an acetyl group at the N-terminal

end and an amine group at the C-terminal end. The peptide was then solvated in a cubic solvation box of TIP4P water molecules [60] with a minimum distance of 1.0 nm between the peptide and any box edge. Na⁺ and Cl⁻ ions were added to a concentration of 0.150 M to neutralize the system, and periodic boundary conditions with the particle mesh Ewald (PME) method [61,62] were used for the treatment of electrostatic interactions (using a 1.0 nm cutoff for short-range interactions). The same cutoff was also used for the Lennard-Jones (LJ) interactions, with a switching function applied between 1.0 and 1.2 nm, and long-range LJ contributions were accounted for using a dispersion correction. The system was then subjected to 5,000 steps of steepest descent and 5,000 steps of conjugate gradient energy minimization, followed by short 500 ps equilibration runs under first NVT and then NPT conditions (1 ns total simulation time), using 1,000 kJ mol⁻¹ nm⁻² position restraints on all non-hydrogen atoms. The temperature was kept at 300K using a Berendsen thermostat [63] with a coupling constant of 1.0 ps⁻¹, and, in the NPT simulations, the pressure was kept at 1 bar using a Parrinello-Rahman barostat [64] with a 0.5 ps coupling constant. A final 1 μ s production was performed for each system at 300K, using the NPT ensemble without position restraints, and the leap-frog integrator [65] with a 2 fs time step with coordinates saved every 10 ps. All bonds were constrained *via* the LINCS method [66,67].

In total, 9 μ s of production time over all systems was accumulated: 3 μ s each for Mn(II), Zn(II) and Cu(II). The root mean square deviation (RMSD) of all backbone atoms of the peptide was monitored during the simulation, and clustering analysis was performed applying the method of Daura et al. [68] as implemented into GROMACS using a cutoff distance of 0.2 nm and considering structures sampled every 50ps.

Solid state atomic force microscopy

Solid state atomic force images were recorded using a Bruker's Scan Asyst (Bruker Corp., USA) operating in peak-force tapping mode with a sample rate of 1.95 Hz and a resolution of 256-256 or 1024-1024 pixels. 100 μ M aggregated A β (1-40) peptide samples with Mn(II) ions added in different concentrations were prepared by incubation at +37 °C and 200 rpm in Eppendorf tubes. After 6 h 3 μ l of the samples were diluted with 100 μ l distilled water and applied on freshly cleaved mica substrates. Excess liquid was removed after 20 minutes of incubation. The mica substrates were then washed with distilled water three times and dried at room temperature.

Circular Dichroism spectroscopy

Circular dichroism (CD) spectra were recorded with a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, U.K.), with a Peltier temperature control system. A quartz cell with a 4 mm optical path length was used to record CD spectra between 190-255 nm with a bandwidth of 1 nm, a step size of 1.0 nm, and a time per point of 1 or 4 s. The measurements were performed with 10 μ M A β (1-40) in 20 mM sodium phosphate buffer, pH 7.35, and the baseline for distilled water was subtracted. All data curves were processed with a smoothing function of ten points in the DPlot version 2.3.3.3 software. To test the direct effect of Mn(II) ions on 10 μ M A β peptide, Mn(II) acetate was titrated to the A β peptide solution with Mn(II) concentration of 5, 10, 15, 25, 40, 65, 95, 150, and 250 μ M and a spectrum was recorded for each titration step (Fig. S6A). For kinetic CD measurements, initial CD scans were first recorded for A β only solutions. Next, Mn(II) ions were added in concentrations of 2, 10, 50, or 100 μ M. Spectra for the resulting A β + Mn(II) solutions were recorded every five minutes for four hours, during which the samples were kept at +37 °C and agitated with a magnetic stirrer inside the CD quartz cell (Fig. S6B and S6C). CD spectra were also recorded (Fig. 8) for the incubated A β samples used for AFM imaging (see above for the preparation protocol – subsamples were taken after incubation and diluted ten times for the CD measurements).

Results

Our results show binding of Mn(II) ions to the A β peptide, albeit without any major effects on the A β aggregation, likely explained by a weak and transient interaction.

Nuclear magnetic resonance spectroscopy

Binding effects of Mn(II) ions to the monomeric A β (1-40) peptide were monitored via residue-specific intensity changes in 2D NMR spectra (Figs. 1 and 2). The intensities of amide crosspeaks in ^1H , ^{15}N -HSQC spectra were unequally affected when Mn(II) ions were titrated to the A β peptide solution (Fig. 1). Upon addition of 231 μM Mn(II) ions (A β :Mn ratio 1:3) the residues in the C-terminal region retained approximately 50-80% of their original signal intensity, while residues in the N-terminal region were reduced to around 0-50% of their initial signal intensity. No large chemical shift changes were observed in any of the recorded spectra. These observations indicate an A β /metal ion interaction where signal attenuation is likely caused by line broadening due to paramagnetic effects. Additionally, chemical exchange on a NMR-intermediate time scale might be involved, as observed in the presence of zinc ions [14]. Because of solvent exchange effects, the amide crosspeaks of the flexible D1, H6, H13, and H14 residues in the N-terminal region part cannot be observed in the ^1H , ^{15}N -HSQC spectrum of A β alone. It is therefore difficult to elucidate from the ^1H , ^{15}N -HSQC data exactly which N-terminal residues might bind the Mn(II) ions. As ^{13}C -bound hydrogens are more protected from solvent exchange, they can provide complementary information to the data obtained for amide hydrogens in ^1H , ^{15}N -HSQC spectra. In the aromatic region of the 2D ^1H , ^{13}C -HSQC spectrum shown in Fig. 2A it is clear that the histidine crosspeak intensities for δ - and ϵ -hydrogens are reduced upon Mn(II) ion titration, for all three histidines. Also in the $\text{C}^\alpha\text{-H}$ region signal reduction was observed (Fig. 2B), most notably for D1 and H13, suggesting that these two residues are involved in Mn(II) binding. In addition, V40 was also affected, and the ^1H , ^{15}N -HSQC spectrum in Fig. 1 indicates a secondary weaker binding site between A β residues 22-25. The presence of a second binding site in the central region is consistent with previous findings for Zn(II) ions [27], with the negatively charged residues E22 and D23 as likely binding ligands. From the Mn(II) effects on crosspeak intensities in the ^1H , ^{13}C -HSQC spectra, a $K_D^{\text{app}*}$ value of $44 \pm 17 \mu\text{M}$ was derived using Eq.1 for residues exhibiting significant signal attenuation (Fig. S1). However, the NMR-derived $K_D^{\text{app}*}$ parameter is only a rough estimate of affinity: it is not equal to K_D^{app} derived from fluorescence studies, nor is it a strictly quantitative measure of binding strength.

Fluorescence spectroscopy

Previous studies have shown that paramagnetic Cu(II) ions quench the intrinsic fluorescence of Y10 in A β peptides [69,70]. The details of the quenching mechanisms remain unknown. The varying dissociation constants (K_D) reported in the literature are highly dependent on experimental conditions such as buffer strength, peptide length and peptide aggregation state. In the present study, an apparent dissociation constant (K_D^{app}) for Cu(II) in relation to A β was found to be $\sim 3 \mu\text{M}$, determined from Eq. 1 (Fig. 3). This number is in agreement with previously published binding constants [19,27,51,70,71]. Although Mn(II) ions are paramagnetic, they did not induce any discernible quenching of the Y10 fluorescence under the present conditions (Fig. 3). Thus, in order to shed light on the binding properties of Mn(II), an experimental setup with Cu(II)/Mn(II) competition binding was devised. As seen in Fig. 3, the quenching effect of titrated Cu(II) ions is virtually unaffected by the presence of varying amounts of Mn(II) ions, as are the A β /Cu(II) K_D^{app} values. This lack of Mn(II)/Cu(II) competition suggests that Mn(II) binding to A β is much weaker than Cu(II) binding, or that the two ions have different binding sites, or both.

Molecular dynamics calculations

The stability of A β (1-16)'s interactions with Cu(II), Mn(II) and Zn(II) were investigated with MD

simulations, using the A β (1-16)/Zn(II) binding conformation present in the NMR structure as the starting point for all simulations. The average RMSD plots of backbone atoms for all conformers of each system are shown in Fig. 4. As can be seen from this figure, the peptide undergoes significant conformational changes during the production runs, which is corroborated by our clustering analysis shown in Fig. 5 of the main text and Figs. S2-S4 of the Supporting Information. In particular, in the case of the simulations of A β (1-16) with Mn(II), the metal ion appears to have dissociated from the initial binding site (formed by H6, E11, H13 and H14), preferring instead to coordinate to negatively charged residues near the N-terminus of the peptide, i.e. D1, E3, D7, and E11. This can also be seen in the metal-ligand distances shown in Fig. 6 and Table S1. In the case of Zn(II) and Cu(II), in contrast, the system was relatively stable over the course of 1 μ s of simulation time, with only slight dissociation of H13 from Cu(II) in the A β (1-16)/Cu(II) complex (Fig. 5B and Table S1).

Thus, the MD results indicate that while favourable for complexes with either Zn(II) or Cu(II), A β histidine coordination does not provide efficient binding to the Mn(II) ion, which instead appears to preferentially interact with negatively charged residues. Additionally, the overview of the top-ranked clusters from our clustering analysis for A β 16 simulations with Mn(II), Zn(II) and Cu(II) shown in Fig. 5 demonstrates that the preferred conformations for Mn(II) involve interactions with combinations of E11, and sometimes also D7 and D1, in contrast to the Zn(II) and Cu(II) simulations (Figures 5A and 5B), which prefer to interact with the central histidine residues. This, in turn, appears to be coupled with significant conformational rearrangement of the overall peptide.

Solid state atomic force microscopy

Solid state AFM images were used to investigate the morphologies of A β fibrils formed with or without varying (sub-, equi- and supra-stoichiometric) concentrations of Mn(II) ions (Fig. 7). The images are shown in higher resolution in Fig. S5. No major morphological changes were observed in the fibrils, for any Mn(II) concentration.

Circular Dichroism

Previous studies have reported the promotion of a structural change in A β in the presence of high concentrations of Cu(II) and Zn(II) ions [24]. Here, no direct structural transitions were observed when Mn(II) ions were titrated to an A β sample (Fig. S6A), and only a small decrease in CD signal was observed due to dilution of the sample volume.

To monitor secondary structural changes when the A β peptide solutions aggregate over time in the presence of different Mn(II) ion concentrations, CD spectra were recorded over a period of four hours (Figs. S6B and S6C). At time zero the monomeric A β peptide was present in a random coil type conformation. Without any Mn(II) present, the peptide converted to a distinct β -structure conformation with an isodichroic point at 206 nm. This transition was followed by CD signal attenuation, likely related to A β precipitation and formation of large A β aggregates with low light transmission (Fig. S6B). In the presence of Mn(II) ions similar structural changes were seen. The kinetics were not significantly affected (Fig. S6C).

CD spectra were recorded at the end of the incubation time for the A β /Mn(II) samples (Fig. 8) used to create fibrillary aggregates for AFM imaging (see above). The resulting spectra indicate that Mn(II) ions do not change the secondary structure transition to β -structure followed by peptide aggregation, Mn(II) ions do however appear to make this aggregation process go slightly faster in a concentration-dependent manner (Fig. 8).

Thioflavin T fluorescence

The aggregation kinetics of A β was monitored by ThT fluorescence experiments (Fig. S7). Preliminary

results indicate that stoichiometric low, equimolar, or stoichiometric high concentrations of added Mn(II) ions do not significantly affect the aggregation process, compared to A β alone (Fig. S7).

Thioflavin T (ThT) fluorescence was measured (data not shown) at the end of the incubation time (directly after the CD measurements) for the samples prepared for AFM imaging (see above). All samples showed the presence of ThT-active aggregates, regardless of the amount of added Mn(II) ions, consistent with the results from the solid state AFM and CD spectroscopy analysis.

Discussion

Alzheimer's disease is a severe and irreversible neurodegenerative disorder, pathologically characterized by senile plaques in the brain parenchyma. Plaque components include A β peptides and metal ions such as iron, calcium, copper and zinc [72]. In this study we demonstrate binding of Mn(II) ions to the monomeric A β peptide *in vitro*, which shows that metal ion binding to the A β peptide is not exclusively limited to physiologically abundant metal ions. Our NMR data indicates an apparent dissociation constant (K_D^{app*}) for Mn(II) of around 45 μ M, more than one order of magnitude weaker than the A β binding affinities for Cu(II) and Zn(II) ions [27,73,74]. Competitive binding experiments employing the intrinsic fluorescence of Y10 showed that Mn(II) ions were not able to compete with Cu(II) ion binding to A β . This might be due to weaker A β binding of Mn(II) ions and possibly at a slightly different binding site than Cu(II) ions. Cu(II) has been suggested to have similar binding ligands as Zn(II), which has been shown to bind to the three A β histidines and the aspartic acid in the N-terminus [24,26,27,75]. The current NMR data indicates that Mn(II) ions bind in the N-terminal part of A β , with interactions suggested for the D1 and H13 residues (Fig. 2). In addition, a secondary binding site can be seen around the negatively charged residues D22 and E23 (Fig. 1), where also Zn(II) ions are known to display specific binding [23,46]. Also V40 appears to be affected by the Mn(II) ions (Fig. 1), likely due to paramagnetic properties of Mn(II) or by a weak interaction. Attenuation of NMR signals in the A β N-terminal region can be explained by line broadening due to paramagnetic effects. Additionally to this paramagnetic line broadening, chemical exchange with an Mn(II)-induced transient structure could contribute to the signal attenuation. Due to the low binding affinity and the lack of chemical shift changes in the NMR spectra, such a possible metal ion induced conformation would however be rather unstable and transient.

The molecular modeling data for a truncated A β (1-16) peptide indicates that Mn(II) ions primarily interact with negatively charged residues such as D1, E3, D7, and E11 (Figs. 5C and S4). The exact details of the A β binding site(s) for Mn(II) cannot be elucidated in this work, although we conclude that Mn(II) ions likely bind different A β residues than Zn(II) and Cu(II) ions, and may display multiple binding modes. This finding is corroborated by the fact that in our simulations with Zn(II) and Cu(II), the ion maintained almost the same binding conformation as in the NMR structure [76], although the Cu(II) ion lost interaction with H13. However, this conformation appears unstable for Mn(II), and when using the position of Zn(II) in the NMR structure as our starting conformation for the Mn(II) simulations, Mn(II) left the binding site preferring instead to interact with E11 and either E3 or D7 depending on starting conformations. Although the theoretically determined binding sites do not coincide with the NMR-derived binding ligands, the variation may be due to general weak binding affinity of Mn(II) compared to either Zn(II) or Cu(II), or the used starting structure of a well-defined Zn(II) binding site in a truncated A β (1-16) peptide structure.

The effect of Mn(II) ions on the kinetics and aggregation of the A β peptide over time was monitored via the CD spectroscopy and ThT fluorescence experiments. Both methods indicated a random coil-like starting conformation followed by a transition to β -structure (Figs. S6C and S7). AFM images revealed

that the morphology of the amyloid fibrils formed by aggregated A β did not significantly vary in the presence or absence of Mn(II) ions during the incubation period (Fig. 7 and S5). These results are compatible with a weak and transient A β /Mn(II) interaction, which is not strong enough to influence the aggregation kinetics and fibril morphologies of A β .

Fragments consisting of 40 or 42 residues are the most common isoforms of A β peptides generated from APP by β - and γ -secretase enzymatic cleavages. *In vivo* the here studied A β (1-40) version is most abundant, while the amyloid plaques consist to a higher degree of more aggregation-prone A β (1-42) peptides. The N-terminal metal binding site in A β peptides is generally assumed to be similar for both A β (1-40) and A β (1-42). The A β (1-16) model here used for MD simulations includes this binding site, although simulations with a full-length A β peptide is motivated for future studies.

The current observations raise the question to what extent the binding of Mn(II) ions to A β has any relevance for AD pathology. Previously, Mn(II) ions have been suggested to induce APP and BACE1 expression [77]. Data on Mn in relation to AD are scarce and still under discussion, but some case reports describe dementia symptoms occurring after severe Mn exposure [78]. Mn has also been suggested to contribute to the pathogenesis of other neurodegenerative disorders such as Parkinson's disease (PD) [79,80] and ALS [81]. In one AD study, higher Mn concentrations in whole blood were correlated to lower AD disease severity scores, and higher Mn concentrations have also been associated with higher A β peptide concentrations in blood [82]. Some studies have however shown reduced Mn concentrations in AD blood compared to controls [83]. Mn, Fe, and Cr were significantly inversely correlated with CSF A β (1-42) concentrations in a human autopsy [84], although Cu and Zn showed the most distinctive correlation. In exposure experiments with non-human primates, Mn induced AD-like pathologies in the form of A β plaques, neuronal nuclear hypertrophy, intracytoplasmatic vacuoles, and other signs of neurodegeneration in the frontal cortex [85]. However, other *in vivo* studies did not show any significant primary effects of Mn(II) exposure or significant effects in AD patients [86–90]. The relation between AD and various metals appears to be complex, and further studies of A β metal binding properties are warranted. In conclusion our data show a weak Mn(II) ion binding to A β peptides, without competing with A β /Cu(II) binding, and with negligible effects on A β aggregation. These results add another piece to the A β /metals puzzle.

Conflict of interest statement

The authors report no conflicts of interest.

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Figure Legends

Fig. 1. NMR 2D ^1H , ^{15}N -HSQC data showing site-specific binding of Mn(II) ions to the A β peptide. Mn(II) acetate was titrated to 77 μM ^{13}C , ^{15}N -labeled monomeric A β (1-40) peptides in 20 mM HEPES buffer, pH 7.2, at +5 °C. Spectra are shown before (blue) and after (red) addition of approximately 3:1 ratio Mn(II)/A β . The 2D ^1H , ^{15}N -HSQC spectrum shows reduced signal intensities for N-terminal amino acids upon added Mn(II), indicating binding in this region. The amide crosspeak intensities are reduced also for E22 and D23, suggesting a secondary binding site for Mn(II). Amino acids designated with an asterisk (*) were not observed in spectra due to spectral overlap or fast solvent exchange effects.

Fig. 2. NMR 2D ^1H , ^{13}C -HSQC data showing site-specific binding of Mn(II) ions to the A β peptide. Mn(II) acetate was titrated to ^{13}C , ^{15}N -labeled monomeric 84 μM A β (1-40) peptides in 20 mM sodium phosphate buffer, pH 7.35, at +5 °C. Spectra are shown before (blue) and after (red) addition of approximately 3:1 ratio Mn(II)/A β . The N-terminal histidines (H6, H13, H14) are clearly affected compared to other aromatic side chains (A). Figure B shows particular effects on the C $^\alpha$ -H crosspeaks for H13 and D1. The amino acids designated with an asterisk (*) were not included in the relative intensity analysis due to spectral overlap.

Fig. 3. Intrinsic Y10 fluorescence intensity of 10 μM A β (1-40) in 20 mM sodium phosphate buffer, pH 7.3, versus metal ion concentration. Titration with Cu(II) ions decreases the fluorescence intensity due to Cu(II)-induced quenching. Fitting a binding curve to the Cu(II) data reveals an apparent dissociation constant (K_D^{app}) of approximately 3 μM . Adding Mn(II) ions does not affect the fluorescence signal, indicating that Mn(II) does not induce quenching. Titrating Cu(II) in the presence of 60 or 90 μM Mn(II) ions produces Cu(II) binding curves and K_D^{app} values similar to that for Cu(II) alone, showing that Mn(II) ions do not significantly interfere with Cu(II) binding to A β . This could be due to low affinity for A β /Mn(II) binding, or/and because Mn(II) and Cu(II) have slightly different binding sites.

Fig. 4. Average RMSD plots of A β (1-16) peptide in complex with (A) Zn(II), (B) Cu(II), and (C) Mn(II) ion in simulations from each NMR conformation - Struct1 (black), Struct2 (red) and Struct3 (blue). As can be seen from this figure, the peptide complexes are disordered and exhibit significant conformational changes along the trajectory.

Fig. 5. Top ranked cluster representations from our molecular dynamics simulations of A β (1-16) in complex with either (A) Zn(II), (B) Cu(II) or (C) Mn(II) ions. The clustering analysis for each metal was performed on the combined trajectories of each of our production runs from each NMR conformation, *i.e.* it corresponds to a total of 3 μs of simulation time for each metal. The divalent metal is coordinated by H6, E11, H13 and H14 in the starting conformations. Water molecules complete coordination with the metal. The Zn(II), Cu(II) and Mn(II) ions are shown in orange, green and purple respectively, and, for visual simplicity, we have converted our octahedral dummy model to a van der Waals sphere.

Fig. 6. Time evolution of metal ligand distances (in Å) during simulations of A β (1-16) in complex with each of (A) Zn(II), (B) Cu(II), and (C) Mn(II) ions. Distances are measured between the metal center and H6 (black), E11 (red), H13 (green) and H14 (blue). All differences are averages over three simulations of 1 μs in length. In all three cases, the metal ion was in complex with the sidechains of H6, E11, H13 and H14 at the start of the simulation. Note that the Y-axis range is not identical for all three panels, due to the large differences in distances involved with the different metals. For a tabulated version of these distances, see Table S1.

Fig. 7. Solid state AFM images of 100 μ M A β (1-40) in 20 mM phosphate buffer, pH 7.35, incubated for 6 h at +37 $^{\circ}$ C and 200 rpm shaking in Eppendorf tubes with no (A), 100 μ M (B), 300 μ M (C), 600 μ M (D), 1000 μ M (E), or 2000 μ M (F) added Mn(II) acetate. The amyloid fibrils formed display no significant morphological changes as a result of the added Mn(II) ions.

Fig. 8. Liquid phase CD spectra recorded at the end of the incubation time for ten times diluted A β samples shown in Fig. 7: 100 μ M A β (1-40) in 20 mM phosphate buffer, pH 7.35, incubated for 6 h at +37 $^{\circ}$ C and 200 rpm shaking in Eppendorf tubes with increasing concentrations of Mn(II) ions.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8