Copper binding to the amyloid-beta (Aβ) peptide associated with Alzheimer’s disease. Folding, coordination geometry, pH dependence, stoichiometry and affinity of Aβ(1-28); insights from a range of complementary spectroscopic techniques.

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Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer’s disease; CD, circular dichroism; CSF, cerebrospinal fluid; EM, N-ethylmorpholine buffer; EPR, Electron Paramagnetic Resonance; NMR, Nuclear Magnetic Resonance; SOD, superoxide dismutase.
ABSTRACT

There is now direct evidence that copper is bound to amyloid–β peptide (Aβ) in senile plaque of Alzheimer’s disease (AD). Copper is also linked with the neurotoxicity of Aβ and free radical damage, and Cu\(^{2+}\) chelators represent a possible therapy for Alzheimer’s disease. We have therefore used a range of complementary spectroscopies to characterize the coordination of Cu\(^{2+}\) to Aβ in solution. The mode of copper binding is highly pH dependent. EPR spectroscopy indicates that both coppers have axial, Type II coordination geometry, square-planar or square-pyramidal, with nitrogen and oxygen ligands. Circular dichroism studies indicate that copper chelation causes a structural transition of Aβ. Competition studies with glycine and L-histidine indicate that copper binds to Aβ(1–28) at pH 7.4 with an affinity of \(K_a \sim 10^7\) M\(^{-1}\). \(^1\)H NMR indicates that histidine residues are involved in Cu\(^{2+}\) coordination but Tyr10 is not. Studies using analogues of Aβ(1-28) in which each of the histidine residues have been replaced by alanine or in which the N-terminus is acetylated suggest that the N-terminus and His13 are crucial for Cu\(^{2+}\) binding and that His6 and His14 are also implicated. Evidence for the link between AD and Cu\(^{2+}\) is growing, and our studies have made a significant contribution to understanding the mode of Cu\(^{2+}\) binding to Aβ in solution.
INTRODUCTION

Alzheimer’s disease (AD) is characterized by innumerable deposits of extracellular amyloid plaques. A small peptide, amyloid–β peptide (Aβ), plays a critical role in the initial build up of these amyloid plaques and is the main constituent of the amyloid deposits (1, 2). In addition genetic alterations underlying familial AD are associated with an increase in the production and/or the deposition of Aβ in the brain (3-6). Amyloid–β peptide can be between 39 and 43 residues in length, of which Aβ(1-40) and Aβ(1-42) are the most abundant fragments. The N-terminal portion of Aβ is hydrophilic, while the C-terminus amino acids 29-42 are rich in hydrophobic residues and represent the transmembrane region in the amyloid precursor protein. The sequence of human Aβ(1-42) is as shown:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Soluble Aβ(1-40) and Aβ(1-42) are found in the cerebrospinal fluid (CSF) and blood plasma of all humans where Aβ(40) has a concentration of 5 nM in CSF (7, 8). It is yet to be established what triggers Aβ to convert from its soluble form to an amyloidogenic form, but it has been shown that physiological levels of Cu$^{2+}$ and Zn$^{2+}$ cause marked aggregation of Aβ. This process is thought to be the prelude to amyloid formation (9). Levels of these metals are elevated in amyloid plaque deposits; 0.4 mM and 1 mM for Cu$^{2+}$ and Zn$^{2+}$ respectively (10). Cu$^{2+}$ induced aggregation of Aβ occurs as the pH is lowered to 6.8. This mildly acidic environment mimics a feature of inflammation found in AD (11). Studies on cerebrospinal fluid indicate that zinc will cause the selective aggregation of endogenous soluble Aβ peptide (12). Metal chelators specific to Cu$^{2+}$ and Zn$^{2+}$ will reverse this aggregation process (13, 14). In addition, the neurotoxicity of Aβ is linked to metal induced oxidative damage and is a
feature of the pathogenesis of AD (15). A dual role as a pro- and anti-oxidant in copper redox cycling in a Fenton-type reaction has been proposed for Aβ (16-22).

There are two commonly expressed objections to the role of copper in AD. The first objection is that AD is not associated with elevated exposure to environmental copper. It is important to clarify this misconception. The total concentration of copper within the brain is potentially more than sufficient to be neurotoxic. As a consequence the brain has efficient homeostatic mechanisms in place to maintain compartmentalization of metal ions, which when compromised cause neurodegenerative diseases such as Wilson’s and Menkes disease. There is evidence to suggest that homeostatic mechanisms for metal ions are impaired in AD patients and AD is characterized by altered metal ion-dependent processes and metal ion concentrations in the brain (23). The second objection is that the affinity of Aβ for Cu^{2+} is too low to bind these metals at their extracellular concentrations. This is also a misconception since extracellular levels of Cu^{2+} may reach as high as 15 μM (10), while we show here that Aβ affinity for Cu^{2+} is at the sub-micromolar level and is reported to be much higher in amyloid plaques (24).

A recent study using Raman spectroscopy has provided direct evidence that copper and zinc are bound via the histidine imidazole rings in isolated senile plaque cores (25). Perhaps one of the most significant pieces of evidence to link copper with AD is the observation that normally insoluble amyloid deposits of post-mortem brain tissue from AD patients can be solubilized in aqueous media by the presence of metal chelators specific to Cu^{2+}(14). It has been shown that the use of copper chelators can markedly inhibit amyloid accumulation in AD transgenic mice and are in trials as potential drug therapies for AD (26, 27). A recent
study has shown that trace amounts of copper in the drinking water of rabbits induces beta-amyloid plaque accumulation (28, 29).

Despite an increasing body of evidence to link Cu$^{2+}$ with AD, the precise coordination geometry and the residues involved in Cu$^{2+}$ ligation are yet to be established. Both monomeric and dimeric species have been proposed (30). In addition, there are disagreements as to the affinity and stoichiometry of binding. For example, both attomolar affinities (24) and micro-molar affinities (31) for Cu-Aβ(1-42) have been reported. In this study, we use a range of complementary spectroscopies to characterize the binding of Cu$^{2+}$ to Aβ and the structural changes induced in Aβ upon copper coordination. To facilitate solution spectroscopy methods we have used the more soluble fragment of Aβ(1-28) which lacks the C-terminal third of the molecule. Residues 29-42 are highly hydrophobic and are not believed to be associated with direct coordination of the metal ion (30, 41). In addition, we have studied a number of analogues of Aβ(1-28) in which each of the three histidine residues have been replaced with an alanine.
EXPERIMENTAL

Peptide Synthesis and Purification:- Peptides representing various fragments of the amyloid–β peptide were synthesized by employing solid phase F-moc chemistry and produced by the ABC facility at Imperial College, London. After removal from the resin and de-protection, the samples were purified using reverse phase HPLC and characterized using mass spectrometry and \(^1\)H NMR.

Titrations:- The pH was measured before and after each spectrum was recorded. N-Ethylmorpholine (EM) buffer was found not to interfere with Cu\(^{2+}\) binding. Typically, 50 mM EM buffer was used for EPR studies while for \(^1\)H NMR and CD studies, samples were prepared in ultra high quality (>18 \(\Omega\).cm resistivity) water and the pH was adjusted using small amounts of 0.1 M NaOH or HCl. The peptide concentrations were determined using the extinction coefficient of 1280 M\(^{-1}\) cm\(^{-1}\) (due to the single tyrosine residue) (32). Typically, the freeze-dried peptides contained 5 to 10 % moisture by weight. The addition of metal ions or competing ligands to the A\(\beta\) peptides was performed using small aliquots from stock aqueous solutions.

Circular Dichroism (CD):- 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 260 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 three scans was recorded and baseline spectra were subtracted from each spectrum. AVIV software was used to smooth data when necessary. Data was processed using Kaleidagraph spreadsheet/graph package. Direct CD measurements (\(\theta\), in mdeg) were
converted to molar ellipticity, $\Delta \varepsilon$ (M$^{-1}$ cm$^{-1}$) using the relationship $\Delta \varepsilon = \frac{\theta}{33,000 \times c \times l}$

where $c$ is the concentration and $l$ the path length.

Absorption Spectroscopy (UV/Vis):- UV/visible electronic absorption spectra were obtained with a Hitachi U-3010 double beam spectrophotometer, using a 1 cm path length quartz cuvette.

Fluorescence Spectroscopy:- Fluorescence spectra were collected using a Hitachi F-2500 fluorescence spectrophotometer. An excitation frequency of 280 nm was used and data collected over the range of 290 – 400 nm. Samples were placed in a four-sided quartz fluorescence cuvette (Hellma) and data recorded at room temperature.

Stability Constants:- The absolute affinity (pH independent) of L-histidine for Cu$^{2+}$ is 1.5 * 10$^{10}$ M$^{-1}$, the apparent affinity, at pH 7.8 is therefore 2.6 * 10$^{8}$ M$^{-1}$ or a dissociation constant of 1.5 nM. (As log $\alpha$= $pK_a$- pH = 9.17-7.8 = 1.37 and log $K_1$ (app) = log $K_1$ – log $\alpha$ = 10.2 – 1.37 = 8.83 or 6.7 * 10$^{8}$ M$^{-1}$). Similarly, the absolute affinity (pH independent) of glycine for Cu$^{2+}$ is 1.2 * 10$^9$ M$^{-1}$, the apparent affinity at pH 7.8 is therefore 1.8 * 10$^6$ M$^{-1}$ or a dissociation constant of 500 nM (33).

EPR:- X-band Electron Paramagnetic Resonance (EPR) data was recorded using a Bruker ELEXSYS 500 spectrometer, operating at a microwave frequency of ~ 9.3 GHz. All spectra were recorded using a microwave power of 0.63 mW across a sweep width of 2000 Gauss (centred at 3200 Gauss) with a modulation amplitude of 10 Gauss. Samples were frozen in quartz tubes and experiments carried out at between 20 and 120 K using a liquid Helium cryostat. A minimum of two scans were recorded per spectrum. All EPR spectra shown have
been background subtracted from a water blank with subsequent baseline correction in the XEPR software package using 3rd or 4th order polynomial splines. In order to analyse EPR data using the method described by Peisach and Blumberg (34), it is necessary to convert values from Gauss (G) to milli-Kaisers (mK) by using the formula $A_{II} \text{(mK)} = 0.046686 \times g \times H$ where $g = 2.0023$ and $H$ is the $A_{II}$ splitting measured in Gauss.

**NMR:** Proton NMR data was collected using a Varian UNITYplus-600 MHz $^1$H frequency spectrometer. Data was processed using the VNMR software package. All spectra were recorded at 25°C in 100% D$_2$O solution, typically at a peptide concentration of 1mM. Proton peak assignments were made by the analysis of two-dimensional TOCSY and ROESY spectra of the apo peptides. TOCSY and ROESY spectra were collected with typical mixing times of ~75 and ~300 ms respectively.

**Design of Peptides:** Typically the N-terminus was left as the native amino group while the truncated C-terminus was blocked as the ethyl ester at the C-terminus. Peptides synthesized of the human sequence included: -(Ac indicates acetylation of the N-terminal amino group.)

| Designation   | Sequence                                      |
|---------------|-----------------------------------------------|
| Aβ(1-28):     | DAEFRHDSGYEVHHQKLVFFAEDVGSNK                 |
| Aβ(H6A):      | DAEFRADSGYEYVHHQKLVFFAEDVGSNK                |
| Aβ(H13A):     | DAEFRHDSGYEVAIHQKLVFFAEDVGSNK               |
| Aβ(H14A):     | DAEFRHDSGYEVHAQKLVFFAEDVGSNK                |
| Aβ(Ac1-28):   | Ac-DAEFRHDSGYEVHHQKLVFFAEDVGSNK             |
| Aβ(1-16):     | DAEFRHDSGYEVHQK                              |
| Aβ(Ac10-16):  | Ac-YEVHHQK                                   |
| Aβ(1-11):     | DAEFRHDSGYE                                  |
RESULTS

**pH dependence of Cu$^{2+}$ binding:** Figure 1 shows the EPR spectra of 0.8 mol equivalent of Cu$^{2+}$ bound to Aβ(1-28) over a range of pH values between 5 and 10. The EPR spectrum at pH 5 gives a single set of signals typical of type II copper, axial (square-planar or square pyramidal) coordination geometry. The $A_{II}$, $g_{II}$ and $g_{\perp}$ values are 177 Gauss (~ 16.5 mK), 2.26 and 2.06 respectively. As the pH is increased a new set of hyperfine peaks are observed to higher field with $A_{II}$, $g_{II}$ and $g_{\perp}$ values of 170 Gauss (~ 15.9 mK), 2.22 and 2.06 respectively. Commensurate with the appearance of a new set of axial signals the spectra observed at pH 5 reduces in intensity. At pH 8, the two sets of EPR signals have comparable intensities, and at pH 9 the high field signals dominate. The variation in peak intensity with pH is shown as an insert in Figure 1. It is clear that at pH 7.4 a mixture of two complexes are observed. Peisach and Blumberg have shown that a combination of $A_{II}$ and $g_{II}$ values can indicate ligand type (34). The $A_{II}$ and $g_{II}$ values at pH 5 are most typical of three nitrogen and one oxygen ligands (3N1O) although 2N and 2O coordination cannot be ruled out. At pH 10 the $A_{II}$ and $g_{II}$ values are more typical for 4N coordination.

We have carried out complementary studies using CD spectroscopy. Figure 2 shows visible CD spectra for Aβ(1-28) with one equivalent Cu$^{2+}$ at various pH values between 4.5 and 10.5. At pH 7.5 a visible absorption band is observed at 600 nm ($\varepsilon_{600\text{nm}} = ~50 \text{ M}^{-1} \text{ cm}^{-1}$) which is typical of a type II Cu$^{2+}$ d-d transition. At pH 7.5 and below, the associated CD band is extremely weak and is not detected, however the accompanying CD band at 312 nm assigned as an amide-to-copper charge transfer band is observed at pH 5.5 and above (35). Only as the pH is raised to above pH 8.5 is a negative CD band at 514 nm observed, arising from Cu$^{2+}$ d-
d transitions. Inserts in Figure 2 show the change in the intensity of CD bands in the presence of 1 equivalent of Cu$^{2+}$ at 252 nm, 312 nm and 514 nm.

Relatively strong CD bands are often observed for d-d transitions of Cu$^{2+}$ tetragonal complexes (36, 37). However these complexes involve main-chain amide coordination as well as histidine coordination via the imidazole ring. In these cases, the dominant contribution to optical activity observed is due to the vicinal contributions resulting when the asymmetric alpha carbon is held in a chelate ring between two chelating donor atoms, for example adjacent main-chain amides (38). At physiological pH and below, the lack of optical activity from the d-d transition of the Cu-A$\beta$ complex suggests that backbone amide coordination is not taking place. It is likely that raising the pH above 8 promotes amide deprotonation and copper coordination by the main chain, resulting in a CD band being observed at 514 nm. In summary, it is clear that A$\beta$ forms a Type II square-planar coordination geometry with Cu$^{2+}$, and both EPR and CD measurements indicate that the coordinating ligands are highly pH dependent, a mixed species is present at physiological pH, and main-chain amide coordination is not present at lower pH values.

**Stoichiometry of Cu$^{2+}$ binding:** EPR spectra of A$\beta$(1-28) with increasing mole equivalents of Cu$^{2+}$ have been collected in order to identify the number of copper ions binding to A$\beta$ and are shown in Figure 3. With increasing amounts of Cu$^{2+}$ up to one equivalent, identical line shapes are observed with a commensurate increase in intensity. However, above one equivalent of copper, there is a clear shift to low field for the $g_\perp$ signal; 2.06 at one equivalent Cu$^{2+}$ and 2.07 at 2 equivalent Cu$^{2+}$. The signal increases linearly in intensity up to two equivalents of copper. Comparison of the EPR signal intensities with a Cu(Gly)$_2$ standard sample, confirms that all the EPR signals for the Cu-A$\beta$ complex are observed and therefore
rules out the possibility of EPR silent spin-coupled Cu$^{2+}$. Addition of a further mole equivalent of copper (and up to five mole equivalents) results in no further increase in the intensity of the EPR spectra. Cu$^{2+}$ ions in water will give a largely EPR silent signal at pH $> 7$ (39). We have confirmed this observation by obtaining EPR spectra of CuCl$_2$ in EM buffer at pH 7.8; copper EPR signals are drastically attenuated relative to Cu$^{2+}$ bound to A$\beta$. Spin integration of the EPR spectra has been plotted versus copper addition as shown as an insert in Figure 3. It is clear from the EPR data that A$\beta$(1-28) binds two Cu$^{2+}$ ions sequentially. After two mole equivalents of Cu$^{2+}$, A$\beta$(1-28) becomes saturated with copper at physiological pH. Similar studies have been performed using CD, both in the UV and visible regions. Monitoring of the band at 205 nm reveals saturation of A$\beta$(1-16) and A$\beta$(1-28) at two mole equivalent of Cu$^{2+}$ at pH 9. In summary both EPR and CD derived Cu$^{2+}$ binding curves indicate Cu$^{2+}$ saturation of binding sites on A$\beta$(1-28) after two mole equivalents.

**Folding of the backbone in the presence of Cu$^{2+}$**- It is believed that amyloid formation in AD and other amyloidogenic diseases such as prion disease are the result of protein or peptide misfolding. The A$\beta$ peptide has a structural transition associated with amyloid formation, with conversion from random coil to an extended beta sheet like conformation (40). CD spectra in the UV region can be used to monitor changes in the main-chain conformation. Figure 4 shows the CD spectra of A$\beta$(1-16) and A$\beta$(1-28) with addition of increasing amounts of Cu$^{2+}$, at various pH values. Cu$^{2+}$ binding to A$\beta$(1-16) occurs as low at pH 5.9, as the negative signal at 198 nm is reduced in intensity as the pH is increased from pH 5.5 to 5.9 in the presence of Cu$^{2+}$. Control experiments with A$\beta$(1-16) in the absence of Cu$^{2+}$ shows that there are no significant differences in the spectra between pH 5.2 and 9.5 (data not shown). At pH 7.5, addition of Cu$^{2+}$ (Figure 4a) causes a loss of the negative CD band at 198
nm and the appearance of a positive band at 225 nm. The intensity of a second positive contribution at 205 nm is pH sensitive and is more apparent at higher pH values. (For comparison, see Figures 4 a and b which show copper titrations at pH 7.5 and 9.5 respectively.) Similar changes in the CD spectra of Aβ(1-28) with Cu$^{2+}$ addition are observed as shown in Figure 4c. A positive band at 225 nm appears and is accompanied by a loss of negative band at 198 nm. The positive contribution at 205 nm with copper is observed but is swamped by the more intense random coil CD band at 200 nm. Apo subtracted difference spectra which illustrate the effect of Cu$^{2+}$ addition to both Aβ(1-16) and Aβ(1-28) are shown as inserts in Figure 4 a, b and c. The difference spectra emphasize the similarity in the changes in the secondary structure with copper addition between Aβ(1-16) and Aβ(1-28). The additional 12 residues to the C-terminus have no effect on the copper-induced conformational transition. The changes in the spectra with Cu$^{2+}$ addition are complete by two mole equivalents of Cu$^{2+}$, which agrees with the stoichiometries determined by EPR (Figure 3). Isodichroic points are observed at 217 nm and 195 nm, and these are maintained between 0 and 1.0 equivalent of Cu$^{2+}$. If a dimeric species were formed, the isodichroic point would be expected to shift at 0.5 equivalent Cu$^{2+}$.

The chirality at 217 nm is largely invariant with the addition of copper between pH values of 5.5 – 9.5. An increased negative CD signal at 217 nm is often attributed to β-sheet or extended conformation. The copper induced structuring of Aβ can therefore not be directly attributed to an increase in β-sheet or extended structure. The appearance of a positive CD contribution at 205 nm is not characteristic of any defined secondary structure but does indicate increased ordering of the main-chain.
**Affinity of copper binding:** Key to the physiological significance of Cu$^{2+}$ binding to Aβ is its affinity. With this in mind, we have used the competitive effects of glycine and L-histidine to measure Cu$^{2+}$ affinity for Aβ by fluorescence spectroscopy. Figure 5a shows that the addition of Cu$^{2+}$ to Aβ(1-28) causes marked quenching of the tyrosine fluorescence signal at 307 nm. As glycine is added, it competes with Aβ for the Cu$^{2+}$ and the tyrosine fluorescence signal reappears, as shown in Figure 5b. Cu$^{2+}$ coordinates to glycine via the amino and carboxylate groups with an apparent (pH adjusted) of $K_a = 1.8 \times 10^6$ M$^{-1}$, and two glycine residues will bind to a single Cu$^{2+}$ ion (33). It takes more than 100 mole equivalents of glycine to cause the tyrosine fluorescence signal to completely return to its maximal strength. Half the maximal quenching is achieved at $\sim$18 ($\pm$ 2) equivalents of glycine. Thus the affinity of Cu$^{2+}$ for Aβ(1-28) is at least an order of magnitude higher than that of glycine, putting the dissociation constant $K_d$ in the sub μM range ($K_d << 0.5 \mu$M). Similar experiments have been carried out using L-histidine as the competing ligand. In this case, tyrosine fluorescence returns to its maximal value with only 2.5 mol equivalents of L-histidine, as shown in Figure 5c. Two molecules of histidine will bind a single Cu$^{2+}$ ion using the amino and imidazole nitrogens as ligands with an apparent $K_d$ at pH 7.8 of 1.5 nM. This indicates that copper will bind to Aβ(1-28) with a lower affinity than L-histidine. This puts the affinity of Cu$^{2+}$ for Aβ greater than $1.8 \times 10^6$ M$^{-1}$ but less than $6.7 \times 10^8$ M$^{-1}$ or a $K_d$ of $<<500$ nM but $>1.5$ nM, i.e. 10-100 nM.

We have obtained similar Cu$^{2+}$ affinities for Aβ(1-28) using CD spectroscopy. CD was used to directly measure copper-Aβ associated absorption band at $\sim$ 314 nm. Using the competitive effects of glycine, copper absorption bands become CD silent when bound to non-chiral glycine (37). We find using the CD band at $\sim$ 314 nm that similar amounts of glycine are
required to remove Cu$^{2+}$ from Aβ(1-28) as is indicated by the fluorescence quenching experiments.

Very high affinities (10$^{15}$ M$^{-1}$) have previously been suggested for Cu-(Aβ)$_2$ (24), (i.e. at 0.5 equivalent Cu$^{2+}$). We note that glycine or L-histidine would have little impact on the binding of copper to such a high-affinity site. This could result in a false plateau for the fluorescence quenching data shown in Figure 5. To rule out this possibility, we have added sub-stoichiometric levels of Cu$^{2+}$ to Aβ (0.3 mol equivalent of Cu$^{2+}$). If there is indeed a very high-affinity site for copper associated with Aβ(1-28) then the addition of L-histidine would have little effect on the fluorescence signal. However, addition of just one equivalent L-histidine to Aβ(1-28) with 0.3 mole equivalent of Cu$^{2+}$ present caused the fluorescence signal to return to its maximal value. We can also rule out apo-Aβ being inadvertently loaded with Cu$^{2+}$ during peptide synthesis and sample preparation since no Cu$^{2+}$ associated signals are observed for apo Aβ(1-28) in EPR or CD spectra.

In summary, using both direct measurements of Cu-Aβ(1-28) from CD absorptions bands and indirect fluorescence quenching methods we have shown that the first mole equivalent of Cu$^{2+}$ ions bind to Aβ with a dissociation constant in the sub-micromolar level; 10-100 nM. The possibility of a high-affinity copper site for Aβ(1-28) has been ruled out.

**Cu$^{2+}$ coordination ligands:** Fragments of Aβ have been used to determine which residues are involved in binding to Cu$^{2+}$. The three histidine residues within Aβ(1-42) are thought to be the most likely candidates for Cu$^{2+}$ coordination under physiological conditions. Aβ(1-28), Aβ(1-16), Aβ(1-11) and Aβ(Ac10-16) (which is N-terminally blocked) were studied. CD
spectra in the visible region and the EPR spectra of Cu-Aβ(1-16) and Cu-Aβ(1-28) are almost indistinguishable. A comparison was made over a range of pH values between 4.5 and 9.5 at one mole equivalent Cu^{2+}. In the presence of 3 mole equivalent of Cu^{2+} the visible CD spectra of Aβ(1-16) and Aβ(1-28) are also very similar at all pHs. From Figure 6 it is clear that residues 17-28 have little influence on the binding of Cu^{2+} to Aβ either at 1 or 3 mole equivalent of Cu^{2+} over a range of pH values. The differences in the spectra of Aβ(1-16) and Aβ(1-28) around 270 nm are due to the presence of two Phe aromatic CD signals in the longer fragment irrespective of the presence of Cu^{2+}.

In contrast, the CD spectra of Aβ(Ac10-16) and Aβ(1-11) are very different from Aβ(1-16) and Aβ(1-28), as shown in Figure 6. In particular, at pH 7.5, Aβ(1-16) and Aβ(1-28) show almost no CD bands associated with d-d transitions above 500 nm while Aβ(1-11) has a negative CD band at 590 nm and Aβ(Ac10-16) has a strong positive CD band at 560 nm. The CD spectra of Aβ(Ac10-16) suggests that the N-terminus and/or His6 are involved in copper coordination. In addition, Aβ(1-11) CD spectra indicate that His13 and/or His14 are key residues in coordinating the Cu^{2+} ion. A difference in the EPR spectra between Cu-Aβ(Ac10-16) and copper complexes of the larger fragments are observed but are less pronounced, as Aβ(Ac10-16) still gives rise to an axial Type II Cu^{2+} spectrum (data not shown). The pH dependence of binding is similar for Aβ(1-16) and Aβ(1-28) although EPR data (see insert of Figure 1) indicates that the transition between the pH 6 and pH 9 coordination mode occurs at a higher pH for Aβ(1-16) with a midpoint of 8.7 rather than 8.0.

Aβ(1-28) analogues:- Aβ(H6A), Aβ(H13A), Aβ(H14A) and Aβ(Ac1-28):- To identify the residues directly involved in the coordination of copper, three analogues have been
synthesized in which each of the histidine residues has been replaced with an alanine residue. An additional peptide has also been synthesized in which the N-terminus is blocked by acetylation. It is believed that nitrogens from these four loci are the most likely candidates for copper coordination. The EPR spectra of all four analogues studied indicate axial Cu$^{2+}$ coordination containing N and O ligands. At pH 6 differences in the spectra between all the analogues and Aβ(1-28) are slight, at pH 9 the Cu-EPR spectra of the analogues are largely unchanged. In contrast, as shown in Figure 1 there is a strong pH dependence to the Cu-EPR spectra of wild-type Aβ(1-28). The EPR data suggest that the complex formed at the higher pH requires the presence of all the His residues and the N-terminal amino group. However we have used a number of further spectroscopic techniques described below to support this assertion. For example, when coordination geometry and ligands are similar, visible CD can potentially be much more sensitive to the coordination sphere around the Cu$^{2+}$ ion compared to EPR.

Visible CD spectra are very sensitive to the relative position of coordinating ligands with changes in the intensity and sign of CD bands (38). The visible CD spectra of the wild-type peptide and CD spectra for the four Aβ(1-28) mutants are shown in Figure 7. Spectra have been obtained at pH 5.5, 7.5 and 9.5 for all four analogues as well as wild-type Aβ(1-28). In addition, spectra have been obtained with both one and three mole equivalents of Cu$^{2+}$ present. CD bands associated with the Cu$^{2+}$ ion are observed at key positions of 514 nm, (assigned to Cu d-d transitions), 360 nm (assigned to N$_{im}$ > Cu$^{2+}$ charge transfer), 312 nm (assigned to N amide to Cu$^{2+}$ charge transfer) and 280 nm (assigned to N$_{im}$ π2> Cu$^{2+}$ charge transfer) (35). Taking each analogue in turn with one equiv Cu(II) at pH 9.5, the N-terminally blocked Aβ(Ac1-28) gives a very different trace to wild-type Aβ(1-28) implying the N-
terminus is essential for Cu(II) binding. In particular, only Aβ(Ac1-28) gives a CD band at 360 nm and the band at 514 nm increases by ~35% relative to the wild-type spectrum. Furthermore, only Aβ(Ac1-28) gives a positive chirality above 580 nm. The spectra of Aβ(H13A) are also perturbed relative to the wild-type spectra. In particular there is a ~40% drop in signal at 514 nm but with an increase in the positive charge transfer band below 280 nm. The copper loaded spectrum of Aβ(H14A) has some differences relative to the wild-type, with small reduction in intensity (~17%) of the band at 514 nm. It is not clear if the small drop in intensity for the band at 514 nm is significant, or due to reduced solubility or small variation in pH. Similarly, the differences between Aβ(H6A) and the wild-type spectrum are slight, with a very small reduction (~4%) in the band at 514 nm. Taking these observations together, it appears that at one equivalent of Cu^{2+}, His13 and the N-terminus certainly coordinate the copper ion. Cu^{2+} coordination to His14 and His6 is less apparent from the visible CD data but cannot be ruled out. Indeed it is apparent from the CD data in the UV region as shown in Figure 8, and described below, that His14 and His6 are involved in coordination.

Copper titrations on each of the analogues were also studied using CD in the UV region and compared to the wild-type spectra. Figure 8 shows the apo subtracted difference CD spectra of each of the 4 analogues and the wild-type spectra after 1 equivalent of Cu^{2+} was added. All four analogues look significantly different from wild-type Aβ(1-28) and Aβ(1-16). For example, none of the analogues show positive chirality at 225 nm. In addition, the other positive CD band at 205 nm is less intense for the analogues relative to the wild-type and is shifted to 200 nm. This data therefore implicates not only the N-terminal amino group in the coordination of the first mole equivalent of Cu^{2+}, but also all three histidine side chains.
In a further attempt to confirm the ligands directly involved in copper coordination, the affinity for each of the analogues was measured using glycine competition and fluorescence (as described previously). Fluorescence quenching in the presence of competing glycine indicates that Aβ(1-16) and Aβ(1-28) have similar affinity for copper with glycine causing a return to half maximal fluorescence signal at ~18 (±2) mole equivalent of glycine. N-blocked Aβ(1-28) shows a sharp reduction in affinity for copper. This supports the CD studies that indicate the N-terminus is a key ligand for copper binding. Aβ(Ac1-28) gives a half maximal fluorescence signal after the addition of between only 5 and 8 equivalent glycine. Similarly, Aβ(Ac10-16) gives a half maximal fluorescence signal at 3 equivalent glycine, implying that Aβ(Ac10-16) has an affinity for copper only comparable to the coordination of glycine. The analogue with the His6 removed also shows a reduction in affinity for Cu²⁺ with only ~11 mole equivalent glycine required to produce a half maximal fluorescence. Aβ(1-28) analogues with His13 or His14 removed show a smaller reduction in affinity relative to the wild-type Aβ(1-28), with a half maximal return of fluorescence with ~14-16 mole equivalent glycine in each case. In agreement with the UV-CD data shown in Figure 8, the affinity of Cu²⁺ for Aβ(1-28) is reduced by blocking of the N-terminus or the removal of any of the three histidine residues.

IH NMR:-  IH NMR has been used to isolate coordinating ligands still further. The apo spectrum of Aβ(1-28) gives line-widths indicative of a monomeric peptide. Paramagnetic Cu²⁺ will broaden IH NMR signals when in close proximity to (≤ 7Å) or directly coordinated to the metal ion. Figure 9 clearly shows profound broadening of the Hε1 and Hδ2 resonances for all three histidine residues of Aβ(1-28) at pH 7.8 relative to the other aromatic side chains. In particular Tyr10 which has been suggested as a potential ligand to Cu²⁺ (30, 41) is
relatively unaffected by Cu$^{2+}$ addition, and for this reason is unlikely to be involved in direct coordination to the Cu$^{2+}$ ion. The Cu$^{2+}$ ions are exchanging sufficiently rapidly between peptide molecules to broaden all the Aβ molecules with only 0.05 equivalents of Cu$^{2+}$ present. The rapid rate of exchange of the copper between peptides means that the broadening observed does not indicate quantitatively what fraction of Cu$^{2+}$ is bound. Unfortunately, due to the fast copper on/off rate, it is not possible to distinguish between high-affinity copper coordination and a transient small fraction of the copper coordinating to for example, a single histidine imidazole ring. It is therefore not clear from the NMR data whether the broadening of each histidine residue is due to a transient effect or a high-affinity binding site. It is however clear the lack of broadening of the Tyr10 resonances indicates that it can be eliminated as a possible ligand in the coordination of copper.

Taking the $^1$H NMR data together with CD data in both the UV and Visible regions, as well as the glycine competition studies of the all the Aβ analogues, we reveal a picture of the essential ligands for Cu$^{2+}$ coordination of the first mole equivalent of Cu$^{2+}$ ions to Aβ. Our data strongly indicates the coordination of the N-terminus of Aβ and His13, His14 and His6 are also implicated, but not Tyr10.

**Bridging Cu-His-Cu dimer:** It has previously been suggested that there is histidine bridging between two Cu$^{2+}$ ions in the Aβ(1-28) Cu$^{2+}$ complex (30). This work was based on the observation that the EPR spectra broaden with Cu$^{2+}$ addition (at pH 6.8) and assumed this was due to electron coupling via a bridging histidine as is observed in EPR spectra of Cu$_2$-SOD (42). However, we have not observed broadening of EPR spectra with increasing copper addition. EPR spectra in which electron coupling is taking place are very temperature dependent, and no such temperature dependence is observed in these spectra. Spectra have
been obtained for Aβ(1-28) with 0.8 mole equivalent Cu$^{2+}$ over a range of temperatures between 20 K and 110 K, but line shapes are not effect by this change in temperature. We suggest that the broadening observed in this previous study (30) were simply due to through-space dipole coupling at high copper ion concentration rather than scalar through-bond coupling in a bridged complex for Cu-Aβ-Cu.

**Model Complex:** A crystal structure of a model compound containing a di-peptide analogue of two adjacent histidine residues has been reported (43). In this compound, copper chelates to the εN of both imidazole rings making a cis arrangement rather than trans coordination (90° rather than 180°) to the copper ion in a square-planar arrangement. Sterically, this type of ligand arrangement could facilitate the coordination of the N-terminus and His6 without any obvious restrictions. Interestingly, coordination to copper from adjacent histidine imidazole rings makes it impossible for main-chain coordination, via the amide or carboxyl, for either Val12, His13, His14 or Gln15. The absence of main-chain coordination is supported by the CD spectra at neutral pH, which shows little chiral optical activity for d-d transitions. The lack of appreciable d-d transition CD bands suggest minimal vicinal effects (38), and this implies there is no main-chain co-ordination at physiological pH and below, as previous discussed. A study of Aβ using Raman spectroscopy indicates Cu-εN at mildly acidic conditions and δN coordination at physiological pH and above (41). Imidazole coordination via the εN makes main-chain coordination less likely than the more stable six-membered chelate ring between the δN and the His main-chain amide (44). This transition between εN and δN coordination with pH is supported by our CD studies that indicate main-chain coordination at higher pH values only. Figure 10 represents the possible ligands
coordinating the Cu$^{2+}$ ion in a square-planar arrangement including the N-terminal amino group, and eN groups of His13 and His14. The fourth ligand is from His6 rather than Tyr10.
DISCUSSION

With increasing interest in the role of Cu$^{2+}$ in Alzheimer’s disease (AD) (24,25,28,29,45) we have used a range of complementary spectroscopic techniques including CD, EPR, NMR and fluorescence to study Cu$^{2+}$ binding to the Aβ peptide. In summary, Cu$^{2+}$ binds to the N-terminus of Aβ and the three histidine imidazole rings but not Tyr10, as shown in Figure 10. Aβ is essentially a random coil peptide in aqueous medium, while the amyloid fibrillar form of the peptide is rich in β-sheet (40). We were therefore interested in the possibility that Cu$^{2+}$ chelation could induce a β-sheet conformation in Aβ. We have shown that the N-terminal tail folds back on itself to facilitate coordination via the N-terminal amino group as well as His13 and His14. However, although copper chelation does induce ordering of the main-chain, CD studies of Aβ(1-28) indicate copper does not induce a typical β-sheet conformation. In addition, CD comparisons of Aβ(1-16) and Aβ(1-28) indicate that residues 17-28 are not directly affected by copper coordination in its soluble form.

We have shown that His13 is a critical residue in coordinating the Cu$^{2+}$ ion. Interestingly, rat-Aβ lacks a histidine at position 13 and copper induced aggregation of rat-Aβ is not observed (11). Furthermore, wild-type rats do not exhibit AD-like pathology, although transgenic rats containing human Aβ can present with AD-like pathology. It has been shown that copper coordination has a profound influence on the solubility of Aβ (11). The fourteen N-terminal amino acids of Aβ contain four carboxylic acid groups and four basic groups, the three histidine residues and the N-terminal amino group. Cu$^{2+}$ coordination may cause the loss of all four positive charges. Thus copper binding will have a profound influence on the electrostatic surface of the Aβ peptide with a reduction in charged groups on Aβ and a change
from an overall neutral charge to a highly negatively charged N-terminal tail. It is likely that this change in charged residues is the cause of the aggregation observed upon Cu$^{2+}$ binding.

We have shown that coordination of Cu$^{2+}$ to Aβ is highly pH dependent. It is clear that there is a transition between two coordination geometries as the pH is raised. In the case of Aβ(1-28) the midpoint is pH 8.0. This observation agrees with a potentiometric study recently published, which suggests a transition between a 3N and 4N complex at ~ pH 8 (46). A study by Atwood et al. showed that Cu$^{2+}$ only induced aggregation of Aβ as the pH is lowered from physiological pHs to 6.8 (11). The change in coordination geometry, perhaps due to the loss of amide main-chain coordination at lower pHs, could be key to the pH dependence of the aggregation observed in the presence of Cu$^{2+}$.

Key to the physiological significance for Cu$^{2+}$ binding to Aβ is its affinity. The concentration of extracellular Cu$^{2+}$ is typically 10 μM in blood plasma with extracellular levels of Cu$^{2+}$ reaching as high as 15 μM (10). Cu$^{2+}$ dissociation constants reported here for Aβ(1–28) are two or three orders of magnitude lower than that of the extracellular copper concentration. This means that Aβ has sufficient affinity to bind copper at physiological levels of Cu$^{2+}$. Previously reported affinities of Aβ for copper differ significantly. Initial studies by Atwood et al. put the Cu$^{2+}$ affinity at 4 μM and 0.3 μM for Aβ(1-40) and Aβ(1-42) respectively (11). This value was revised using competitive metal capture analysis to an attomolar affinity ($10^{15}$) for Aβ(1-42) (24). Others have reported a more modest affinity of 2.0 +/- 0.8 μM for Aβ(1-42) (31). Our K$_d$ of 10-100 nM, lies between these two values, but is considerably closer to that determined by Garzon-Rodriguez et al. (31).
Copper binding curves obtained from EPR data strongly suggest copper saturation of Aβ at 2 mole equivalent of Cu²⁺. We are aware of only two previous reports of the stoichiometry of copper binding to Aβ, one of which suggests a 1:1 stoichiometry (31). However, in agreement with our studies, Atwood et al. have reported copper saturation at ~2 mole equivalent for both Aβ(1-40) and Aβ(1-42), with a high-affinity site at 0.5 mole equivalent of Cu²⁺ (24). With no change in the appearance of spectra between 0.5 mole equivalent and 1 equivalent of Cu²⁺, both our EPR and CD data indicate that Aβ(1-28) does not form a dimeric Cu(Aβ)₂ species under these conditions. In addition, from our EPR data we observe no evidence of Aβ(1-28) using bridged histidine coordination to form a dimeric species as previously suggested (30).

The very different affinities of Cu²⁺ for Aβ described by Atwood et al. (24) could reflect the differences in the complexes formed. We suggest that the Cu²⁺ binding affinities described by Garzon-Rodriguez et al. for Aβ(1-42) and ourselves for Aβ(1-28) reflect the soluble form of Cu-Aβ. The mode of binding copper in solution may be different from that found in plaques. Atwood et al. report a dimeric species Cu(Aβ)₂ while we see no evidence of a dimeric species forming for Aβ(1-28) in solution. Dimerization is a concentration dependent phenomenon. The strong tendency for Aβ(1-42) to form aggregates may facilitate cross-linking and a Cu(Aβ)₂ species. It is likely that soluble Aβ(1-42) initially forms a one-to-one complex as is seen for Aβ(1-28). The hydrophobic C-terminal residues 29-42 do not directly coordinate to copper. Indeed, apart from Met35, which has been ruled out by a study using Raman spectroscopy (41) in which a S-Cu band was not observed, residues 29-42 contain only aliphatic side-chains, which do not possess lone-pairs to facilitate Cu²⁺ ion coordination. Rather the C-terminal residues may promote aggregation that facilitates a cross-linked dimeric copper complex of Aβ.
There have been relatively few structural studies of the Cu-Aβ complex. The paramagnetic copper centre has hampered the determination of an NMR solution structure. Studies using Raman spectroscopy have suggested that Cu$^{2+}$ binds to the Nδ of the histidine imidazole and will coordinate via the Ne nitrogen under mildly acidic conditions (41, 47). Our CD studies support this pH dependent transition. In agreement with our CD and NMR data and glycine competition studies, the Raman spectroscopic study suggests that all three histidines coordinate to copper. However, in contrast with our observations, Tyr10 but not the N-terminus was implicated in coordination (41). In agreement with our work, potentiometric studies of Aβ fragments (46) do implicate the N-terminus in binding Cu(II).

Recent studies now present strong evidence to link Cu$^{2+}$ with AD, for example, Raman microscopy has provided direct evidence that copper and zinc are bound via the histidine imidazole rings of Aβ in isolated senile plaque cores (25). In addition, copper chelators can solubilize amyloid plaques and represent possible therapies for Alzheimer’s disease, as copper chelators can inhibit amyloid accumulation in transgenic mice (26). Copper is also linked with the neurotoxicity of Aβ and free radical damage associated with Alzheimer’s disease (16). Finally, trace amounts of copper in the diet have been shown to induce beta-amyloid plaques and learning deficits in a rabbit model of Alzheimer's disease (28). To conclude, we believe our studies have made a significant contribution to understanding Cu$^{2+}$ binding of Aβ in solution by identifying the key ligands coordinating the Cu$^{2+}$ ion and the effect of pH on the binding mode and structuring of the main-chain of Aβ.
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Figure Captions

Figure 1. Effect of pH on the EPR spectrum of Cu-Aβ(1-28). Aβ(1-28) at ~50 μM in H₂O with 0.8 molar equivalent of Cu²⁺ recorded at 20 K. Changes in the hyperfine splitting features at low field (2600-3200 Gauss) indicate that the coordinating ligands of the bound Cu²⁺ centre changes with pH, with a mixture of two complexes existing at physiological pH. Peak heights at positions I and II are plotted against pH for both Aβ(1-16) and Aβ(1-28) (insert). The crossover point (50:50 mixture of complexes) is indicated.

Figure 2. Effect of pH on the visible CD spectrum of Cu-Aβ(1-28) a) Aβ(1-28) at 70μM in H₂O with 1.0 mole equivalent Cu²⁺ over a range of pH values from pH 4.5 to 10.0 b) CD intensity variation at 252 nm as a function of pH. c) CD intensity variation at 312 and 514 nm as a function of pH.

Figure 3. EPR spectra of Aβ(1-28) - Cu²⁺ titration. Aβ(1-28) 50 μM in aqueous solution at pH 7.8, 20 K. Double integration of EPR spectra plotted versus mole equivalent of Cu²⁺ to peptide (insert) indicates that saturation occurs at 2 mole equivalent of Cu²⁺. EPR spectra for 4.0 and 5.0 mole equivalent Cu²⁺ are not shown for clarity, as they are virtually identical to the 3.0 mole equivalent spectrum.

Figure 4. UV CD spectra of Aβ(1-16) and Aβ(1-28) - Cu²⁺ titration. Main plots show the CD spectra in the region (190-260 nm) with increasing mole equivalence of Cu²⁺. The insert plots are apo subtracted difference spectra, illustrating more clearly the similarity in structural changes of the peptides on addition of up to 2.0 mole equivalent Cu²⁺. a) Aβ(1-16) at ~0.1
mM in H$_2$O at pH 7.5; b) A$\beta$(1-16) at ~0.1 mM in H$_2$O at pH 9.5; c) A$\beta$(1-28) at ~70 $\mu$M in H$_2$O at pH 7.8.

**Figure 5.** Fluorescence spectra of A$\beta$(1-28) with Cu$^{2+}$, and glycine and L-histidine competition. a) Increasing mole equivalence of Cu$^{2+}$ causes quenching of the fluorescence signal using 50$\mu$M of A$\beta$(1-28) in water at pH 7.8  b) The effect of increasing additions of glycine or histidine on the fluorescence maximum at 307 nm versus molar equivalence of the competing ligand.

**Figure 6.** Visible region CD spectra of Cu$^{2+}$-A$\beta$ fragments. One mole equivalent Cu$^{2+}$ for each fragment; A$\beta$(1-28); A$\beta$(1-16); A$\beta$(1-11) and A$\beta$(Ac10-16) (a) at pH 7.5. b) at pH 9.5

**Figure 7.** Visible region CD spectra of Cu$^{2+}$-A$\beta$ analogues. A$\beta$(H6A), A$\beta$(H13A), A$\beta$(H14A), A$\beta$(Ac1-28) with the addition of one mole equivalent of Cu$^{2+}$ at pH 9.5.

**Figure 8.** UV region difference CD spectra of A$\beta$(1-28) analogues A$\beta$(H6A), A$\beta$(H13A), A$\beta$(H14A), A$\beta$(Ac1-28) and wild-type A$\beta$(1-28) with the addition of one mole equivalent of Cu$^{2+}$, pH ~7.6. CD spectra are apo-subtracted difference spectra.

**Figure 9.** 1D proton NMR spectra of A$\beta$(1-16). Spectra obtained at 1 mM A$\beta$(1-16), pH 7.4 in 100% D$_2$O at 25$^\circ$C, apo (lower trace) and with 0.05 mole equivalence (~50$\mu$M) Cu$^{2+}$ added (upper trace). Selective paramagnetic broadening of $^1$H resonances of all three His $\varepsilon$H and $\delta$H resonances are observed. Resonances for Tyr10 are relative unaffected by Cu$^{2+}$ addition. * = His6, 13 and 14 $\varepsilon$H; * = His6, 13 and 14 $\delta$H; † = Tyr10.
Figure 10: Model of Aβ coordinating Cu$^{2+}$. Coordinating ligands include the N-terminal amino group and the imidazole rings of His6, His13, and His14 in a square-planar geometry. The model illustrates the dominant coordination geometry of the first mole equivalent of copper at physiological pH. Coordination at higher pH (pH 8.5 and above) probably involves main-chain amide coordination. A MolMol representation was obtained using distance geometry (DYANA), in which the metal-ligand bond lengths (1.9 Å) and angles (square-planar geometry) were constrained.
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Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

![Fluorescence vs. wavelength](image)

(a) Fluorescence spectra for different concentrations of Cu$^{2+}$.

(b) Change in $F_{\text{max}}$ (307 nm) with mol eq Glycine.

(c) Change in $F_{\text{max}}$ (307 nm) with mol eq Histidine.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Copper binding to the amyloid-beta (Aβ) peptide associated with Alzheimer's disease. Folding, coordination geometry, pH dependence, stoichiometry and affinity of Aβ(1-28); insights from a range of complementary spectroscopic techniques

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