Metal-Binding Properties of the Peptide APP$^{170–188}$: A Model of the Zn^{II}-Binding Site of Amyloid Precursor Protein (APP)

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Abstract: Amyloid precursor protein (APP) plays a key role in Alzheimer's disease (AD), although the function of this membrane protein is still unclear. Metal ions are implicated in AD and they also interact with APP. APP possesses a strong Zn^{II} binding site, which is evolutionary conserved. In this paper a synthetic peptide, APP$^{170–188}$, with a sequence corresponding to the conserved Zn^{II}-binding domain of APP, was synthesised and its metal-binding properties analysed. Titration experiments pointed to the binding of a stoichiometric amount of divalent ions. Further studies indicated that the binding of divalent metals like Zn^{II}, Cd^{II} and Co^{II} induces the dimerisation of the peptide. This dimer contains a dinuclear cluster in which the two divalent metals are bridged by two thiolate ligands from cysteine residues. The other two ligands of the tetrahedral coordination sites of each metal ion are terminal thiolate ligands. This structure was supported by the following arguments. The complex formed with Co^{II} presents the characteristic features for tetrahedral tetrathiolate coordination in its UV-visible spectrum. The sequence of APP$^{170–188}$ contains only three cysteine residues, which is incompatible with a monomeric Co^{II}–APP$^{170–188}$ complex. EPR measurements of the complex with one equivalent of Co^{II} show almost no signal at 4 K, which is compatible with an antiferromagnetic spin-coupling of the metal ions in a cluster structure. Size-exclusion chromatography indicated that the elution time for the complexes with Zn^{II} and Cd^{II} corresponds to the expected molecular weight of a dimer. The circular dichroism (CD) spectrum of the complex with one equivalent of Cd^{II} shows a band at 265 nm(+), and an ellipticity similar to those observed for similar Cd^{II}–thiolate clusters. Possible biological implications of the Zn^{II} binding site and the metal-induced dimerisation are discussed.

Keywords: Alzheimer's disease · amyloid precursor protein (APP) · circular dichroism · metalloproteins · zinc

Introduction

The membrane protein known as amyloid precursor protein (APP) plays a central role in Alzheimer's disease (AD). The peptide amyloid-β (Aβ) is one of the APP cleavage products. Aβ is the major constituent of one of the hallmarks of AD—the amyloid plaques. According to the amyloid cascade hypothesis,[1] an increased Aβ production and accumulation leads first to the formation of Aβ oligomers and then to amyloid plaques. These oligomers are thought to provoke neuronal disfunction, and later-on dementia, probably due to the production of reactive oxygen species. There are also indications that the entire APP (or at least fractions other than Aβ) also forms part of the amyloid plaques.[2] Moreover, several mutations in the APP gene have been linked to familial early-onset AD. This agrees with the fact that transgenic mice overexpressing human APP form amyloid plaques similar to those found in AD patients. These mice are used as animal models for AD.[3]

Studies in vitro, in cell cultures and AD model mice, indicate an important role for metals in this context.[4,5] It has been shown that high concentrations of Zn, Cu, and Fe are found in the amyloid plaques (~mM), and that copper and zinc metabolism is linked to the APP/Aβ metabolism, that is, they influence each other.[6–8] In vitro studies have revealed that these metals promote the aggregation of amyloid-β.[4,9] Moreover, a metal chelator called clioquinol successfully reduces the amyloid plaque burden in transgenic mice. Clioquinol is currently being tested in humans (clinical phase II trials).

APP is a transmembrane protein existing in different isoforms containing 695–770 amino acids. The location of the Aβ is partly in the transmembrane region (amino acids 597–

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639 for the isoform APP 695). APP has been shown to interact with metals and may even play a role as a metal chaperone.[4,5] APP contains a Cu-binding site that consists of four ligands provided by the amino acids His-147, His-151, Tyr-168 and Met-170.[10] Bush et al. have shown that APP is able to bind Zn^{II} relatively strongly, with a *K*d of 765 nM, and that the cysteine residues at positions 186 and 187 in APP are essential for Zn^{II} binding.[11] Further studies have demonstrated a similar Zn^{II}-binding behaviour for the APP-homologue proteins APLP1 and 2,[12] in which the two cysteine residues proposed to be the Zn ligands are conserved.

All this indicates that Zn^{II} binding to APP could play an important role in the metabolism of APP and hence in AD aetiology. To get a deeper insight into the nature of the Zn^{II}-binding site of APP, we have investigated in the present work the metal-binding properties of a synthetic peptide consisting of 19 amino acids corresponding to the sequence of amino acids 170 to 188 of APP. This peptide corresponds to the conserved Zn^{II}-binding domain of APP and its homologues APLP1 and 2. It contains the two key Cys ligands (Cys186 and 187), but also other possible ligands, for example Cys-174, Met-170, Asp-177 and Glu-184.

Results and Discussion

Zn^{II}–$APP^{170–188}$: Figure 1 (top) shows the absorption spectra of the titration of $ZnCl_2$ against the peptide $APP^{170–188}$. The $apoAPP^{170–188}$ curve shows the typical tailing of the absorption of the polypeptide backbone and a weak absorption band at 257 nm for the two Phe moieties. Upon addition of Zn^{II} an increase of the absorption at around 220 nm was observed up to the addition of one equivalent of Zn^{II}. Further addition of Zn^{II} caused no additional change in the spectrum. This is better seen in the difference spectra depicted in the lower part of Figure 1, in which the absorption of $apoAPP^{170–188}$ has been subtracted. The addition of Zn^{II} induces the formation of a band centred at about 220 nm. This change in absorption is typical for the ligation of thiolates to Zn^{II} and is due to ligand-to-metal charge transfer (LMCT) bands.[13,14] The fact that the absorption at 220 nm rises up to the addition of one equivalent of Zn^{II} and then reaches a plateau (see inset in Figure 1) implies that all three cysteine residues (Cys) are involved in the binding of the first equivalent of Zn^{II}. This is confirmed by the intensity of the absorption band at around 220 nm, which exhibits an extinction coefficient (ε) of about $20\times10^3\,M^{-1}cm^{-1}$. Based on an extinction coefficient of $6\times10^3\,M^{-1}cm^{-1}$ per Zn-bound Cys,[13] approximately 3.2 cysteine residues are bound to Zn^{II} in Zn^{II}–$APP^{170–188}$. Titrations performed at pH 7.5 and 8.4 in phosphate and TRIS buffer, respectively, revealed no significant differences. However, the fact that the LMCT bands of Cys–Zn^{II} overlap with the peptide bands makes the analyses more difficult, since Zn^{II} binding could also affect the properties of the absorption of the peptide backbone. Therefore Zn^{II} was substituted by Cd^{II}, a strategy often applied to Zn^{II}–Cys moieties in peptides/proteins, and also to other ligands.[15,16]

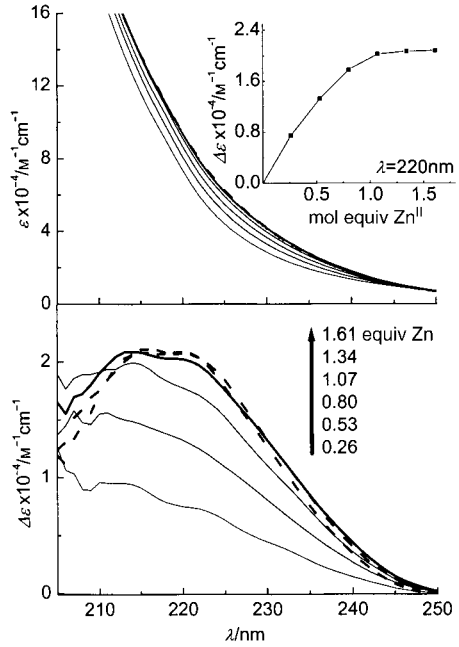

Figure 1. Absorption spectra of Zn^{II} binding to the peptide $APP^{170–188}$. Top: Electronic absorption spectra of $APP^{170–188}$ at pH 8.4, recorded as a function of the addition of increasing equivalents of Zn^{II}. The inset shows the effect of increasing amounts of Zn^{II} on λ_{220} nm plotted as a function of the Zn to peptide ratio. Bottom: Difference absorption spectra, obtained by subtracting the spectrum of $apoAPP^{170–188}$ from the spectra obtained after addition of Zn^{II} to $APP^{170–188}$.

Cd^{II}–$APP^{170–188}$: The titration curve of $APP^{170–188}$ with Cd^{II} shows the steady intensity increase of the typical LMCT band of the Cys–Cd^{II}[15,17] bond at 250 nm (Figure 2). As in the case of Zn^{II}, this band increases with the addition of up to one equivalent of Cd^{II} (Figure 2, inset). Above one equivalent a slight shift to the red was observed, but no further increase in intensity. This behaviour is similar to that observed with Zn^{II} and indicates that all three cysteine residues are bound to the Cd^{II} after the addition of one equivalent of metal ion. As in the case of Zn^{II} this is confirmed by extinction coefficient of the band at 250 nm, about $16\times10^3\,M^{-1}cm^{-1}$, which is in line with three cysteine residues bound to Cd^{II} based on the well-established extinction coefficient of about $5500\,M^{-1}cm^{-1}$ per Cys bound to Cd^{II}.[17]

The red shift of the shoulder at 250 nm upon the addition of more than one equivalent Cd^{II} could be due to Cd^{II}-induced aggregation and formation of large Cd^{II}–thiolate assemblies. This is supported by the large tailing into the visible region, which is probably due to light scattering (Figure 2). Again, the spectroscopic features do not differ significantly between pH 7.5 and 8.5. A Gaussian analysis of the absorption increase upon binding of one equivalent of Cd^{II} reveals a satisfying fit, with two bands centred at about 225 nm and 245 nm (see Figure 3, upper panel). These bands are similar to LMCT bands of other Cd–thiolate moieties.[15]

The circular dichroism (CD) spectra of $apoAPP^{170–188}$ and Cd^{II}-$APP^{170–188}$ are shown in Figure 3. Below 240 nm the CD

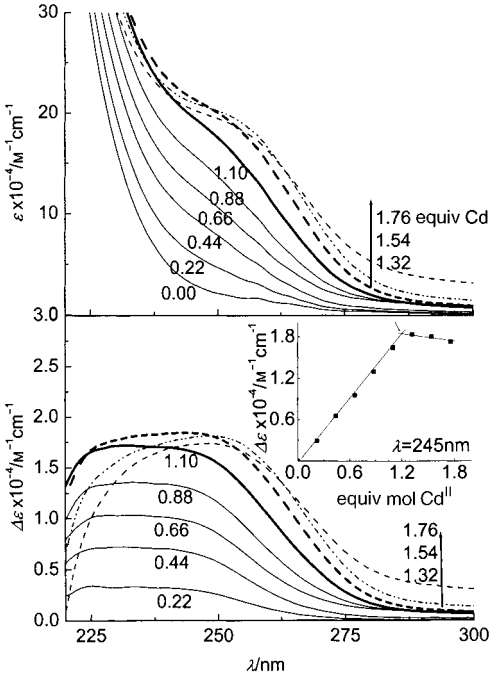

Figure 2. Absorption spectra of Cd^{II} binding to the peptide $APP^{170-188}$. Top: Electronic absorption spectra of $APP^{170-188}$ at pH 7.5, recorded as a function of the addition of increasing equivalents of Cd^{II}. Bottom: Difference absorption spectra, obtained by subtracting the spectrum of $apoAPP^{170-188}$ from the spectra obtained after addition of Cd^{II} to $APP^{170-188}$. The inset shows the effect of increasing amounts of Cd^{II} on λ_{245} nm plotted as a function of the Cd^{II} to peptide ratio.

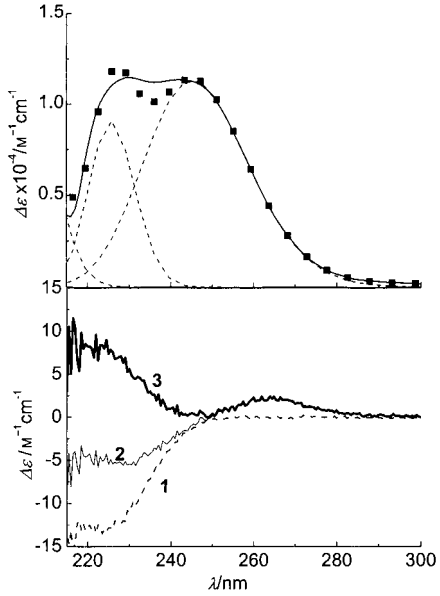

Figure 3. Top: Difference absorption spectra of the complex Cd–$APP^{170-188}$ (1:1) at pH 8.4 (solid line) with Gaussian analysis: individual bands (dashed line) and sum (filled squares). Bottom: Circular dichroism (CD) spectra of 40 μM of $apoAPP^{170-188}$ (dash line, curve 1) and of the complex Cd-$APP^{170-188}$ (1:1) (thin line, curve 2) at pH 8. Curve 3 represents the difference CD spectra obtained by subtracting $apoAPP^{170-188}$ from Cd–$APP^{170-188}$ (1:1).

features give information about the secondary structure of the peptide backbone. In the present case the CD spectrum is mostly in line with a predominantly random-coil structure (negative band at ~198 nm, not shown) and a significant contribution of an α-helix (negative band at 222 nm whose intensity corresponds to approximately 7% α-helix). Upon addition of one equivalent of Cd^{II} to $apoAPP^{170-188}$ the negative band at 222 nm becomes smaller and a new band appears at about 264 nm. Since LMCT transitions (Cys–Cd) also occur at 222 nm (see above), and are likely to be optically active, it is not certain that the α-helical content of the peptide decreases. However, the peptide backbone does not absorb above 240 nm and, hence, the features in this region can be attributed to the Cd binding, that is, to the Cd–Cys LMCT transitions. The first LMCT band in the absorption spectrum of Cd^{II}–$APP^{170-188}$ is centred at 245 nm (see above). It does not correlate with the first band observed at 265 nm in the CD spectrum of Cd^{II}–$APP^{170-188}$. Such a mismatch has also been observed in Cd^{II}–Cys clusters, in which the absorption band is split into two bands in the CD spectrum due to excitonic coupling of the transition dipole moments.[18,19] In the case of metallothionein the first absorption band is at 250 nm and the CD spectrum shows a positive band at 260 nm and a negative band at 240 nm.[18] In contrast, in the mononuclear Cd^{II}–Cys_4 centre of Cd-substituted rubredoxin[17] the absorption and CD bands coincide at 245 nm. The splitting of the first LMCT band in the CD spectrum has been assigned to an excitonic coupling of the transition dipole moments due to the cluster structure. In this context, it is possible that Cd^{II}–$APP^{170-188}$ also contains a Cd–Cys cluster structure, which would explain the location of the first CD band. However, the second, negative CD band of the splitting is not observed for Cd^{II}–$APP^{170-188}$, although it could be possible that this band is masked by the next transition at about 220 nm.

Co^{II}–$APP^{170-188}$: Co^{II} has often be used to probe Zn^{II} sites in proteins and peptides. The UV-visible spectrum of Co^{II} contains a lot of information about the coordination chemistry.[20] The spectra of $APP^{170-188}$ with 0.5, 1 and 1.5 equivalents of Co^{II} are shown in Figure 4. The complex with one

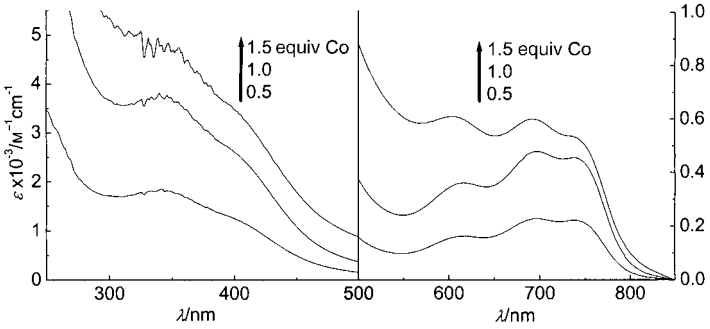

Figure 4. Electronic absorption spectra of the complex Co^{II}–$APP^{170-188}$ at pH 8.6, obtained for addition of 0.5, 1.0, and 1.5 equivalents of Co^{II}, as indicated. Left: charge-transfer region. Right: d–d ligand-field transition region.

equivalent of Co^{II}–$APP^{170–188}$ shows d–d transitions in the region between 550 and 850 nm, with bands at 612 ($\varepsilon = 337$), 696 ($\varepsilon = 458$) and 742 nm ($\varepsilon = 430\,M^{-1}cm^{-1}$). These d–d transitions are characteristic of a high-spin Co^{II} ion in a tetrahedral tetrathiolate coordination environment (i.e. $[Co^{II}(S\text{-}Cys)_4]^{2-}$).[13,20–22] Octahedral and pentacoordinate Co^{II} complexes have smaller extinction coefficients, that is, $\varepsilon < 50$ and $50 < \varepsilon < 300\,M^{-1}cm^{-1}$, respectively. The d–d bands of tetracoordinate Co^{II} complexes show a strong dependence on the type of ligands,[20] with a steady decrease of the wavelength upon the replacement of a thiolate by a nitrogen ligand. The relatively high wavelengths of the d–d transitions in Co^{II}–$APP^{170–188}$ are in line with coordination by four thiolates. Very similar bands have been observed in proteins with an established tetrahedral tetrathiolate coordination.[13,21,23,24]

The right-hand figure in Figure 4 depicts the bands at 340 nm and a shoulder at 387 nm, which have been assigned to the thiolate-to-Co^{II} charge-transfer transitions.[20] A molar absorptivity of $1.1 \pm 0.2 \times 10^3\,M^{-1}cm^{-1}$ per Co^{II}–thiolate bond is well established.[25] This implies that all three cysteine residues are bound to the Co^{II} ion in Co^{II}–$APP^{170–188}$.

Although $APP^{170–188}$ contains only three cysteine residues, its spectroscopic data strongly indicate a tetrahedral tetrathiolate coordination of the Co^{II} centre. Thus, a dimeric model has been proposed (see Figure 5). In this model the

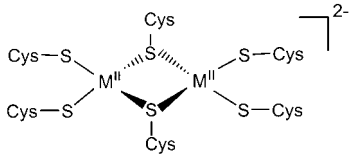

Figure 5. Tentative model of the binding of one equivalent of divalent metal ion to $APP^{170–188}$.

stoichiometry of one Co^{II} ion per $APP^{170–188}$ is maintained and the Co^{II} ion is in a tetrahedral tetrathiolate environment, in line with the spectroscopic data.

It has been shown in metallothionein that these bands undergo a red shift upon going from a monomeric (band at 305 nm, shoulder at 370 nm) to a Co^{II}–thiolate cluster (band at 320 nm, shoulder at 400 nm).[26] The positions of the LMCT band/shoulder at 340 and 387 nm, respectively, in Co^{II}–$APP^{170–188}$ indicate a cluster structure rather than a monomeric centre.

The addition of 0.5 equivalents of Co^{II} to $APP^{170–188}$ yielded identical d–d and LMCT bands as for one equivalent but with half the intensity (See Figure 4). This indicates that the dimeric Co^{II}–$APP^{170–188}$ complex is formed in a cooperative rather than a stepwise (sequential) manner. The addition of 1.5 equivalents of Co^{II} to $APP^{170–188}$ did not increase the intensity of the the d–d and LMCT bands (Figure 4), indicating that all three cysteine residues are bound to the Co^{II} ion after addition of only one equivalent (see also above), and that this additional half equivalent is not bound in a tetrahedral geometry. However, the addition of more than one equivalent (1.5 equivalents in total) induces a featureless exponential increase, which we assigned to light scattering due to aggregation. Indeed, aggregation could be observed in the cuvette.

In the proposed dimeric Co^{II}–$APP^{170–188}$ complex shown in Figure 5, in which the two Co^{II} ions are connected by two bridging thiolates, a strong coupling of the two paramagnetic Co^{II} centres would be expected. In order to test this EPR measurements were performed; these are shown in Figure 6.

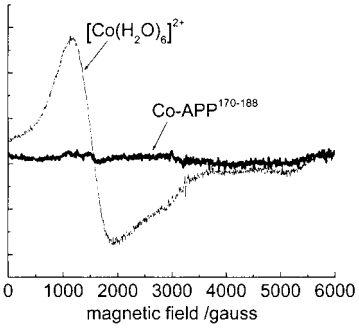

Figure 6. EPR spectra of the complex Co^{II}–$APP^{170–188}$ (thick line) and $[Co(H_2O)_6]^{2+}$ (thin line). Conditions: 300 μM Co–$APP^{170–188}$ (1:1) in 20 mM TRIS·HCl buffer (35 vol.-% CH_3CN), pH 8.6; 300 μM $[Co(H_2O)_6]^{2+}$; microwave power: 20 mW; microwave frequency: 9.25 GHz; temperature 4 K.

Co^{II}–$APP^{170–188}$ shows almost no EPR signal (thick line) when compared to a Co^{II} hexaaquo complex of identical concentration (thin line) measured under the same conditions. The absence of an EPR signal is compatible with an antiferromagnetic spin coupling of the metal ions mediated by bridging cysteine thiolate ligands. Precedents for such a situation have been reported for various metallothioneins[16,26] as well as for other ligand-bridged dinuclear Co^{II} centres.[27]

Apparent mass of M^{II}-$APP^{170–188}$: Since the spectroscopic data suggested the dimeric nature of Co^{II}-$APP^{170–188}$, and some evidence for a dimer was also found for Cd^{II}–$APP^{170–188}$, it was reasonable to assume that all the divalent metal-ion complexes of $APP^{170–188}$ are dimers. In order to test this hypothesis the apparent molecular weight was determined by subjecting the complexes to size-exclusion chromatography (see Experimental Section for details). Zn^{II}–$APP^{170–188}$ and Cd^{II}–$APP^{170–188}$ exhibited elution times corresponding to an apparent molecular weight of 4.5 ± 0.5 kDa, which corresponds to the expected molecular weight of a dimer. In contrast, apo$APP^{170–188}$ elutes with a much lower apparent molecular weight of about 1.4–2.5 kDa (depending on the conditions), which is compatible with the mass of 2.17 kDa of a monomer.

Apparent binding constant of Zn^{II}-$APP^{170–188}$: Apparent binding constants can be estimated by competition with a chelator whose binding constant is known and in the same

range. The zinc chelator 4-(2-pyridylazo)resorcinol (PAR) has been shown to be appropriate for Zn–thiolate protein complexes.[28,29] The titration experiment (see Experimental Section) showed that PAR and $APP^{170-188}$, at similar concentrations, compete for Zn^{II}, and that PAR is a stronger ligand (not shown). The binding constant of Zn^{II}-$APP^{170-188}$ was estimated to be $3\times10^9\,M^{-1}$. This value is similar to other structural eukaryotic Zn^{II} binding sites in proteins. Values in the range from 1.6×10^8 to $2\times10^{12}\,M^{-1}$ have been reported, with most of them around $10^{10}\,M^{-1}$.[28–31] This supports the importance of the Zn^{II}–APP interaction. The Zn^{II} binding constant to the entire APP has been estimated to be about $1.3\times10^6\,M^{-1}$.[11] This discrepancy can be explained by the fact that Bush et al. measured the Zn^{II} binding constant in the presence of 50 mM TRIS, which is known to bind only weakly to Zn^{II}.[11] Thus, their binding constant reflects the relative Zn^{II}–APP binding constant. In the present measurements competition for Zn^{II} binding is between an excess of two strong ligands ($APP^{170-188}$ and PAR), meaning that weak Zn^{II} binding to the buffer can be neglected. To compare the two numbers, the reported binding constant has to be corrected by the binding of Zn to TRIS at pH 7.5. An association constant of $10^4\,M^{-1}$ has been reported for Zn^{II} binding to TRIS.[32] This leads to a calculated apparent binding constant of about 2×10^3 at pH 7.5 when taking into account the pK_a (8.2) of TRIS. This yields a corrected apparent binding constant of about $3\times10^9\,M^{-1}$ for Zn^{II} to the entire APP, which is identical, within the experimental limits, to the binding constant of Zn^{II}–$APP^{170-188}$. Thus, the model peptide $APP^{170-188}$ reproduces well the binding constant of the entire APP; this is of biological relevance.

Conclusion

In the present work we have analysed the binding of the divalent ions Zn^{II}, Cd^{II} and Co^{II} to $APP^{170-188}$, which is a peptide corresponding to the amino-acid sequence 170 to 188 of amyloid precursor protein (APP). This is the conserved domain of the formerly identified Zn-binding site.[11] The obtained data suggest that the binding of divalent metals induces a dimerisation of the peptide. This dimer contains a dinuclear cluster in which the two divalent metals are bridged by two thiolate moieties from cysteines. The two metals are coordinated in a tetrahedral geometry, which is completed by two terminal thiolate ligands for each metal (see Figure 5). The spectroscopic titration experiments are more in line with a cooperative binding of the metals than with a sequential (stepwise) binding.

Possible biological consequences: Dimerisation of proteins is an important process in biology and it is known that the dimerisation of several membrane proteins is involved in the functioning of receptors.[33] There is also evidence that APP dimerisation occurs in vivo and that it plays an important role in the function of APP.[34] A precedent whereby Zn is involved or even regulates the dimerisation of proteins and is important to their function has been described for the DNA-binding protein Rad50,[35] as well as the Tat protein from the human immunodeficiency virus.[36a] In the latter case Zn-induced dimerisation has been mimicked successfully by a model peptide.[36b] Hence, it is possible that Zn could induce dimerisation of APP in vivo, and hence be important for the function of APP (which is still not known), or it could influence the processing of APP, and thus Aβ production, which is a key process in Alzheimer's disease.

Zn binding has also been reported for the secreted form of APP (consisting of the extracellular part, that is, amino acids 1–596/612 in APP695 form). It has been shown that this secreted form aggregates in the presence of Zn (at lower concentrations than needed for Aβ aggregation).[37] Moreover, APP has been found in the amyloid plaques of AD patients[2] along with high concentrations of Zn.[38] Thus, it is conceivable that the Zn–APP interaction and the tendency of the complex to dimerise could play a role in the formation of amyloid plaques (e.g., as a nucleation site for Aβ). However, these considerations are limited by the use of a model peptide in the present study, because the tertiary structure of the entire protein could cause differences of the metal-binding properties with respect to $APP^{170-188}$, which is mostly randomly structured (as determined by circular dichroism spectroscopy; see above). Thus, Zn-induced dimerisation of the entire APP has yet to be shown.

An important feature of Zn–thiolate moieties in biology is their connection with the redox reaction of cysteines [Eq. (1)].[31]

$$Zn^{II} + CysS{-}SCys \overset{2e^-}{\rightleftharpoons} CysS^- {-} Zn^{II} {-}{}^-SCys \qquad (1)$$

Zn binding is only strong when the cysteine group is reduced, and oxidation of the coordinating cysteine groups leads to ejection of the Zn ion. On the other hand the binding of Zn to the reduced cysteine residues augments the redox potential of Cys. From a biological perspective this can be regarded in two ways: either the binding of Zn regulates the oxidation state of the cysteine residues, or the redox state of the cysteine residues regulates the Zn binding. These regulations, called a Zn–cysteine redox switch, have been described for different proteins.[31,39,40] Such a Zn–cysteine redox switch would also explain the apparent contradiction of the redox state of the cysteine groups in APP. In the initial work,[11] the cysteines 186 and 187 were identified as ligands for a strong Zn-binding site, which is very likely to be reduced. In contrast, in one of the subsequent studies[10] the structure of the isolated domain from amino acids 124 to 189 was resolved by NMR spectroscopy; the three cysteines 174, 186 and 187 were involved in disulfide bonds and thus not available for Zn binding. This apparent contradiction could be explained by the Zn–sulfur chemistry described above.[39]

Experimental Section

Source and analysis of synthetic peptide: The synthetic peptide (purity >95%), with the sequence Ac-MLLPCGIDKFRGVEFVCCP-NH_2 ($APP^{170-188}$), was purchased from Advanced ChemTech Company. It was acetylated at the N terminus and amidated at the C terminus in order to mimic the peptide bonds occurring in the entire protein. Electrospray ionisation mass spectrometry (ESI-MS) measurements revealed a mass of 2168.55, which is in agreement with the calculated mass of 2168.69 for the peptide with all three thiols of the cysteines reduced.

The stock solutions of the peptide were prepared by dissolving the peptide in a mixture of CH_3CN/H_2O (35:65 v/v). Peptide concentrations were determined by two parallel methods: 1) by absorption measurements at 257.5 nm using the molar extinction coefficient $390\,M^{-1}\,cm^{-1}$, calculated from the contribution of the aromatic residues (two phenylalanines), which have an established molar extinction coefficient of $195\,M^{-1}\,cm^{-1}$;[41] 2) by the determination of the concentration of sulfhydryl groups with 2,2′-dithiopyridine. The concentration (3 Cys per peptide) was determined using $\varepsilon_{343}=7600\,M^{-1}\,cm^{-1}$.[42] The spectra were recorded with a Perkin–Elmer Instruments Lambda 35 UV-Vis Spectrometer.

Metal derivatives of $APP^{170-188}$: The generation of metal–peptide derivatives was performed by the addition of aliquots containing the desired number of equivalents of a metal-ion ($ZnSO_4$ or $CdCl_2$) stock solution to $APP^{170-188}$, either by the addition of the metal-ion solution to the peptide at pH 2 and raising the pH, or first raising the pH and then adding the metal-ion solution to the peptide. In both cases the spectroscopic absorption signatures showed no significant differences. The pH was raised by the addition of a stock solution of buffer at the desired pH (TRIS/HCl for pH 8.4, HEPES/NaOH or phosphate for pH 7.4). However, at pH 8.4 the cysteines in $apoAPP^{170-188}$ started to oxidise after a few minutes and, hence, in the case of addition of the metal at higher pH, this was done immediately after raising the pH. Once the peptide had bound to one equivalent of Zn or Cd the cysteines were not oxidised. The reduced cysteine content of the peptide was systematically verified with the 2,2′-dithiopyridine test at the beginning and end of the measurements in order to make sure that no oxidation occurred. The metal content of the stock solutions and the metal–peptide complexes was verified by ICP-mass spectrometry with a quadrupole Elan 6000 (Perkin Elmer) spectrometer.

The solubility of $APP^{170-188}$ in the buffer was not very high, probably due to the low number of charged amino acids in the peptide. It was found that the solubility is higher in the absence of salt in the buffer. To avoid precipitation, up to 8% acetonitrile was present in the samples with $APP^{170-188}$ concentrations below 100 μM; for higher concentrations 35% acetonitrile was necessary.

The preparation of the Co^{II}–$APP^{170-188}$ complexes was carried out in a nitrogen-purged glove box. All solutions were degassed and then saturated with argon on a vacuum line prior to their transfer into the glove box. For the titration of the peptide with Co^{II}, three independent Co^{II} samples were prepared that contained 0.5, 1 and 1.5 equivalents of Co^{II}. The $APP^{170-188}$ concentrations were between 180 and 300 μM in water/35% acetonitrile (the pH of this solution was about 2). Co^{II} was added from a $CoCl_2$ stock solution and then the pH was adjusted to 8.6 with 1 M TRIS/HCl buffer.

For spectroscopic measurements the Co^{II}–$APP^{170-188}$ complexes were transferred into a cuvette in the glove box and sealed. The spectroscopic measurements were performed immediately after the sample preparation. For the EPR measurements, the Co^{II}-$APP^{170-188}$ complexes were frozen immediately in liquid N_2. After the EPR measurement they were reanalysed by absorption spectroscopy. The spectra were unchanged, meaning that no oxidation occurred during the EPR measurements. EPR spectra were recorded on a Bruker ESP 300E spectrometer at 4 K (microwave power 20 mW, microwave frequency 9.25 GHz, modulation amplitude 10.515 G).

Size-exclusion chromatography (apparent molecular weight): The apopeptide and its complexes with Zn^{II} and Cd^{II} were analysed by size-exclusion chromatography in order to estimate the molecular weight. The separations were performed with a Waters Instrument equipped with an Amersham Biosciences Superdex 75 10/300 GL size-exclusion column (300×10 mm) under isocratic conditions. For the Me^{II}–$APP^{170-188}$ peptides a buffer solution of 20 mM of HEPES/NaOH at pH 8.0 containing 100 mM NaCl was used. Since the cysteines of $apoAPP^{170-188}$ were oxidised under these conditions and formed polymers due to intermolecular disulfide bridges two other methods were used. In the first case the generation of intermolecular disulfide bridges was prevented by the reaction of $apoAPP^{170-188}$ with DTP (see above). In the second case the chromatography was run at lower pH (10 mM HCl at pH 2 with 100 mM NaCl), which prevented the oxidation of the cysteines. The flow rate was $0.4\,mL\,min^{-1}$ and the separation was followed spectrophotometrically at 250 nm and 280 nm. Molecular weights were estimated from a calibration plot derived from bovine serum albumin ($M_r=67000$), cytochrome C ($M_r=13600$), aprotinin ($M_r=6512$), vitamin B12 ($M_r=1355$) and tyrosine ($M_r=181.2$) (all Sigma–Aldrich reagents)

Circular dichroism: Circular-dichroism spectra were recorded with a Jobin–Yvon Mark VI circular dichrograph at a scan speed of $0.2\,nm\,s^{-1}$ in 1 cm path-length cuvettes. $apoAPP^{170-188}$ and Cd^{II}–$APP^{170-188}$ (stoichiometry 1:1) were measured at a concentration of 40 μM in 2 mM TRIS/HCl at pH 8.0.

Mass spectrometry: ESI mass spectra were recorded on an API-365 quadrupole mass spectrometer from Perkin–Elmer Sciex. The samples were prepared at a concentration of 40 μM in 10 mM $CH_3COO^-NH_4^+$/NH_3 buffer at pH 8.0 and 35 vol% CH_3CN. The Zn^{II} and Cd^{II} complexes of $APP^{170-188}$ exhibited measured masses (based on the peaks of singly and doubly positive charged species) of 2233.4 and 2279.2, respectively. These fit well with the calculated masses of 2232.1 and 2279.1. However, in all cases the spectra also showed peaks corresponding to $apoAPP^{170-188}$. We interpreted this to mean that the metal–peptide complex breaks up during the measurement, as is known for several other metal–peptide complexes.[36b,43] This probably also precluded the detection of the proposed dimeric metal–peptide complexes (see above). The dissociation of dimeric metal–peptide complexes during ESI-MS measurements has already been reported.[36b]

Apparent binding constant of Zn^{II}-$APP^{170-188}$: The apparent binding constant was estimated by a competition assay with the colourimetric Zn chelator 4-(2-pyridylazo)resorcinol (PAR).[28] $Zn(PAR)_2$ shows a distinct absorption band at 500 nm ($\varepsilon=66000\,M^{-1}\,cm^{-1}$) at pH 7.5. The apparent binding constant of $Zn(PAR)_2$ has been reported to be 4×10^{12} at pH 7.5.[29] As PAR forms both 1:1 and 2:1 complexes with Zn^{II}, an excess of PAR must be used to bind >99% of the Zn^{II} to PAR in the 2:1 $Zn(PAR)_2$ complex. Thus, concentrations of 240 μM PAR and 10 μM Zn^{II} were titrated with 2–40 μM $APP^{170-188}$.[28] Under these conditions the binding equilibrium of Zn^{II} between $APP^{170-188}$ and PAR may be expressed as Equation (2).:

$$Zn^{II}-APP^{170-188} + 2\,PAR \rightleftharpoons APP^{170-188} + Zn(PAR)_2 \quad (2)$$

The apparent binding constant of $Zn-APP^{170-188}$ can be calculated by resolving Equation (3) for $K_{app(Zn-APP^{170-188})}$.

$$\frac{[Zn-APP^{170-188}][PAR]^2}{[APP^{170-188}][Zn(PAR)_2]} = \frac{K_{app(Zn-APP^{170-188})}}{K_{app(Zn(PAR)_2)}} \quad (3)$$

The absorption band at 500 nm due to the $Zn(PAR)_2$ complex decreased upon addition of $APP^{170-188}$(not shown). This decrease reflected the transfer of Zn^{II} from PAR to $APP^{170-188}$, which yielded $[Zn(PAR)_2]$ and $[Zn^{II}-APP^{170-188}]$ for Equation (3). [PAR] and $[APP^{170-188}]$ could be calculated by subtracting the Zn-bound fraction from the initial concentration (i.e. $[PAR]=[PAR]_{total}-2[Zn(PAR)_2]$; $[APP^{170-188}]=[APP^{170-188}]_{total}-[Zn-APP^{170-188}]$). By taking into account the reported binding constant of $Zn(PAR)_2$ (see above), the apparent binding constant of Zn^{II}–$APP^{170-188}$ could be calculated.[28]

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