Characterization of the Zn^{II} Binding to the Peptide Amyloid-β^{1-16} linked to Alzheimer's Disease

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Aggregation of the human peptide amyloid-β (Aβ) is a key event in Alzheimer's disease (AD). Zinc ions play an important role in AD and in Aβ aggregation. In vitro, Zn^{II} binds to Aβ and accelerates its aggregation. In this work we have investigated Zn^{II} binding to the synthetic peptide $A\beta^{1-16}$, which contains the metal-binding domain of Aβ. Cd^{II} was used to probe the Zn^{II} site. $A\beta^{1-16}$ bound one equivalent of Zn^{II} with an apparent dissociation constant (K_d) of 10^{-4} M. This K_d value is in the same range as the Zn concentration needed to precipitate Aβ. Circular dichroism and NMR indicated predominantly random-coil secondary structures of apo-$A\beta^{1-16}$, Zn^{II}–$A\beta^{1-16}$ and Cd^{II}–$A\beta^{1-16}$, which were all highly dynamic and flexible. The three histidines at positions 6, 13 and 14 were suggested to be ligands to Zn^{II} and Cd^{II}. Evidence that the aspartate at position 1 served as a fourth ligand to Zn^{II} and Cd^{II} was found at pH 8.7. $^{111}Cd^{II}$ NMR showed a resonance at 84 ppm, in line with a mixed oxygen-/nitrogen-ligand environment. The tyrosine at position 10 could be excluded as a ligand.

Introduction

Amyloid plaques are a characteristic feature in the brains of Alzheimer's disease (AD) victims.[1] These plaques are mainly composed of an aggregated protein called amyloid-β (Aβ),[2] which originates from a membrane protein called amyloid precursor protein (APP) and is present in healthy brains in a soluble form.[3,4] Since the amyloid plaques occur only in AD patients, the aggregation process from Aβ to the plaques is considered to be a key event. According to the amyloid-cascade hypothesis, increased Aβ accumulation and aggregation lead first to the formation of Aβ oligomers and then to amyloid plaques.[5] These oligomers are believed to provoke neuronal disfunction and, later on, dementia, probably through the production of reactive oxygen species.[5] In this context, conditions influencing this aggregation are of great interest.[6] Studies in vitro, in cell cultures and in AD model mice all indicate an important role for metals (Zn, Cu and Fe) in this process.[7–10]

In the case of Zn, a large body of evidence pointing to an important role of this metal ion in the metabolism of APP and Aβ linked to Alzheimer's disease has been accumulated. Zn has been found at high concentrations (≈mM) in the amyloid plaques, and treatment with chelators partially solubilized the plaques.[11] APP possesses a high-affinity Zn-binding site, located outside of the Aβ-region.[12,13] Transgenic mice expressing human APP serve as a model for Alzheimer's disease, due to the pathology based on amyloid plaque formation. In such mice, the lack of the Zn-transporter ZnT3 (which transports Zn into synaptic vesicles) reduced the plaque load, so it was concluded that endogenous Zn contributed to amyloid deposition in transgenic mice.[14] A chelator called clioquinol, known to bind Zn and Cu, successfully reduced the amyloid plaque burden in transgenic mice. Clioquinol is currently undergoing testing in humans (clinical phase II).[15]

In vitro studies revealed that Zn promotes the aggregation of amyloid-β.[6,16,17] However, the Zn concentrations needed to induce Aβ aggregation differed. It is necessary to distinguish between the different forms used—$A\beta^{1-40}$ or $A\beta^{1-42}$, or the truncated $A\beta^{1-28}$ and $A\beta^{1-16}$—because the propensity for aggregation increases with the length of the peptide (all are believed to contain the Zn-binding site; see below). For $A\beta^{1-40}$, values from 5 μM to 100 μM have been reported to provoke significant precipitation (initially a value of 100 nM was reported,[17] but this was corrected to 5 μM in a following publication).[18]

Values of the same order were obtained for the dissociation constant of Zn–Aβ, but they differed widely in the conditions and methods used. Initially Bush et al. reported a substoichiometric binding of Zn to $A\beta^{1-40}$ with K_d values of ≈100 nM and, to a second site, 5 μM from a displacement assay with radioactive and cold Zn binding to blotted $A\beta^{1-40}$ (slightly lower K_d values of 334 nM and 15 μM were reported for $A\beta^{1-28}$).[17] In a subsequent study, also using blotted peptide, Clements et al. found no evidence for submicromolar binding, but confirmed a K_d of ≈5 μM for $A\beta^{1-40}$ with a value of 3.2 μM.[19] Higher K_d values of 300 μM for $A\beta^{1-40}$ and 57 μM for $A\beta^{1-42}$ were deduced

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from increased fluorescence of the single Tyr10 upon addition of Zn.[20]

It has been reported that the metal-binding site is in the N-terminal hydrophilic region composed of amino acids 1–16 of Aβ (called $A\beta^{1-16}$) and that the three histidines at positions 6, 13 and 14 were involved.[21] In more detail, replacement of His13 by an Arg diminished the Zn affinity and the Zn-induced aggregation in $A\beta^{1-28}$.[22] Yang et al. reported that replacement of either His13 or His14 by Ala eliminated the Zn-induced conformation change to β-sheet and aggregation of $A\beta^{1-28}$.[23] Raman spectroscopy studies on Zn-induced aggregates of $A\beta^{1-40}$ and $A\beta^{1-16}$ generated by addition of two- to fourfold excesses of Zn to the peptide have been reported.[24] The analysis suggested that all three histidines residues (6, 13 and 14) provide the primary metal binding sites and that Zn is bound to the N(tau) of His. The data also indicated that Tyr10 acted as a ligand at pH 7.4 (at least partially). It has also been suggested that the peptide aggregates through intermolecular His-(N(tau))–Zn^{II}–His(N(tau)) bridges.[24] The involvement of the three histidines as ligands has also been found by ^{1}H NMR measurements of Zn addition to $A\beta^{1-28}$, which gave rise to broadening of the C2-H and C4-H resonances of His6, His13 and His14.[25] Recently, ESI-mass spectrometric analysis of Zn–$A\beta^{1-16}$ investigated by collision-induced dissociation confirmed the three histidines as ligands and proposed Arg5, but not Tyr10, as a fourth ligand.[26] In conclusion, the three histidines were implicated in Zn binding in the soluble and aggregated forms of Aβ and its model compounds $A\beta^{1-16}$ and $A\beta^{1-28}$, but with other ligands it seems to be dependent on the aggregation state and the conditions.

In this study we investigated the interaction between Zn and $A\beta^{1-16}$, used as a model for the interaction between Zn and Aβ in the soluble form. The binding of Zn to soluble Aβ corresponds to the first step, before the Zn–Aβ complex starts to aggregate. $A\beta^{1-16}$ contains the Zn binding site (see above), and its low propensity to aggregate allows studies of the soluble form at high concentrations.[21]

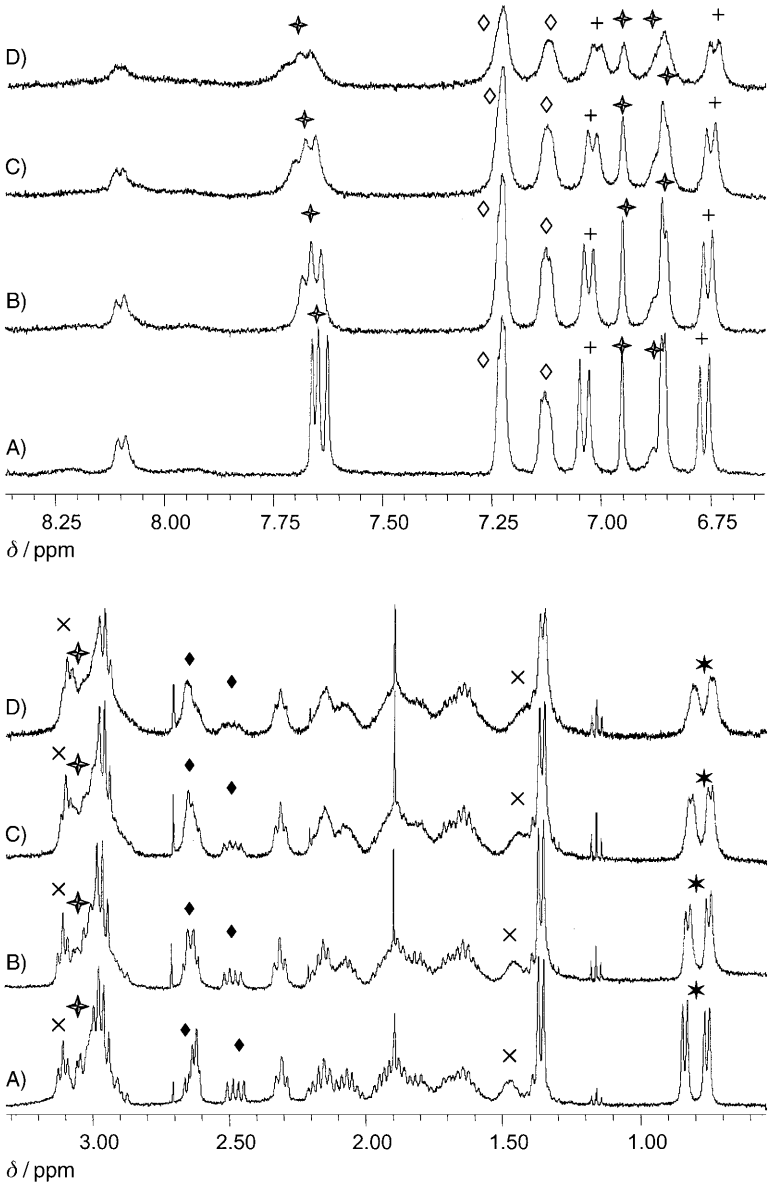

Figure 1. ^{1}H NMR of apo- and Zn^{II}–$A\beta^{1-16}$ at pH 8.7. The spectrum obtained from $A\beta^{1-16}$ (1 mM) in [D_{11}]Tris (pH 8.7, 50 mM)/D_2O (10%) at 293 K is shown (apo: trace A), together with spectra at 0.25, 0.5 and 1 mol equiv of added Zn^{2+} (traces B, C and D, respectively). The labelled resonances are: the three His units (✦: ^{2}H and ^{4}H resonances), Asp1 (◆: $^{\beta}$CH), Phe4 (◇: $^{3-5}$H), Arg5 (×: $^{\gamma}$CH and $^{\delta}$CH), Tyr10 (+: ^{2,6}H and ^{3,5}H) and Val12 (✶: $^{\beta}CH_3$).

Results and Discussion

The solubility of the peptide and its metal complexes at the concentrations to be used later for NMR were investigated first. Up to 1 mM $A\beta^{1-16}$, no precipitation—as estimated by the loss of the Tyr absorption at 275 nm after centrifugation (see Experimental Section)—occurred between pH 7.1 and 8.7. Addition of up to 1 equivalent of either Zn or Cd at pH 7.1 and 8.7 did not yield significant precipitation. At more than 1 equivalent, however, precipitation readily occurred. (This was also in line with NMR measurements, which showed a loss of intensity and a general broadening of the signals.) In order to be sure that no precipitation would occur, metals were added at slightly substoichiometric levels (0.95 equivalents).

^{1}H NMR

apo-$A\beta^{1-16}$: In order to investigate the effect of metal binding on $A\beta^{1-16}$, apo-$A\beta^{1-16}$ first had to be studied. TOCSY, COSY and NOESY experiments allowed the attribution of the resonances to the different protons in the $A\beta^{1-16}$ peptide. The assignment was performed at pH 8.7 and 7.1. 2D-NOESY experiments did not yield any medium- or long-range cross-peak (NOE); this indicates that $A\beta^{1-16}$ possessed a random structure (also con-

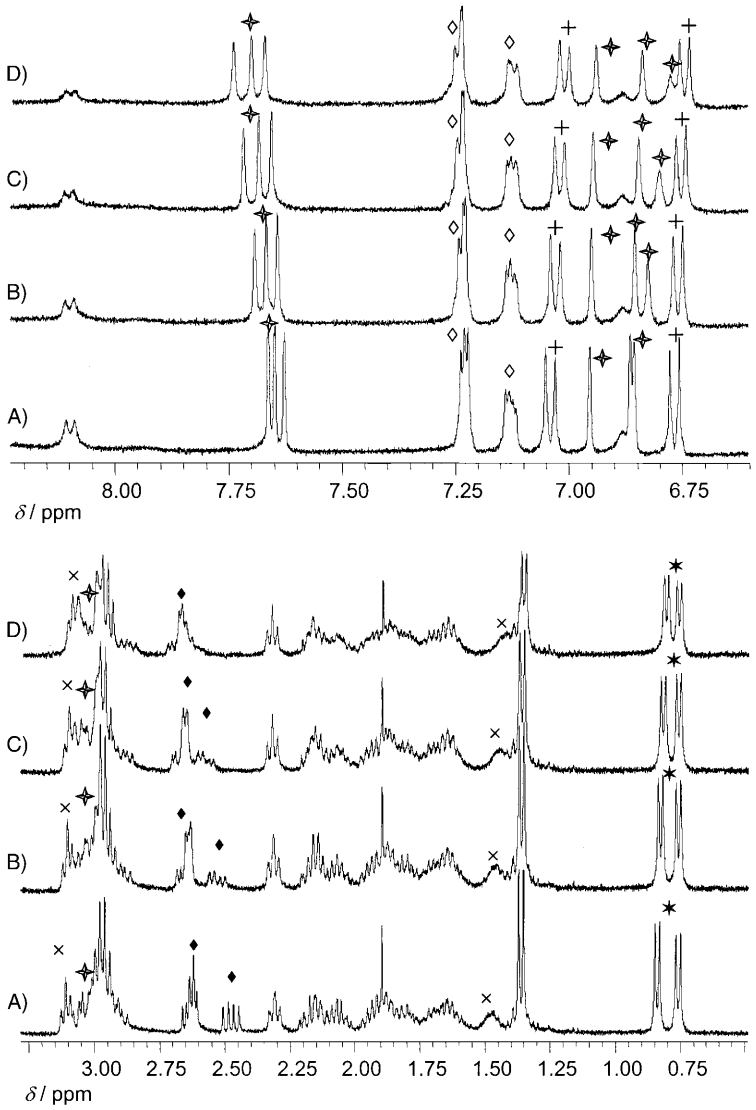

Figure 2. ^{1}H NMR of apo- and Cd^{II}–Aβ^{1-16} at pH 8.7. A spectrum obtained from Aβ^{1-16} (1 mM) in [D_{11}]Tris (pH 8.7, 50 mM)/D_2O (10%) at 293 K (apo: trace A) is shown, together with spectra at 0.25, 0.5 and 1 mol equiv of added Cd^{2+} (traces B, C and D, respectively). The labelled resonances are: the three His units (✦: ^{2}H and ^{4}H resonances), Asp_1 (◆: $^{\beta}$CH), Phe4 (◇: $^{3-5}$H), Arg5 (×: $^{\gamma}$CH and $^{\delta}$CH), Tyr10 (+: ^{2,6}H and ^{3,5}H) and Val12 (✶: $^{\beta}CH_3$).

firmed by the circular dichroism spectrum (see below and Figure 7).

Zn^{II}–Aβ^{1-16} at pH 8.7: The chemical shifts of the histidine side-chain protons are very sensitive to pH, due to the pK_a (about 6.5) of the imidazole H–N. In order to be sure that observed changes in the His resonances were due to the addition of the metal (and not to very slight changes in pH; see Experimental Section), the first experiments were recorded at pH 8.7, in a range in which His is completely deprotonated and the side-chain resonances are thus not very sensitive to slight pH changes. Figure 1 shows the ^{1}H NMR spectrum of apo-Aβ^{1-16} at pH 8.7 and the effect of the subsequent addition of 0.25, 0.5 and 0.95 equivalents of $ZnCl_2$. In general, broadening of the resonances could be observed with addition of $ZnCl_2$, but the extent of this broadening differed significantly, with much larger broadening for His6, His13, His14, Asp1, Tyr10 and Val12 than for the rest of the amino acids. Such intense broadening is likely to be due to the proximity to Zn^{II}, as is the case for ligands or other close-by residues. The Val side chain is aliphatic and can thus be excluded as a Zn^{II} ligand. Tyr residues are known to bind metals and Tyr10 has been suggested as a ligand in Aβ under certain conditions.[24,27] However, we provide evidence below that Tyr10 is not a ligand for Zn^{II} in Aβ^{1-16}, so the most likely ligands are Asp1 and the three His moieties. The large broadenings of the resonances of Tyr10 and Val13 were probably due to their proximity to the two His groups.

The progressive addition of Zn^{II} to Aβ^{1-16} also gave rise to small shifts of some resonances (not shown). Most affected were those residues that had also shown the greatest broadening (see above): that is, Asp1, His6, 13, 14, Tyr10 and Val12. The only exception was Arg5, which also showed a slight upfield shift but no broadening. Arg5 has been proposed as a ligand to Zn^{II}–Aβ^{1-16} and Aβ^{1-28} (from rat).[26,28] In general, the Arg side chain is not considered to be a ligand to metal ions, due to its high pK_a of $\approx$12.5. To the best of our knowledge there is no arginine side chain that has been clearly identified as a metal ligand for a protein or peptide. Even if it were conceivable that Arg ligation could be imposed by a protein, this would seem less likely in such a flexible peptide as Aβ.

Cd^{II}–Aβ^{1-16} at pH 8.7: The same type of experiment was also performed with Cd^{II} instead of Zn^{II}. Cd has often been used to probe Zn^{II} sites and has the advantage that it has two isotopes with spin $^1/_2$, exploitable by NMR (see below). In general, the addition of Cd affected the same resonances (Figure 2). However, the shifts of the resonances upon Cd addition were larger and the broadenings were smaller. His6, 13, 14 and Asp1 showed the largest downfield shifts and so are most consistent with being the ligands. Arg5 and Tyr10 showed upfield shifts less consistent with their being ligands. However, Cd^{II} binding to Aβ^{1-16} also produced slight downfield shifts in the two possible ligands Asp7 and Glu11, but these were much smaller than for Asp1. The four most likely ligands (His 6, 13, 14 and Asp1) also showed the largest broadening of the resonances.

Zn^{II}– and Cd^{II}–Aβ^{1-16} at pH 7.1: Similar experiments were also performed at pH 7.1, which better reflects physiological conditions. Addition of Zn to Aβ^{1-16} is shown in Figure 3. The three His moieties showed large broadenings upon addition of Zn, consistent with the Zn addition at higher pH and with His being involved in coordination. However, Asp1 was not significantly affected relative to the other residues (apart from Arg5 and Val12). There is thus no evidence for involvement of Asp1 as a ligand. However, no other resonance of any potential ligand was affected in return.

Zn^{II}–Aβ^{1-16} at pH 6.4: Investigation of Zn binding to Aβ^{1-16} at the lower pH of 6.4 did not reveal any further information

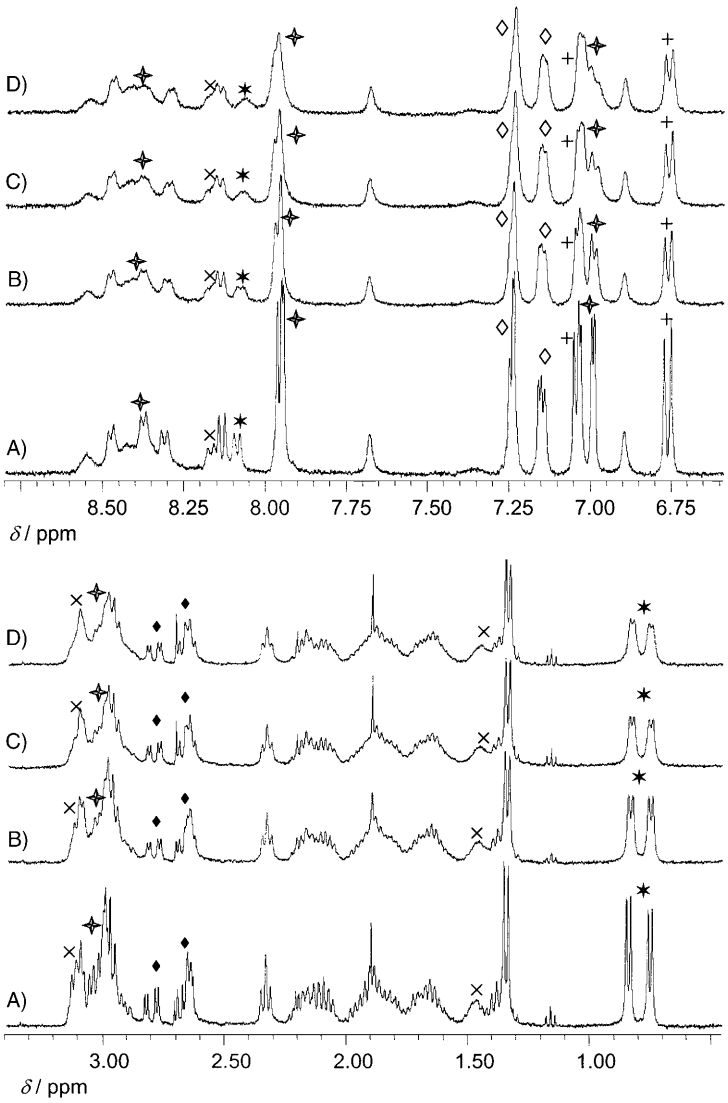

Figure 3. ^{1}H NMR of apo- and Zn^{II}–Aβ^{1-16} at pH 7.1. A spectrum obtained from Aβ^{1-16} (1 mM) in phosphate buffer (50 mM, pH 7.10)/D_2O (10%) at 293 K (apo: trace A) is shown, together with spectra at 0.25, 0.5 and 1 mol equiv of Zn^{2+} added (traces B, C and D, respectively)). The labelled resonances are: the three His units (✦: ^{2}H and ^{4}H resonances), Asp1 (◆: $^{\beta}$CH), Phe4 (◇: $^{3-5}$H), Arg5 (×: $^{\gamma}$CH and $^{\delta}$CH), Tyr10 (+: ^{2,6}H and ^{3,5}H) and Val12 (✶: $^{\beta}CH_3$).

concerning a fourth ligand. Again, the three His moieties were affected most, whilst the other resonances were either not affected (including Asp1) or showed the same behaviour as at high pH (e.g., broadening of Val12; not shown).

$^{111}Cd^{II}$–Aβ^{1-16}: ^{111}Cd and ^{113}Cd are both spin $^{1}/_{2}$ nuclei with similar magnetic properties and have proven to be useful probes for Zn^{II}-binding sites in proteins. Their chemical shifts are dependent on the number and types of bound ligands,[29] and with >90% enrichment can readily be detected at ≈millimolar concentrations. The ^{111}Cd NMR spectrum at pH 7.1 and 293 K showed at least one broad peak at ≈100 ppm, but the signal-to-noise ratio was to small to be reliable (Figure 4). The spectrum was therefore recorded at a higher temperature (323 K), and this is also shown in Figure 4 and features a single sharp peak at 84 ppm. This behaviour indicated dynamic behaviour in the Cd–Aβ^{1-16} complex, such as a metal exchange implicating different conformations, averaged out by the rising temperature. The chemical shifts of ^{111}Cd and ^{113}Cd correlate most strikingly with the type and number of ligands.[29,30] When the chemical shift of 84 ppm was compared to those of known Cd-substituted metalloproteins from the literature, it was found to fall into the region of mixed ligands consisting of oxygen and nitrogen. The two closest ^{113}Cd substituted proteins were: i) carboxypeptidase A, with a chemical shift δ_{Cd}= 120 ppm (ligands: two His, a bidentate Asp and a water molecule in a bipyramidal geometry) and ii) alkaline phosphatase B site, with a chemical shift δ_{Cd}=62 ppm (ligands: one His, two monodentate Asp and a serine in a tetrahedral geometry). According to calculations of ^{113}Cd shifts from first principles,[30] both the number of ligands and, to a lesser extent, the geometry—that is, bond lengths and angles—affect the chemical shift. In general, a higher coordination number produces a downfield shift (i.e., higher ppm). Thus, for the case of ^{111}Cd–Aβ^{1-16}, with three His moieties assumed as ligands (see above), it then fits best if no other additional ligand is present.[30] However, additional oxygen ligands—such as from Asp1—cannot be excluded and would be more in line with Cd^{II} coordination chemistry, which prefers a higher number of ligands (typically between four and six).

Circular dichroism

The circular dichroism spectrum of apo-Aβ^{1-16} (Figure 5) showed features typical of a predominantly random coil structure, in agreement with the 2001 study by Kozin et al.[21] Unlike in that study, however, the Aβ^{1-16} did not precipitate upon addition of up to 1 equivalent Zn or Cd, perhaps due to the different conditions (concentration of Aβ^{1-16}= 0.28 mM, 5 mM Tris/HCL, pH 7.3, 50 mM NaCl, whereas Kozin et al. used concentration of Aβ^{1-16}= 0.200 mM, 10 mM sodium phosphate buffer pH 6.5 and no salt).[21] The addition of up to 1 equivalent of either Zn^{II} or Cd^{II} did not change the CD spectrum significantly, indicating that Aβ^{1-16} stayed predominantly in a random coiled structure when bound to these metals. This is also in agreement with the fact that only small changes in a restricted number of residues were observed by ^{1}H NMR upon metal addition (see above).[21] It is interesting to note here that significant changes from random coil to a more structured state (β-structure) were observed in the CD study by Kozin et al. upon addition of Zn to Aβ^{1-16} with an acetylated N terminus and an amidated C terminus.[21] This could imply that either the N terminus or the C terminus plays an important role in binding of Zn to Aβ^{1-16}. The N terminus consists of the residue Asp1, which has also been shown to be affected by Zn, at least at higher pH (see above). It is thus possible that the N terminus acts as a ligand

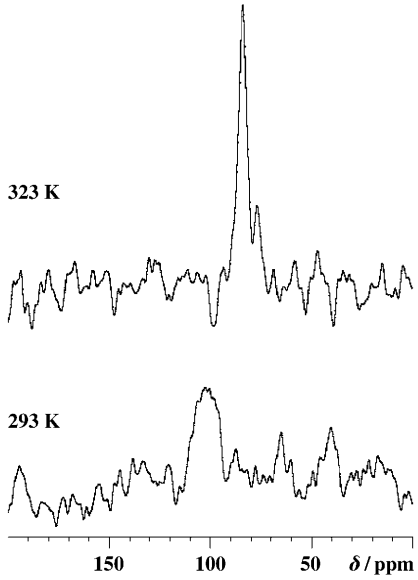

Figure 4. ^{111}Cd NMR spectrum of enriched ^{111}Cd–Aβ^{1-16} at 323 K (top) and 293 K (reference $Cd(ClO_4)_2$) in [D_{11}]Tris (50 mM, pH 7.1)/D_2O (10% vol).

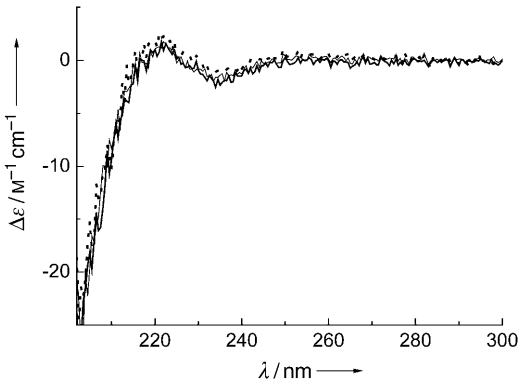

Figure 5. Circular dichroism spectra of apo-Aβ^{1-16} (····), Zn–Aβ^{1-16} (-----) and Cd–Aβ^{1-16} (——): [Aβ^{1-16}] = 284 μM, Tris/NaOH (pH 7.3, 10 mM).

or at least is important for Zn-ligation, and that this is abolished by acetylation of the N terminus.

Zn^{II}- and pH-dependent absorption of Tyr10 in Aβ^{1-16}

Tyr10 has been suggested to play an important role in the chemistry and biology of Aβ. In particular it has been proposed to serve as a ligand for Cu^{II}, Fe^{II} and Zn^{II}, but also implicated in radical reactions of Cu^{II}–Aβ, for example, forming dityrosines etc.[31,32] In order to investigate the role of Tyr10 in the complexation of Zn^{II}, the pH dependence of the absorption of Tyr10 was measured in the presence and in the absence of Zn^{II} (Figure 6). At physiological pH the addition of up to four equivalents of Zn^{II} did not significantly influence the absorption spectrum of the tyrosine. Note that Tyr10 is the only Tyr in the sequence and that no Trp is present, so the absorption above 250 nm is almost exclusively due to the phenolic side

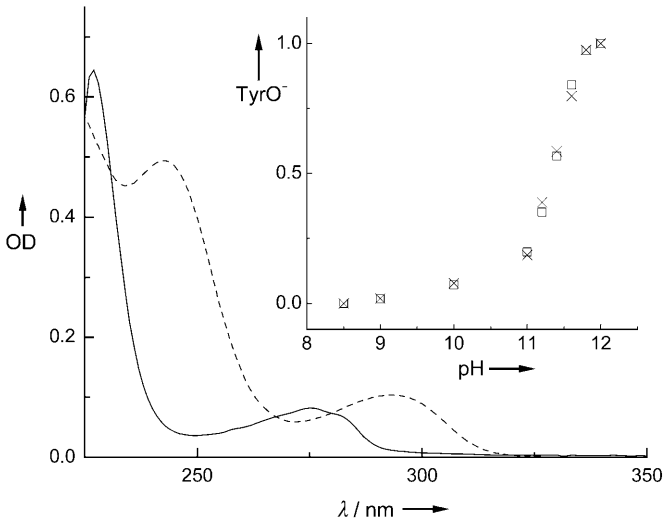

Figure 6. Absorption spectra of Aβ^{1-16} at pH 7.5 (——) with Tyr10 as tyrosine and pH 12 (-----) with Tyr10 as tyrosinate. Conditions: 60 μM Aβ^{1-16}. Inset: pH dependence of the protonation state of the phenolic proton of Tyr10 in the absence (□) and in the presence (×) of 1 equiv of Zn^{II}, estimated from the absorption of tyrosinate at 290 nm.

chain of Tyr10. If the Tyr10 were a ligand to Zn^{II}, deprotonation of the phenol side chain, and hence a change in the absorption spectrum, would be expected. This is shown by the spectrum of apo-Aβ^{1-16} at pH 11 (Figure 6, dashed line). The tyrosine absorption maximum has shifted from 275 nm to 290 nm, as is typical for tyrosinates. This suggests that Tyr10 does not form a tyrosinate ligated to Zn^{II}. However, it could also be possible that Tyr10 coordinates Zn^{II} (at least weakly) without losing its phenolic proton. In order to investigate this we measured whether Zn binding to Aβ^{1-16} would have an effect on the acidity of the phenolic proton of Tyr10, determining the pH dependence of the absorption spectrum of Aβ^{1-16}. From the fully protonated and fully deprotonated spectra of tyrosine (see above) the pK_a of Tyr10 in apo-Aβ^{1-16} was determined and was found to $\approx$11.3. In the presence of one to four equivalents of Zn the apparent pK_a did not change significantly (Figure 6; inset). This indicates that Tyr10 was not acting as a ligand to Zn^{II} (even when several equiv were present) and that the binding of Zn^{II} does not influence the pK_a of Tyr10, through a structural change, for example (in agreement with NMR data; see above).

Size-exclusion chromatography

In order to address the question of whether Aβ^{1-16} dimerises or oligomerises upon Zn binding, apo-Aβ^{1-16} and Zn–Aβ^{1-16} were analysed by size-exclusion chromatography. apo-Aβ^{1-16} and Zn–Aβ^{1-16} each exhibited a single peptide peak at about the same elution volume (Zn–Aβ^{1-16} had the tendency to elute later). The elution volume corresponded to an apparent molecular mass of about 4–5 kDa. This suggests that the Zn binding to Aβ^{1-16} does not augment the apparent molecular weight; that is, that Zn–Aβ^{1-16} is most probably monomeric, like the apo-peptide.[33]

Apparent binding constant of Zn^{II}–$A\beta^{1-16}$

Key to the physiological significance of Zn^{II} binding to Aβ is its affinity. Apparent binding constants can be estimated by competition with a chelator with a known binding constant in the same range. The Zn chelator Zincon (Zi) has been shown to be appropriate for Zn–protein complexes.[34,35]

Figure 7 shows the titration experiments (see Experimental Section). The upper panel showed the absorption at 620 nm from the Zn–Zi with addition of increasing amounts of $A\beta^{1-16}$. The decrease in the absorption at 620 nm reflects the transfer of Zn^{II} from Zi to $A\beta^{1-16}$. A concentration of about 13 μM $A\beta^{1-16}$ was necessary to reduce the band at 620 nm by half, indicating that half of the 5 μM Zn bound to 10 μM Zi had been transferred to $A\beta^{1-16}$ (dotted lines, upper panel). The binding constants were thus globally very similar, Zi being slightly stronger then $A\beta^{1-16}$. The calculations give an apparent binding constant (K_{app}) of 6.1×10^4 for Zn–$A\beta^{1-16}$ (the spectra of some titration points are depicted in the inset).

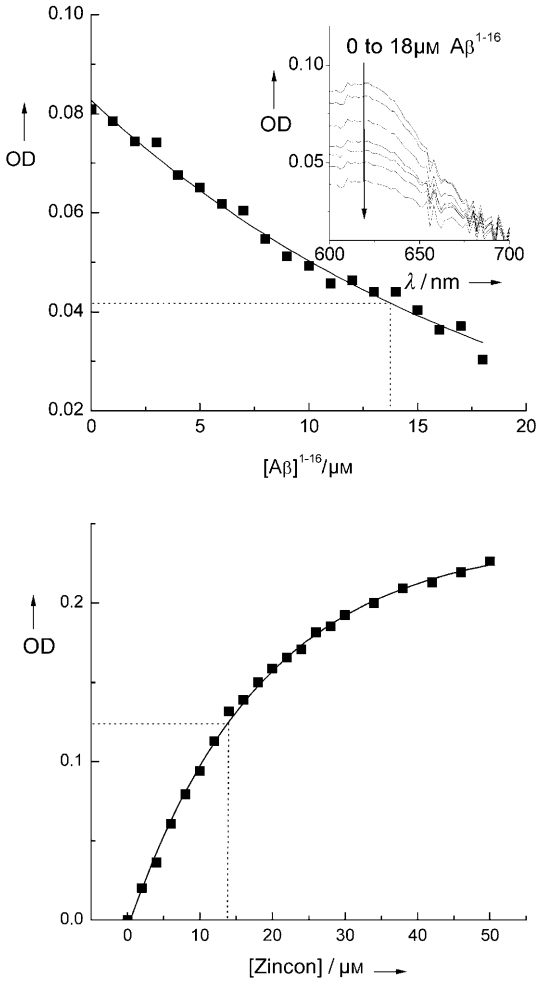

Figure 7. Estimation of the Zn^{II} binding constant by competition with the Zn^{II} chelator Zincon: Upper panel: absorption (at 620 nm) of the Zn–Zincon complex with addition of increasing amounts of $A\beta^{1-16}$. Conditions: Zincon (10 μM), Zn (5 μM), pH 7.4, HEPES (50 mM), NaCl (100 mM). Inset: corresponding absorption spectra of Zn–Zincon (10 μM) upon addition of 0, 3, 6, 9, 12, 15 and 18 μM $A\beta^{1-16}$ (in direction of the arrow). Lower panel: increasing concentrations of Zincon were added to the complex of Zn–$A\beta^{1-16}$, and the absorption (at 620 nm) of the Zn–Zincon was followed. Conditions: $A\beta^{1-16}$ (20 μM), Zn (10 μM), pH 7.4, HEPES (50 mM), NaCl (100 mM).

The lower panel shows the inverse experiment, in which Zi was added to Zn–$A\beta^{1-16}$. Here, half of the 10 μM Zn was bound to Zi after addition of ≈14 μM Zi.[36] This confirmed the above experiment and showed that, globally, the binding constants were in the same range with Zi a little stronger than $A\beta^{1-16}$. The calculation yielded a K_{app} of 5.5×10^4 for Zn–$A\beta^{1-16}$. The fact that the two approaches yielded the same K_{app} (within experimental limits) indicated that the Zn exchange reaction had reached equilibrium, a prerequisite for the calculation of K_{app}.

Dissociation constants have been measured for $A\beta^{1-40}$ and $A\beta^{1-28}$. In each case two constants were deduced: 104 nM and 5.2 μM for $A\beta^{1-40}$, and 334 nM and 15 μM for $A\beta^{1-28}$. The stronger constants in $A\beta^{1-40}$ and $A\beta^{1-28}$ were for substoichiometric binding of 0.7 and 0.25 respectively.[17] The binding studies were performed by use of a displacement assay with peptide blotted on a polyvinylidene difluoride membrane, so it is conceivable that blotting changed the structure of the peptide or that the peptide was partially aggregated, thus exhibiting a higher apparent binding constant. In a subsequent study with a similar method but other conditions, Clements et al. found no evidence for such submicromolar binding but confirmed a K_d value of ≈5 μM for $A\beta^{1-40}$ with their value of 3.2 μM.[19] The peptide was also blotted on a membrane (nitrocellulose) in this case. The K_d value of Aβ in solution (not blotted) has been deduced from increased fluorescence of the single Tyr10 upon Zn addition. Values of 300 μM for $A\beta^{1-40}$ and 57 μM for $A\beta^{1-42}$ were found.[20]

Our studies for $A\beta^{1-16}$ match well with the low μM K_d (3 to 15 μM), but no indication of stronger submicromolar binding was observed. It is very possible that the low submicromolar K_d was due to partially aggregated $A\beta^{1-40}$, whereas $A\beta^{1-16}$ is more soluble and thus no submicromolar K_d was observed. This would also explain why the submicromolar K_d binding site had a stoichiometry of 0.7 in $A\beta^{1-40}$ and only 0.25 in $A\beta^{1-28}$, which is less aggregating. It can be proposed that the Zn binding to Aβ in the soluble form is in the $K_d = 10$ μM range, but the apparent binding may become stronger when Zn–Aβ is aggregated. (It would have also to be shown that the apparent binding is thermodynamic and not kinetic trapping.) In the case of the higher K_d reported by fluorescence measurements, it has to be considered that these were carried out in Tris buffer (10 mM). Tris is known to ligate Zn modestly, which could be responsible for the higher apparent K_d of 300 μM for $A\beta^{1-40}$. In the current study, competition between two stronger ligands (Zincon and $A\beta^{1-16}$) was used, so the buffer should interfere less and a second HEPES buffer, which is a much weaker ligand for Zn than Tris, was used.

A dissociation constant in the μM range is not very strong relative to other Zn-binding proteins such as Zn-fingers, metallothionein etc.[35,37] However, it has been estimated that Zn concentrations can reach up to 300 μM in certain regions of the brain,[38] indicating that this binding site could be occupied by Zn. It is interesting to note that the Zn concentrations found to initiate precipitation of Aβ were of the same order as the binding constant (that is, ≈5 μM).[18,39] It is therefore very

possible that Zn binding to this site is related to the precipitation.

Conclusion

The Zn–Aβ^{1-16} complex showed an apparent dissociation constant (K_d) of about 15 μM, in line with previous observations of 3–5 μM for Aβ^{1-40} (and 300 μM in Tris buffer) and 15 μM for Aβ^{1-28}, and thus is consistent with Aβ^{1-16} being a minimal binding fraction of Aβ in its soluble form. The reported lower K_d values could be due to more aggregated forms of Zn–Aβ. Size-exclusion chromatography suggests that Zn–Aβ^{1-16} is monomeric.

Aβ^{1-16} bound Zn (and its substitute Cd) through the three His moieties. Evidence for Asp1 as a ligand has been found at higher pH values (8.5), but this is less clear at lower pH, although no evidence for another amino acid replacing Asp1 as ligand at lower pH was observed. The other previously proposed ligands, Arg5 and Tyr10, were not consistent with the data obtained, neither were the rest of the potential amino acids.

The fact that Zn and Cd binding affect the NMR resonances of Asp1 only at high, and not at low, pH is more consistent with binding to the amine group of Asp1 (i.e., the N terminus of the peptide) rather than to the carboxylate side chain. In contrast, the chemical shift seen in the ^{111}Cd NMR is more in line with an oxygen than a nitrogen ligand, but the latter cannot be excluded.

The peptide structures in the forms both of apo-Aβ^{1-16} and of Zn–Aβ^{1-16} were mostly random and likely to be very flexible, so it is conceivable that Asp1, likely to be a ligand at pH 8.7, also serves as a ligand at neutral pH, but attaching to and detaching from the Zn, binding through its amine and carboxylate group or being in competition with other ligands such as water or hydroxide.

It is interesting to note that a recent report by Hou et al. showed that the same ^{1}H resonances—that is, the three His moieties and the aspartate region—were affected upon aggregation of Aβ (without addition of metals).[40] They found evidence that the histidines are engaged in electrostatic interactions with the aspartate, thus forming a loop with a turn in the region of Ser8 to Val12. Another possibility for the formation of such a loop is the binding of a metal to the His and Asp, which would explain the promoting effect of metals on the Aβ aggregation.

Experimental Section

Sample preparation: The peptide Aβ^{1-16} (sequence Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys) was synthesized by standard F-MOC chemistry and purified by HPLC on a C8 column (Brownlee labs). ESI-MS showed masses of 1954.95 and 978.55 for the mono- and dicharged species, respectively, which was in agreement with calculated masses of 1954.88 and 978.44, respectively. Most samples were used for multiple experiments and were stored by refrigeration. Under these conditions, neither degradation nor aggregation of the samples was observed. The peptide Aβ^{1-16} concentration was determined by absorption spectroscopy, by use of the well established extinction coefficient of Tyr at 275 nm ($\varepsilon = 1410\ cm^{-1}\,M^{-1}$).[41] (Note that the peptide is predominantly in a random coil conformation (see below) and so the Tyr is likely to be surrounded by water as for the free Tyr.)

The metals were added from concentrated metal solutions of $ZnCl_2$ or $CdCl_2$ in HCl (10 mM). The HCl was used to keep the metals in solution, so the pH was monitored after each addition of this metal stock solution to the peptide and adjusted if necessary.

NMR spectra: NMR spectra were recorded on Bruker AMX 400 and DMX 500 spectrometers fitted with 5 mm triple resonance inverse Z-gradient probes in H_2O (90%)/D_2O (10%). All chemical shifts for ^{1}H are relative to TMS, and Cd(ClO_4) was used as an external reference for ^{111}Cd. ^{1}H NMR spectra were recorded at 293 K and ^{111}Cd NMR spectra at 293 and 323 K.

The ^{1}H signals of the free peptide were assigned by conventional homonuclear methods based on 2D TOCSY, NOESY and gs-COSY45 experiments. Suppression of the water signal was achieved with a WATERGATE sequence. The NOESY spectrum was acquired with a mixing time of 200 ms and TOCSY was recorded with a spin-lock time of 80 ms. Typically, 4096 t_2 data points were collected for 512 t_1 increments. ^{111}Cd with inverse gated ^{1}H decoupling was acquired with 50 000 transients, an acquisition time of 0.5 s and a recycle delay of 1 s. Spectra processing was performed on a Silicon Graphics O2 workstation with use of XWINNMR 2.5 software.

The addition of Zn^{2+} or Cd^{2+} to Aβ^{1-16} was carried out at 293 K. The peptide (1 mM) was dissolved in [D_{11}]Tris (0.5 mL, 50 mM) or phosphate (0.5 mL, 50 mM) buffer depending on the pH selected. Zn^{2+} or Cd^{2+} were added as chloride salts from concentrated stock solutions (40 mM) in order to keep the concentration of peptide almost constant (volume changes were below 5%). The pH was checked after each addition (and readjusted if necessary).

For ^{111}Cd NMR measurements ^{111}Cd was added from a concentrated aqueous solution of $^{111}CdCl_2$, generated by dissolving ^{111}CdO (95.11% isotopic purity, Oak Ridge National Laboratory, Oak Ridge, TN) in HCl (1 M) and then washing with pure water several times. The pH was checked after each addition. Chemical shifts given are with reference to $Cd(ClO_4)_2$.

As a control, ^{111}Cd in the buffer without the peptide was measured under the same conditions. A signal was clearly shifted to 60 ppm (as compared to the presence of peptide), which was in agreement with Cd^{II} complexed (at least partially) to the Tris buffer.

The ^{1}H NMR spectrum was recorded after each addition to determine the observed chemical shifts of the peptide at a given concentration of the corresponding Zn^{II} or Cd^{II}.

Circular dichroism: CD spectra were recorded on an AVIV Circular Dichroism model 202 spectrometer at 25 °C. Typically, a cell with a 0.1 cm path length was used for spectra recorded between 185 and 400 nm, with sampling points every 0.5 nm. A 1 cm cell path length was used for data between 240 and 800 nm, with a 2 nm sampling interval. A minimum of two scans were averaged, and baseline spectra were subtracted from each spectrum. AVIV software was used to smooth data when necessary. Data were processed with the aid of an origin spreadsheet/graph package. Direct CD measurements (θ, in millidegrees) were converted to molar ellipticity, $\Delta\varepsilon$ ($M^{-1}\,cm^{-1}$) by use of the relationship $\Delta\varepsilon = \theta/33\,000 \times c \times l$, where c represents the concentration and l is the path length. The Aβ^{1-16} concentration was 284 μM in Tris/NaOH (10 mM) at pH 7.3 and pH 8.7. Aliquots (0 to 1 mol equivalent) of 40 mM $CdCl_2$ or $ZnCl_2$ were added.

Absorption spectroscopy: UV/visible absorption spectra were obtained with a Cary 2300 spectrometer with use of a 1 cm path length quartz cuvette.

Size-exclusion chromatography: The apopeptide and its complexes with Zn^{II} were analysed by size-exclusion chromatography. The separations were performed with an AKTA Instrument (Amersham) fitted with an Amersham Biosciences Superdex 75 10/300 GL size exclusion column (300×10 mm) under isocratic conditions. For the Zn–Aβ^{1-16}, HEPES/NaOH buffer solution (pH 7.4, 20 mM) containing NaCl (100 mM) was used. Since the Zn–Aβ^{1-16} complex lost the Zn under these conditions, the same experiments were repeated in the presence of $ZnCl_2$ (10 or 100 μM) in order to ensure that Zn was always bound to Aβ^{1-16}. The Aβ^{1-16} peak was collected and quantified by absorption (see above), which showed that Aβ^{1-16} had passed through the column quantitatively. This excludes the possibility of polymerisation of Zn–Aβ^{1-16} having occurred but having escaped our detection by the peptide's getting stuck on the column. Molecular weights were estimated from the calibration plot utilizing bovine serum albumin (M_r = 67 000), cytochrome C (M_r = 13 600), aprotinin (M_r = 6512), vitamin B12 (M_r = 1355) and tyrosine (M_r = 181.2; Sigma–Aldrich reagents).

Apparent binding constant of Zn^{II}–Ab^{1-16}: The apparent binding constant was estimated by competition assay with the colorimetric Zn-chelator Zincon (2-carboxy-2′-hydroxy-5′-(sulfoformazyl)benzene.[42] It has been reported in the literature that Zincon (Zi) forms a complex with Zn^{II} in a 1 to 1 stoichiometry (Zn^{II}–Zi), showing a distinct absorption band at 620 nm (ε = 23 500 $cm^{-1}\,M^{-1}$) at pH 7.4 and having an apparent binding constant (K_{app}) of 7.9×10^4.[34] We verified this parameters by titration of $ZnCl_2$ to different concentrations of Zi (10–20 μM) under our conditions (pH 7.4, 50 mM HEPES, 100 mM NaCl). Very similar parameters, within the experimental limits, were found (i.e., $\varepsilon \approx 25\,000$ $cm^{-1}\,M^{-1}$; $K_{app} \approx 8.5\times10^4$).

The binding equilibrium of Zn^{II} between Aβ^{1-16} and Zi can be expressed as Equation (1):

$$Zn^{II}-A\beta^{1-16}+Zi \rightleftharpoons A\beta^{1-16}+Zn^{II}-Zi \quad (1)$$

The apparent binding constant of Zn^{II}–Aβ^{1-16} can be calculated by resolving Equation (2) for K_{app}(Aβ^{1-16}).

$$\frac{[Zn^{II}-A\beta^{1-16}]\,[Zi]}{[A\beta^{1-16}]\,[Zn-Zi]} = \frac{K_{app}(Zn-A\beta^{1-16})}{K_{app}(Zn-Zi)} \quad (2)$$

In the case of titration of Aβ^{1-16} to the Zn–Zi complex, the absorption band at 620 nm was due to the Zn–Zi complex, which decreased upon addition of Aβ^{1-16}. This decrease reflected the transfer of Zn^{II} from Zi to Aβ^{1-16}, which yielded [Zn^{II}–Zi] and [Zn^{II}–Aβ^{1-16}] for Equation (A). [Zi] and [Aβ^{1-16}] could be calculated by subtracting the Zn-bound fraction from the initial concentration (i.e., $[Zi] = [Zi]_{total} - [Zn–Zi]$ and $[A\beta^{1-16}] = [A\beta^{1-16}]_{total} - [Zn–A\beta^{1-16}]$). By taking the reported binding constant of Zn^{II}–Zi (see above) into account, the apparent binding constant of Zn^{II}–Aβ^{1-16} could be calculated.[28] In order to make sure that most Zn was bound to the ligand, an excess of Zi over Zn (2:1 ratio) was used as a starting point of the titration.

An analogous approach was used for the inverse titration: that is, the addition of Zi to Zn^{II}–Aβ^{1-16}. Both approaches yielded similar apparent binding constants of Zn^{II}–Aβ^{1-16}, indicating that reaction 1 had reached equilibrium.

Mass spectrometry: ESI-MS were performed on an API-365 quadrupole mass spectrometer (Perkin–Elmer Sciex). The samples were prepared at a concentration of 75 μM in $CH_3COO^-NH_4^+/NH_3$ buffer (pH 8.7 or 7.1, depending on the conditions, 50 mM) to 30% vol. MeOH. Apo-Aβ^{1-16}, Zn^{II}–Aβ^{1-16} and Cd^{II}–Aβ^{1-16} exhibited double positive measured peaks at 978.55 Da, 1009.75 Da and 1033.15 Da, respectively. These masses fit well with the calculated masses of 978.44, 1009.90 and 1033.89 Da, respectively; the isotope distribution was also in agreement with the coordination of the different metals. In all cases, however, the spectrum also showed peaks corresponding to the apo-Aβ^{1-16}. We interpret this as indicating that Zn^{II} and Cd^{II} each formed a 1:1 complex with Aβ^{1-16}, but this was disrupted during the measurement, as is known for several other metal–peptide complexes in the literature.[43,44] The formation of a 1:1 complex of Zn^{II} and Aβ^{1-16} has already been shown by mass spectrometry.[26]

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