Durham Research Online

Deposited in DRO:
21 May 2018

Version of attached file:
Published Version

Peer-review status of attached file:
Peer-reviewed

Citation for published item:
Yako, Nineveh and Young, Tessa R. and Cottam Jones, Jade M. and Hutton, Craig A. and Weld, Anthony G. and Xiao, Zhiguang (2017) 'Copper binding and redox chemistry of the A16 peptide and its variants: insights into determinants of copper-dependent reactivity.', Metallomics, 9 (3). pp. 278-291.

Further information on publisher’s website:
https://doi.org/10.1039/C6MT00299D

Publisher’s copyright statement:
This article is licensed under a Creative Commons Attribution 3.0 Unported Licence.

Additional information:

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a link is made to the metadata record in DRO
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the full DRO policy for further details.
Copper binding and redox chemistry of the Aβ16 peptide and its variants: insights into determinants of copper-dependent reactivity†

Nineveh Yako, Tessa R. Young, Jade M. Cottam Jones, Craig A. Hutton, Anthony G. Wedd and Zhiguang Xiao*‡

The metal-binding sites of Aβ peptides are dictated primarily by the coordination preferences of the metal ion. Consequently, Cu(I) is typically bound with two His ligands in a linear mode while Cu(II) forms a pseudo-square planar stereochemistry with the N-terminal amine nitrogen acting as an anchoring ligand. Several distinct combinations of other groups can act as co-ligands for Cu(II). A population of multiple binding modes is possible with the equilibrium position shifting sensitively with solution pH and the nature of the residues in the N-terminal region. This work examined the Cu(II) chemistry of the Aβ16 peptide and several variants that targeted these binding modes. The results are consistent with: (i) at pH<7.8, the square planar site in Cu(II)–Aβ16 consists primarily of a bidentate ligand provided by the carboxylate sidechain of Asp1 and the N-terminal amine supported by the imidazole sidechains of two His residues (designated here as component IA); it is in equilibrium with a less stable component IB in which the carboxylate ligand is substituted by the Asp1-Ala2 carbonyl oxygen. (ii) Both IA and IB convert to a common component II (apparent transition pK_B 7.8 for IA and ~6.5 for IB, respectively) featuring a tridentate ligand consisting of the N-terminal amine, the Asp1-Ala2 amide and the Ala2-Pro3 carbonyl; this stereochemistry is stabilized by two five-membered chelate rings. (iii) Component IA is stabilized for variant Aβ16-D1H, components I (both IA and IB) are imposed on Aβ16-A2P while the less stable IB is enforced on Aβ16-D1A (which is converted to component II at pH ~6.5); (iv) components IA and IB share two His ligands with Cu(I) and are more reactive in redox catalysis than component II that features a highly covalent and less reactive amide N–C0 ligand. The redox activity of IA is further enhanced for peptides with a His1 N-terminus that may act as a ligand for either Cu(I) or Cu(II) with lower re-organization energy required for redox-shuttling. This study provided insights into the determinants that regulate the reactivity of Cu–Aβ complexes.

**Significance to metallomics**

Copper binding to Aβ peptides has been subjected to recent intensive studies due to its possible relevance to the aetiology of Alzheimer’s disease. Multiple metal binding ligands are present in the soluble and unstructured N-terminal section of Aβ(x–16), where x > 1 indicates truncation. The nature of the copper binding modes is dictated by the coordination preference of the copper oxidation state and by the medium pH, as is the reactivity of the Cu–Aβ complexes. This study clarifies current controversy regarding several important Cu(n) binding modes and provides an improved understanding of the complex copper chemistry of Aβ peptides.

**Introduction**

Disruption of metal homeostasis has been linked to Alzheimer’s disease and undesirable interactions between Aβ peptides and copper ions may play roles in the promotion of toxic Aβ aggregation and/or in the catalytic production of reactive oxygen species (ROS).1,2 Monomeric Aβ peptides are flexible and unstructured and the copper binding sites and binding modes
change dynamically with the oxidation state and pH, as does the reactivity of the metal centres towards ligand substitution and redox catalysis.

Previous studies have demonstrated that Aβ peptides bind Cu(i) in dynamic linear two-coordinate modes utilizing the three different combinations of the available His-6, -13 and -14 sidechains (Fig. 1a). In contrast, they typically bind Cu(ii) in a square planar-based stereochemistry. The N-terminal amine nitrogen acts as a primary anchoring ligand. Depending on the conditions, several distinct combinations of other groups can act as co-ligands. The speciation depends sensitively on the medium pH and on the nature of the available co-ligands including the His residues, the sidechain of Asp1 and the carbonyl oxygen or deprotonated amide nitrogen of the Asp1-Ala2 peptide link (Fig. 1b). Note the presence of stabilizing six- or five-membered chelate rings in each Cu(ii) complex.

The three distinct complexes in Fig. 1b are designated as components IA, IB and II in this work. Notably, they each can incorporate different combinations of His ligands as they appear to be conformationally available. Structure IA has frequently been proposed as a coordination mode at lower pH. However, recently IB has been preferred, based mainly on EPR studies.

A transition from components IA/IB to component II requires deprotonation of the peptide amide of Ala2 and is promoted by elevated pH. Any modification or truncation to the N-terminal residues, as occurs in native Aβ peptides, can alter the Cu(ii) binding modes dramatically. For example, both the substitution of Asp1 with Asn and deletion of Asp1 to impose Ala2 as the N-terminal residue in the Aβ16 peptide lead to a decrease of over one pH unit in the pKₐ of transition I ↔ II. Deletion of the first three residues, as detected in native Aβ peptides in high proportion, generates a classic N-terminal H₂N-X-X-His₃ motif known as the ATCUN (amino terminal copper and nickel) binding motif: the Cu(ii) centre is stabilized by three chelate rings in a tetradentate ligand.

The different binding modes sensitively affect the reactivity of Cu–Aβ complexes towards ligand substitution and redox catalysis, especially for Cu(ii). This study undertook a range of chemical and spectroscopic studies of the copper chemistry of the Aβ16 peptide to provide improved insight into the relationship between binding mode and reactivity. In particular, several variants that targeted the Cu(ii) binding site specifically were examined. The work was facilitated by the recent development of a set of peptide-based fluorescent dansyl probes DP1–4 that are able to quantify Cu(ii) binding stoichiometries and affinities from micromolar to femtomolar concentrations on a unified scale. The combined data demonstrated that (i) the Aβ16 peptide binds Cu(ii) dominantly as component IA in equilibrium with less stable IB at low pH, while at higher pH, component II predominates (Fig. 1b); (ii) component I is more reactive than component II in redox catalysis.

**Experimental section**

**Materials and general procedure**

Materials purchased from Sigma included the ligands Ferene S (Fs), Ferrozine (Fz) and bicinechonic anion (Bca) (as their sodium salts Na₄Fs, NaHFs and Na₂Bca), and the reductants NH₂OH (as its H₂SO₄ salt) and ascorbic acid (Asc). They were all used as received. A standard Cu(ii) solution was prepared by dissolving CuSO₄ in Milli-Q water and the concentration calibrated via reaction with excess of the Cu(i)-binding ligand Bca in Mops buffer containing reductant NH₂OH. Under such conditions, all copper ions are converted quantitatively to the well-defined chromophoric complex anion [Cu²⁺(Bca)₃]⁻ with characteristic absorbance at 562 nm (ε = 7900 M⁻¹ cm⁻¹). The peptides and probes listed in Table 1 were purchased from GL Biochem (Shanghai) except for Aβ16-A2P and Aβ16-D1H/H14A which were synthesized on site by standard solid phase peptide techniques as
of each sample was re-checked after recording each spectrum.
The samples were snap-frozen in liquid nitrogen and the
spectra recorded at 77 K in a liquid-nitrogen finger Dewar.
The EPR parameters (g values and A values) were extracted
directly from the recorded spectra without simulation.

Quantification of Cu(II) binding

Cu(II) binding to Aβ peptides was quantified with a series of DP
probes as reported recently.21 These probes feature a dansyl
group and emit intense fluorescence at $\lambda_{\text{max}} \approx 550$ nm upon
excitation at $\sim 330$ nm. They bind one equiv. of Cu(II) with varying
affinities and Cu(II) binding to the probes is detected sensitively via
proportional fluorescence quenching. The quantification is based
on the following relationships:

\begin{align}
\text{Cu}^{\text{II}}\text{DP} + \text{A}^\beta &\rightleftharpoons \text{Cu}^{\text{II}}\text{A}^\beta + \text{DP} \quad (1) \\
K_{\text{ex}} &= \frac{[\text{Cu}^{\text{II}}\text{A}^\beta][\text{DP}]}{[\text{Cu}^{\text{II}}\text{DP}][\text{A}^\beta]} = \frac{K_{\text{D}}}{K_{\text{D}} + [\text{DP}]} \quad (2) \\
\frac{[\text{A}^\beta]_{\text{tot}}}{[\text{DP}]_{\text{tot}}} &= \frac{[\text{Cu}^{\text{II}}\text{DP}]}{[\text{Cu}^{\text{II}}\text{DP}][\text{A}^\beta]} \\
&\quad + \frac{K_D[\text{Cu}^{\text{II}}\text{A}^\beta]}{K_D(\text{Cu}^{\text{II}}\text{DP})} \left( 1 - \frac{[\text{Cu}^{\text{II}}\text{DP}]}{[\text{DP}]_{\text{tot}}} \right) \quad (3) \\
\frac{[\text{Cu}^{\text{II}}\text{DP}]}{[\text{DP}]_{\text{tot}}} &= \frac{F_0 - F}{F_0 - F_1} = \frac{\Delta F}{\Delta F_1} \quad (4)
\end{align}

where $K_D(\text{Cu}^{\text{II}}\text{A}^\beta)$ and $K_D(\text{Cu}^{\text{II}}\text{DP})$ are the respective dissociation
constants under the same conditions. Eqn (3) is derived from
eqn (2) with incorporation of mass balance relationships for an
effective competition described by eqn (1) and is a convenient
format for curve-fitting of the experimental data to derive $K_D(\text{Cu}^{\text{II}}\text{A}^\beta)$
with known $K_D(\text{Cu}^{\text{II}}\text{DP})$. Under the conditions of the fluorescent
emission of the dansyl probes ($\lambda_{\text{ex}} = 330$ nm; $\lambda_{\text{em}} = 550$ nm), the Aβ
peptides are fluorescence-silent. Consequently, the Cu(II) speciation
in eqn (1)–(3) may be analysed conveniently and reliably by
monitoring the response of the probe to binding of Cu(II) via eqn (4),
where $F_0$, $F$ and $F_1$ are the fluorescence intensity of the DP probe
upon binding 0, <1.0 and 1.0 equiv. of Cu(II), respectively. The
experiments were performed via reported protocols.21

The $K_D$ values for each peptide were determined similarly at
a range of pH values. The pH-dependent affinities of the Cu(II)
probes DP–4 have been reported recently.21 If the Cu(II) binding
site remains unchanged within a pH range and the ligand
compositions are known, then the conditional $K_D$ within that
pH range may be correlated with the absolute dissociation
constant ($K_D^{\text{abs}}$) and the proton ionization constants ($K_{a,n}$) of each
ligand function of the site according to eqn (5):

$$K_D = K_D^{\text{abs}} \left( 1 + \sum_{n=1}^{N} 10^{\left( \sum_{i=1}^{N} k_{i,n} - \text{pH} \right)} \right)$$

The experiments were conducted in buffers (50 mM) Mes
(pH 6.0–6.6), Mops (pH 7.0–7.4), Heps (pH 7.8–8.2) and Ches

---

**Table 1** Sequences of Aβ16 peptides and probes

| Peptide or probe | Sequence | Molar mass [Da] |
|-----------------|----------|----------------|
| Aβ16           | DAEFRHDSGYEVHHQK | 1955.0 1954.5 |
| Ac-Aβ16        | Ac-DAEFRHDSGYEVHHQK | 1960.5 1967.0 |
| D1A            | EAERFDSDGNYEVHHQK | 1911.0 1910.7 |
| D1E            | EAERFDSDGNYEVHHQK | 1969.0 1970.4 |
| D1N            | NAEFRHDSGYEVHHQK | 1954.0 1954.9 |
| D1H            | HAEFRHDSGYEVHHQK | 1970.0 1978.0 |
| D1H/H14A       | HAEFRHDSGYEVHHQK | 1911.0 1911.4 |
| A2P            | DPEFRHDSGYEVHHQK | 1981.0 1981.5 |
| D2P            | DA[K(\text{DNS})]HDHH | 1240.3 1240.9 |
| D3P            | HP[K(\text{DNS})]DHDHH | 1118.2 1118.0 |
| D4P            | H[K(\text{DNS})]HH | 790.8 790.4 |

* The residues changed are underlined in bold.
(pH 8.5–9.2). On the other hand, the relationship between the determined $K_D$ and pH may also be employed to model the most likely binding site using eqn (5) to derive $K_D^{\text{obs}}$ for the Cu(n) complex and $K_{\text{unb}}$ for the possible binding ligands.

### Quantification of Cu(i) binding

Competition for Cu(i) with a suitable probe ligand $L$ ($L = \text{Fs or Fz}$) was set up according to eqn (6) and (7):\(^{26}\)

$$[\text{Cu}^{\text{I}}L_2]^{3-} + \text{A}\beta \rightleftharpoons \text{Cu}^{\text{I}}\text{A}\beta + 2L^{2-} \quad (6)$$

$$\frac{[\text{A}\beta]_{\text{tot}}}{[\text{Cu}^{\text{I}}]_{\text{tot}}} = 1 - \frac{[\text{Cu}^{\text{I}}L_2]}{[\text{Cu}^{\text{I}}]_{\text{tot}}} + K_D^B \left( \frac{[L]_{\text{tot}}}{[\text{Cu}^{\text{I}}L_2]} - 2 \right)^2 \left[ \frac{[\text{Cu}^{\text{I}}L_2]}{[\text{Cu}^{\text{I}}]_{\text{tot}}} \right] \quad (7)$$

where $\beta_2$ is the known formation constant for the chromophoric probe complex $[\text{Cu}^{\text{I}}L_2]^{3-}$ whose equilibrium concentration may be determined directly from the specific solution absorbance. The dissociation constant $K_D^B$ for the Cu(I)–$L$ complex was determined by curve-fitting of the experimental data to eqn (7). Probe ligands Fs and Fz were employed and they bind Cu(i) with different affinities ($\log \beta_2 = 13.7$ for L = Fs and 15.1 for L = Fz).\(^{26}\) The experiments were performed \textit{via} reported protocols.\(^{26}\)

### Catalytic aerobic oxidation of ascorbate

Experiments were conducted in air-saturated Mops buffers (50 mM; pH 6.6 and 7.4) and Ches buffer (50 mM, pH 8.7). To minimize the catalytic effect from unbound copper, a stock solution of each Cu–peptide complex was prepared with one-fold excess of the corresponding apo-peptide and the total copper content quantified accurately with the Cu(I) probe Bcs as a stable chromophoric complex $[\text{Cu}^{\text{II}}(\text{Bcs})]^{3-}$ ($\log K_{\text{obs}} = 19.9$; $\varepsilon_{403} = 13\,000\ \text{M}^{-1}\ \text{cm}^{-1}$).\(^{27}\) The reactions were conducted using a quartz mixing cuvette where one half-cell was loaded with Asc ($\sim 100\ \mu\text{M}$) and the other half-cell with the Cu(I)–$A\beta$ complex (varying in concentration between 2 and 40 $\mu$M, dependent on the catalytic activity). After recording the background reaction (if any), the catalytic reaction was started by quick mixing (note: the concentration of each component in the half-cells becomes half after the mixing) and followed in real time by the change in absorbance $A_{365}$ of the reduced Asc. The oxidation rate was expressed as $\mu$M min$^{-1}$ and calculated using the extinction coefficient $\varepsilon_{269} = 14\,500\ \text{M}^{-1}\ \text{cm}^{-1}$ for Asc.\(^{28}\) The catalytic activity of each Cu–A$\beta$ complex was expressed as molar turn-over number per min (MTN/min) and was obtained from the slope of a linear plot of the Asc oxidation rate ($\mu$M min$^{-1}$) \textit{versus} the concentration ($\mu$M) of each Cu–A$\beta$ complex. The background oxidation rate without added Cu has been removed from the determined oxidation rate.

### Formal reduction potential

The formal reduction potential of the copper centres in each Cu–A$\beta$16 complex was estimated from the relative affinities of the peptides for Cu(i) and Cu(n) under the same conditions \textit{via} the Nernst relationship of eqn (8):

$$E^0 (\text{mV}) = E^0 + 59 \log \left( \frac{K_D^{\text{Cu}(n)}}{K_D^{\text{Cu}(i)}} \right) \quad (8)$$

where $E^0 = 153\ \text{mV}$ (vs. SHE) is the standard reduction potential of Cu$^{2+}$/Cu$^+$.\(^{29}\)

### Results and discussion

The N-terminal residues of A$\beta$ peptides have a major impact on Cu(n) binding

Processing of the amyloid precursor protein (APP) by proteases $\beta$- and $\gamma$-secretase generates the A$\beta$ peptides 1–(36–43) whose interaction with transition metal ions, and with copper, in particular, appears to be a source of toxicity \textit{in vivo}.\(^{30}\) The possible effects include catalytic generation of reactive oxygen species (ROS) by toxic oligomers.\(^{31,32}\) All major Cu ligands are located within the first 16-residue water-soluble fragment and thus A$\beta$16 is a valid model for the study of the copper chemistry of full length A$\beta$.\(^{3}\) In particular, the N-terminal amine nitrogen and nearby residues play a critical role in Cu(n) coordination and in imposing both binding affinity and coordination modes.

In this study, the two N-terminal residues Asp1 and Ala2 of A$\beta$16 were changed systematically \textit{via} generation of the following variants (Table 1):

(i) D1A eliminates the sidechain of residue 1 as a potential ligand (cf. IA of Fig. 1b).

(ii) D1E, D1N, and D1H provide alternative residue 1 side-chains as potential ligands; D1H/H14A also removes His14 as a potential ligand.

(iii) A2P removes the amide of residue 2 as a potential anionic ligand (cf. II of Fig. 1b).

In addition, the N-terminus was acetylated (Ac-A$\beta$16) to perturb its metal binding properties. Detection of fine differences in properties is possible with a set of peptide-based dansyl fluorescent probes DP2–4 (Tables 1 and 2).\(^{21}\)

A control titration of standardized CuSO$_4$ solution with the DP2 probe alone generated a titration curve with an endpoint at $[\text{Cu}(n)]_{\text{tot}}/\text{[DP2]} = 1.0$ (Fig. 3a and b, black trace), confirming that the probe binds a single Cu(n) ion of the highest affinity ($\log K_D = -9.9$).\(^{21}\) The equivalent titration into a 1 : 1 mixture of DP2 and A$\beta$16 (2.0 $\mu$M) in Mops buffer (50 mM, pH 7.4) generated a titration curve with an apparent endpoint at $[\text{Cu}(n)]_{\text{tot}}/\text{[DP2]} \sim 2.0$ (Fig. 3b, red trace). The essentially linear nature of the curve indicated that the peptide can bind one equiv. of Cu(n) with an affinity similar to that of DP2. Indeed, the Cu(n) binding sequence in the DP2 peptide was designed as a mimic of A$\beta$16 (Table 1).\(^{21}\)

Equivalent titrations for A$\beta$16-D1A and A$\beta$16-D1H also displayed apparent titration endpoints at $[\text{Cu}(n)]_{\text{tot}}/\text{[DP2]} \sim 2.0$ (Fig. 3b, green, blue), consistent with these peptides also binding a single Cu(n) ion. However, the different shapes of the titration curves are consistent with the affinity of A$\beta$16 being higher than that of A$\beta$16-D1A but lower than that of A$\beta$16-D1H. In fact, the probe
does not compete effectively with the latter peptide until 
[\text{Cu}(ii)_{\text{obs}}]/[\text{DP2}] \sim 1.

A more quantitative approach is possible with the fluorescence 
recovery analysis of titrations of apo-peptides into solutions of a 
copper–probe complex of appropriate affinity. Preliminary testing 
indicated that the appropriate experiments involved sub-
stoichiometric Cu(ii) in the systems Cu\text{II}_{1.5}–DPx (x = 2 for \text{Aβ16–}
D1A and 3 for \text{Aβ16–D1H} and x = 2 or 3 for \text{Aβ16}). Analysis of the 
results suggested that, at pH 7.4, the Cu(ii) binding affinity of 
\text{Aβ16–D1A} is 5-fold weaker than that of \text{Aβ16} while the affinity of 
\text{Aβ16–D1H} is \sim 100 times stronger (Fig. 3c–f and Table 2).

Equivalent experiments and analyses for the remaining 
peptides, with their respective optimal DP probes, lead to an 
increasing order of relative Cu(ii) binding affinities at pH 7.4 as 
follows (Table 2):

\text{Ac-}\text{Aβ16} (50) < \text{Aβ16–D1A} (5.0) < \text{Aβ16–D1E} (3.2)

< \text{Aβ16–D1N} (2.0) < \text{Aβ16} (1.0) \sim \text{Aβ16–A2P} (1.0)

< \text{Aβ16–D1H/H14A} (0.02) < \text{Aβ16–D1H} (0.01) \quad (9)

The numbers in the brackets refer to the dissociation constants 
normalized to that of \text{Aβ16} and decrease in the order of increasing 
Cu(ii) binding affinity. It is apparent that the residues in the 
N-terminal region have a major impact on the binding affinity 
and likely on the binding mode as well. A more complete 
analysis follows.

Component IA is favoured by the presence of a Hist1-based 
chelate ring

The two most significant changes in Cu(ii) binding affinity are 
seen for Ac-\text{Aβ16} and \text{Aβ16–D1H} (eqn (9)). The N-terminal 
amine nitrogen was blocked by acetylation in Ac-\text{Aβ16}, leading to 
a significant decrease in affinity under all conditions (Table 2), 
confirming the primary anchoring role of the N-terminal nitrogen 
in Cu(ii) binding for each of the components IA, IB and II. 
Replacement of the carboxylate sidechain of Asp1 with the 
imidazole sidechain of Hist1 in \text{Aβ16–D1H} led to an increase in 
affinity of almost two orders of magnitude at pH 7.4 (Table 2).

---

**Table 2** Log $K_D$ for Cu(ii) of Aβ16 peptide and variants at different pH values$^a$

| Aβ16 peptide or DP probe | log $K_D^{\text{Aβ16}}$ | $K_D^{\text{Aβ16}/k_D^{\text{wt}}}$ (wt) |
|--------------------------|------------------------|---------------------------------|
|                          | pH 6.2 | pH 7.4 | pH 9.4 | pH 6.2 | pH 7.4 | pH 9.4 | Affinity standard |
| WT                       | −8.1   | −9.8   | −12.9  | 1.00   | 1.00   | 1.00   | DP2          |
| D1A                      | −7.2   | −9.1   | −12.9  | 7.94   | 5.01   | 1.00   | DP2          |
| D1E                      | −7.3   | −9.3   | −12.9  | 6.31   | 3.16   | 1.00   | DP2          |
| D1N                      | −7.9   | −9.5   | −12.7  | 1.58   | 2.00   | 1.58   | DP2          |
| A2P                      | −8.3   | −9.8   | −12.4  | 0.63   | 1.00   | 3.16   | DP2          |
| D1H                      | −9.7   | −11.7  | −13.3  | 0.03   | 0.01   | 0.40   | DP3          |
| D1H/H14A                 | −9.4   | −11.5  | −12.8  | 0.05   | 0.02   | 1.26   | DP3          |
| Ac-Aβ16                  | nd$^b$ | −8.1   | −12.1  | nd$^b$ | 50     | 6.3    | DP2          |
| DP2$^c$                  | 8.1    | −9.9   | −12.9  |     |       |       | Gly/His      |
| DP3$^c$                  | 9.9    | −12.2  | −13.5  |     |       |       | Gly/His      |

$^a$ The log $K_D^{Aβ16}$ values were determined via ligand competition for Cu(ii) between each peptide and the specified DP probe ligand in respective buffer (50 mM) of Mes (pH 6.2), Mops (pH 7.4) and Ches (pH 9.4). See the Experimental section for details. $^b$ Too weak to be determined reliably. $^c$ Following relationships demonstrated from a previous study$^21$ were used to calculate the log $K_D^{Aβ16}$ values given in the table: log $K_D^{Aβ16} = 1.15–1.49$ pH for DP2 and eqn (5) with $K_D^{Aβ16}$ and each $pK_a$ value given in Table 3 for DP3.

---

**Fig. 3** Quantification of Cu(ii) binding to Aβ16 peptides in Mops buffer (50 mM, pH 7.4). (a) Illustrative quenching of the fluorescence spectrum of a Cu(II) probe (2.0 μM) upon titration with Cu(II) solution (λem = 330 nm; λex = 550 nm); (b) Cu(II) titration curves at 550 nm for 1:1 mixtures (2.0 μM) of DP2 and Aβ16 peptides plus control with DP2 only; (c and d) recovery of the Cu(II)-quenched fluorescence intensity of mixtures (c) Cu(II)–DP2 or (d) Cu(II)–DP3 (2.0 μM) upon titration with Aβ16 peptides; (e and f) correlation of (e) [Cu(II)–DP2] versus [Aβ16 peptide]tot/[DP2]tot or (f) [Cu(II)–DP3] versus [Aβ16 peptide]tot/[DP3]tot. The solid traces in (e and f) are the fits of eqn (3) to the experimental data, allowing estimation of the conditional log $K_D^{Aβ16}$ for the Cu(ii) complex of each Aβ16 peptide in the range of pH values listed in Table 1.
The presence of His1 allows the formation of a stabilizing bidentate ligand comprising the N-terminal amine and the His1 sidechain within a six-membered chelate ring. Other known examples of this binding mode include the DP3 probe employed in this work (Fig. 2a) and the bacterial copper binding proteins PcoC and CopC.24,33–35 The Cu(n) coordination sphere in Cu(II)(Aβ16-D1H) may be depicted schematically by Fig. 4a(i), noting its similarity to the proposed IA site in Aβ16 (Fig. 1b). There are three other His residues (His-6, -13, -14) in the Aβ16-D1H sequence, but only two of them are required to constitute the proposed Cu(n) site. In fact, replacing one of them (His14) with Ala in Aβ16-D1H/1H14A only marginally affects the Cu(n) affinity (Fig. 5a and Table 2).

The Cu(n) sites proposed above for Cu(II)(Aβ16-D1H) and Cu(II)(Aβ16-D1H/H14A) are supported by the fact that the pH-dependence of their affinities for Cu(n) (expressed as pK_D = −log K_D) can be modeled satisfactorily over the pH range 6.0–8.7, assuming that the dependence is driven by the basicity of the proposed ligands (Fig. 5a and eqn (5)). Their derived ligand pK_a values are listed in Table 3. The I → II transition is not evident in these peptides and the pH dependence of the affinities is comparable to those of several documented examples including DP3 and several CopC proteins that adopt a DP3-type Cu(n) binding mode, shown in Fig. 2a.21,33

In contrast, the absence of His1 in the WT, D1A and A2P peptides leads to very different pH-dependencies that could not be modelled over the same pH range with any single set of ligands or a single binding mode (Fig. 5). The behaviour is attributed to the influence of the I → II transition, even for the A2P peptide at pH > 8.5 (see Fig. 6c). The Cu(n) site of component II features a deprotonated peptide amide ligand (Fig. 1b).

pH-Dependent EPR spectra provide further support for the proposed binding models. The EPR signal of Cu(II)(Aβ16-D1H) shows no change within the pH range 5.9–8.9 and essentially superimposes that of Cu(II)–DP3 (Fig. 6a and b). It differs significantly from the component I EPR signals of Cu(II)(Aβ16) or Cu(II)(Aβ16-A2P) (cf., Fig. 6a–d and Table 4). This supports a Cu(n) site in Cu(II)(Aβ16-D1H) of composition [NH_3, 3N_im] that features the stabilizing bidentate ligand comprising the N-terminal amine and the His1 sidechain (Fig. 2a and 4a(i)).

This particular His1 sidechain binding mode is suppressed in Cu(II)–DP4. Although its four-residue peptide sequence (His-Lys-His-His) features a His1 residue, the presence of His3 provides the more favourable ATCUN binding mode that includes the N-terminal amine and two intervening deprotonated backbone amides (Fig. 2b).21 This conclusion is supported by the dramatic pH-dependence of its Cu(n) binding affinity and the unique EPR signal: both are readily distinguishable from those of components I and II (Fig. 5b and 6f).21 This same ATCUN binding mode is adopted by the N-truncated Aβ16 peptide, Aβ4-16 (sequence: FRHDSGYEVHHQK) that exhibits Cu(n) affinities and EPR spectra equivalent to those of DP4 (Table 4).6,21–23

Component IB is favoured by an Ala1 N-terminus at low pH but is less stable than IA

The Cu(n) site of Aβ16-D1A has a five-fold lower affinity for Cu(n) than Aβ16 at pH 7.4 (eqn (9); Fig. 5a; Table 2). The I → II transition in Cu(II)(Aβ16) with pK_a ~ 7.8 is characteristic of deprotonation of the peptide amide of Ala2. The non-coordinating methyl sidechain of Ala1 eliminates the IA binding mode, enforcing a IB mode (Fig. 4a(ii)) that is converted to component II at pH ~ 6.5, i.e., > 1 pH unit lower than that for the equivalent transition of Cu(II)(Aβ16) (Fig. 6d and e). However, at higher pH values, the affinities of Aβ16 and Aβ16-D1A for Cu(n) become indistinguishable (Fig. 5a), consistent with a common tridentate chelate ([–NH_2, N_im, CO] as part of the square planar array [–NH_2, N_im, CO, N_im]) characteristic of component II (Fig. 1b). The binding affinity of the latter shows a different pH dependency and EPR signal to those of DP4, a Cu(n) ligand of the ATCUN binding mode (Fig. 2b, 5 and 6).

The equivalent data for variants D1N and D1E provide further support for a role for Asp1 as an equatorial ligand: their Cu(n) binding properties are intermediate between those of Aβ16 and
Deletion of Asp1 in Aβ generates a truncated peptide with an Ala N-terminus (sequence: AEFRHDSGYEVHHQK). Its qualitative Cu(ii) binding properties are comparable to those of D1A: weaker binding affinity for Cu(ii) and a lower I → II transition pKa. Similar effects were also observed for D1N,17–19 although the decreased pKa was attributed to the change of a putative hydrogen bond to the Cu(ii) ligand.17

Component I is favoured in CuII(A2P)

The I → II transition relies on the availability of the Asp1-Ala2 peptide amide (Fig. 1b). Substitution of Ala2 with Pro eliminates this possibility and all properties observed for CuII(A2P) are consistent with the binding mode being locked in component I. Most obviously, Kd is comparable to that of CuII(Aβ16) at lower pH values but becomes larger (i.e., lower affinity) at higher pH with the point of deviation occurring above pH 7.8, in line with the I → II transition for CuII(Aβ16) around this pH (Fig. 5a and Table S1, Ref.NH2 NIm). Similar effects were also observed for D1N,17–19 although the decreased pKa was attributed to the change of a putative hydrogen bond to the Cu(ii) ligand.17

At lower pH, component IA is preferred for Aβ peptides with an Asp1 N-terminus

The Cu(ii) affinities of component I of CuII(Aβ16) and CuII(Aβ16-A2P) are similar. They are about one order of magnitude higher than that of component IB, which is favoured for CuII(Aβ16-D1A) at lower pH values such as pH 6.2 (Fig. 5a and Table S1, ESI†). This implies that component IA rather than IB is the preferred binding mode for both CuII(Aβ16) and CuII(Aβ16-A2P) at lower pH values (Fig. 1b). This is consistent with the

Table 3  Thermodynamic data of Cu(i) sites derived from correlation of log Kd and pH

| Peptide or protein | Cu(i) binding site | Log Kd
|-------------------|--------------------|------|
| Aβ16-D1H         | NH2 (H1); 3 NIm (H1,6,13/14) | −12.7 |
| Aβ16-D1H/H14A    | NH2 (H1); 3 NIm (H1,6,13) | −12.3 |
| DP3 probe        | NH2 (H1), 3 NIm (H1,5,7) | −13.6 |
| Ps-CopC          | NH2 (H1); 2 NIm (H1,91); COO− (D89) | −14.4 |
| Ec-PcoC          | NH2 (H1); 2 NIm (H1,92); COO− (D90) | −14.0 |
| Aβ16             | NH2 (H1); 3 NIm (H1,6,13) | −12.3 |

a See Fig. 5 for experiments; parameters obtained by curve-fitting to eqn (5).

Table 4  EPR parameters derived from frozen solution spectra

| Ligand for the Cu(i) complex | Component I | Component II | pKa for I ↔ II |
|------------------------------|-------------|--------------|---------------|
|                | gI | g⊥ | A1 (G) | gJ | g⊥ | A| (G) | pKa |
| DP3            | 2.242 b | 2.060 b | 182 b | — | — | — | — | — |
| Aβ16-D1H       | 2.243 2.051 170 | 2.204 2.041 161 | ~ 8.8 |
| Aβ16-A2P       | 2.269 2.051 170 | 2.222 2.041 161 | ~ 6.5 |
| Aβ16-D1A       | 2.249 2.051 170 | 2.204 2.041 161 | ~ 8.8 |
| Aβ16           | 2.264 2.051 170 | 2.222 2.041 161 | ~ 6.5 |
| Aβ4-16         | — | — | 2.178 2.050 200 b | — | — | — | — |
| Aβ4-16         | — | — | 2.178 2.049 206 b | — | — | — | — |

a Estimated directly from the respective spectrum, see Fig. 6, Fig. S1–S7 (ESI) for details. b Data adopted from ref. 21. c Data for CuII(Aβ16-D1H/H14A) were indistinguishable (Fig. S5 and S6, ESI). d Data adopted from ref. 16. e Minor component at pH 7.4.

References
negatively charged harder carboxylato ligand favouring the harder metal ion Cu(II) over a neutral peptide carbonyl oxygen for similar stereochemistries (a six-membered chelate ring in IA versus a five-membered chelate ring in IB; see Fig. 1b).

However, the possibility for a IB binding mode for either Cu(II)(Aβ16) or Cu(II)(Aβ16-A2P) at low pH cannot be excluded. The EPR signals for both components IA and IB are indistinguishable (Fig. 6c-e and Table 4) and cannot be used to differentiate the binding modes at low pH. It is possible that components IA and IB are in fast exchange at lower pH for those peptides with an Asp1 N-terminus but with IA being the preferred binding mode. At higher pH, both components IA and IB are converted to component II: the Kf values of Cu(II)(Aβ16) and Cu(II)(Aβ16-D1A) become indistinguishable at pH 9.4 (Fig. 5a). But the I → II conversion is restricted for Cu(II)(Aβ16-A2P), weakening its Cu(II) binding affinity at high pH relative to other Aβ16 peptides (Fig. 5a).

It is unlikely that the differences in the properties of Cu(II)(Aβ16), Cu(II)(Aβ16-A2P) and Cu(II)(Aβ16-D1A) at lower pH values are due to axial coordination by the Asp1 sidechain due to the following reasons:

(i) Given the possibility of axial coordination, mutation of Asp1 by Ala should lower the affinity across the whole pH range. However, there is no difference in the Cu(II) affinity for component II between WT and D1A.

(ii) A bidentate [NH₂, COO⁻] binding mode for peptides with N-terminal aspartyl residues has been well-documented in many cases, including various Aβ peptides.

(iii) The IA binding mode is confirmed for many peptides and proteins with a His1 N-terminus including Aβ16-D1H, DP3 probe, CopC and PcoC proteins, as surveyed above.

(iv) Contributions from axial coordination at Cu(II) centres are usually negligible due to the Jahn–Teller effect. There are many possible carboxylate sidechains (Asp1, Asp7, Glu3 or Glu11) which have been proven to be redundant. For example, an E3Q mutation showed no significant effect on the EPR spectrum.

The IA coordination mode for Aβ16 has been proposed frequently as one of the two possible coordination modes of Asp1 at lower pH for component I (Fig. 1b). However, component IB has been preferred recently, based mainly on pulsed EPR studies. The chemical and spectroscopic study presented in this work, supported by previous Cu(II) binding studies for those peptides with N-terminal aspartyl residues, provides strong evidence that the preferred binding mode for component I in Cu(II)(Aβ16) is IA, and not IB (Fig. 1b). It is recommended that the possibility of the IB binding mode can be re-investigated by pulsed EPR using Cu(II)(Aβ16-D1H) and Cu(II)(Aβ16-D1A) as controls.

**Impact of N-terminal modification on Cu(II) binding**

The first evidence of Cu(I) binding to Aβ peptides was implicated in an early study of Cu(I) potentiation of Aβ neurotoxicity. We now know that Aβ peptides bind Cu(I) mainly via a combination of two histidine residues within the first 16 residues. The Cu(I) binding stoichiometries and affinities of various Aβ16 peptides generated in this work were examined using Cu(I) probe ligands Ferene S (Fs) and Ferrozine (Fz) according to a recent protocol. Both probe ligands react with Cu(I) under reducing conditions to yield respective chromophoric Cu(I) complexes [Cu(I)L₂]⁻ with different formation constants (log β₁ = 13.7 for L = Fs and 15.1 for L = Fz). They were used to estimate the binding stoichiometries of Aβ16 peptides according to eqn (6) under weakly competing conditions and the binding affinities according to eqn (6) and (7) under strongly competing conditions. As demonstrated previously for Aβ16, mostly peptides listed in Table 1 bind a single equiv. of Cu(I) under Cu(II) limiting conditions imposed by a slight excess of the weak probe ligand Fs (Fig. 7a). The exception was Aβ16-D1H that appeared to bind more than one equiv. Cu(I) under the same conditions (Fig. 7b).

Increasing Fs ligand concentration or employing the stronger ligand Fz created more effective competing conditions for eqn (6) that allowed analysis and definition of Cu(I) binding affinity of each peptide with eqn (7) (Fig. 7c and d). There are only marginal differences in Cu(I) binding affinities for those peptide variants with substitution of a non-His residue in the sequence. But, the Cu(I) binding affinities of Aβ16-D1H and Aβ16-D1H/H14A are slightly higher than those of the other peptides (Table 5).

The data are consistent with previous observations: (i) the non-His residues in the N-terminal region play little role in Cu(I) binding; (ii) two and only two His ligands are required for the flexible Aβ16 peptides to constitute a Cu(I) binding site (Fig. 1a); (iii) the Cu(I) bound by Aβ16 peptides is...


Table 5  Log $K_D$ for Cu(i) of Aβ16 peptide variants at different pH values$^a$

| Aβ16 peptide | log $K_D$ | $K_D$ (wt) | Probe-affinity std |
|--------------|----------|------------|--------------------|
|              | pH 6.2  | pH 7.4    | pH 9.4             | Probe-affinity std |
| WT           | -9.6     | -10.6     | -11.0              | Fs                |
| D1A          | -9.3     | -10.6     | -11.0              | Fs                |
| D1E          | -9.5     | -10.4     | -10.9              | Fs                |
| D1N          | -9.5     | -10.4     | -10.8              | Fs                |
| A2P          | -9.9     | -10.7     | -11.0              | Fs                |
| D1H          | -10.2    | -11.1     | -11.5              | Fs                |
| D1H/H14A     | -10.1    | -10.9     | -11.3              | Fs/Fz             |
| Ac-Aβ16      | -10.4    |           |                     | Fs/Fz             |

$^a$ The log $K_D$ values were determined via ligand competition for Cu(i) between each peptide and the specified probe ligands Fs and/or Fz in the respective buffers (50 mM) Mes (pH 6.2), Mops (pH 7.4) and Ches (pH 9.4). See the Experimental section for details.

populated between different pairs of His ligands in a fast exchange mode that tends to increase the apparent binding affinities.$^7,8$ There are three different combinations of His pairs for most peptides examined in this work (Fig. 1a) and little difference in their Cu(i) binding properties, except that for Aβ16-D1H/H14A is slightly higher (Table 5).

Aβ16-D1H contains an extra His residue to provide four possible His ligands. Its Cu(i) affinity is the highest of all the peptides. Theoretically, it may bind two equiv. of Cu(i) with similar affinities, but this does not seem to be the case (Fig. 7b). Binding of the first equiv. of Cu(i) appears to discourage the binding of a second equiv. of Cu(i) with the same affinity (Fig. 7b). Certainly, the first equiv. of Cu(i) bound in Aβ16-D1H may be populated among six different combinations of His pairs with considerable entropic gain, but binding of a further equiv. of Cu(i) is expected to restrict the binding sites to two His pairs only with considerable entropic loss.

Impact of the N-terminal modifications on the catalytic aerobic oxidation of ascorbate

A common feature in Alzheimer’s disease is oxidative stress, a component of which may result from undesirable catalytic reduction of dioxygen by cellular reductants such as ascorbate (Asc) mediated by Cu complexes of Aβ peptides.$^2,31,32,38,46–48$ The dioxygen reduction product $\text{H}_2\text{O}_2$ is liable to undergo further reduction via Fenton-type reactions to generate the hydroxyl radical that can cause damage not only to the direct copper ligands,$^49$ but also to the surrounding residues.$^50$ Indeed, Asc is abundant in the brain as an important neuromodulator and/or a neuroprotective agent and its concentration fluctuates dynamically in the range 200–400 μM in certain synaptic clefts.$^51,52$ In reality, the oxidation rate of Asc by dioxygen is slow and must be catalysed by redox-active couples such as Cu(ii)/Cu(i). It has been proposed that metal dyshomeostasis in the Alzheimer brain may result in binding of copper to Aβ peptides to initiate catalytic roles.$^53$

Aβ peptides can bind Cu(i) with the distinct binding modes of components I (including IA and IB) and II at physiological pH (Fig. 1b). Recent studies suggest that the different components available in Cu–Aβ16 systems (Fig. 1) display different reaction kinetics in both metal substitution and redox chemistry.$^5,6$ This study has identified the critical role of the first two N-terminal residues in determining these Cu(i) binding modes and an integrated study of the thermodynamic and kinetic contributions of these binding modes in the generation of $\text{H}_2\text{O}_2$ via catalytic aerobic oxidation of Asc was also undertaken. It should be noted that the reaction scheme in Fig. 8a is presented as an overall two-electron reaction process, but it may proceed via two consecutive one-electron steps with $\text{O}_2^{•−}$ as an immediate reduction intermediate that may be reduced further to $\text{H}_2\text{O}_2$ by a second Cu–Aβ complex.$^54$

The experiments were performed in air-saturated buffers (50 mM) at three different pH values (Mops buffer at pH 6.6 and 7.4; Ches buffer at pH 8.7). The catalytic aerobic oxidation of Asc (50 μM) was undertaken with each Cu–Aβ16 complex (prepared with a one-fold excess of apo-peptide) at a range of concentrations (1.0–20 μM). The reaction was followed by the rate of decrease in absorbance at 265 nm for the reduced form of Asc (Fig. 8a). The catalytic activity of each Cu–Aβ16 peptide complex was determined from the slope of a linear correlation

![Fig. 8](image_url)
of the initial oxidation rates versus the catalyst concentrations and expressed as \( \mu M \) Asc oxidized/\( \mu M \) catalyst per minute, i.e., molar turnover number min\(^{-1}\) (see the Experimental section for details).

As observed previously,\(^8,55\) Asc is stable in metal-free air-saturated buffers within the experiment timescale (1–2 h) and all apo-peptides are inactive (at least after demetallation with EDTA). Free \( \text{Cu}^{2+}\) and \( \text{Cu}^{+} \) have robust catalytic activities that are suppressed, but not silenced, by apo-peptides. The Cu–A\(\beta\)16 complexes are redox-active catalysts, but less active than ‘free’ copper. A one-fold excess of apo-peptide over each Cu–peptide was sufficient to minimize the catalytic contribution from ‘free’ Cu and to allow reliable detection of the catalytic activity of the Cu–complex. The results are summarized in Fig. 8, Fig. S8 (ESI\(^\dagger\)) and Table 6.

At pH 7.4, the molar catalytic activities (min\(^{-1}\)) of the different Cu–A\(\beta\)16 complexes for Asc oxidation are given below in increasing order of their activity:

\[
\text{D1A (0.08 min}^{-1}\text{) } \sim \text{D1E (0.10) } \sim \text{D1N (0.14) } < \text{ wt (0.38) } \sim \text{A2P (0.66) } \sim \text{D1H (0.78) } \sim \text{H14A (0.9) } \tag{10}\]

As discussed above, the binding modes and affinities of these peptides are similar for Cu(i) (with minor difference for A\(\beta\)16-D1H and A\(\beta\)16-D1H/H14A) but differ considerably for Cu(ii). On the one hand, replacement of Asp1 with Ala, Glu or Asn leads to destabilization of component IA inducing the I \(\rightarrow\) II transition at lower pH (Table 4 and Fig. 6). On the other hand, substitution of Ala2 with Pro suppresses the transition while substitution of Asp1 by His1 further stabilizes the IA mode (Fig. 4a(i)). The observed reactivity order of eqn (10) was sufficient to minimize the catalytic contribution from ‘free’ Cu and to allow reliable detection of the catalytic activity of the Cu–complex. The results are summarized in Fig. 8, Fig. S8 (ESI\(^\dagger\)) and Table 6.

To separate these two opposing effects, the observed oxidation rates were normalized to that of Cu(A\(\beta\)16-D1H) under the same conditions (Table 6). The normalized data indicate: (i) the redox activities of A\(\beta\)16 and variants A\(\beta\)16-D1A, -D1E and -D1N decrease uniformly with increasing pH, consistent with the influence of the I \(\rightarrow\) II transition (Fig. 6d, e and Fig. S1–S4, ESI\(^\dagger\)); (ii) the redox activity of Cu(A\(\beta\)16-A2P) is lowest at pH 8.7, consistent with a partial transition of the Cu(ii) centre in this complex from component I to unknown Cu(ii) centre(s) (Fig. 6c) at high pH; (iii) the redox activities of the two Cu complexes with a His1 N-terminus are higher than all others and notably, the one with peptide A\(\beta\)16-D1H/H14A has the highest activity consistently at all pH values (Table 6). This high redox activity is attributed to a combined redox advantage of the Cu centres in both oxidation states and this is further analysed and discussed below.

Determinants that regulate the redox activity of Cu–A\(\beta\) peptides

In order to be an efficient redox catalytic centre for a particular process of oxidation and reduction, the formal reduction

### Table 6

| A\(\beta\)16 peptide | Catalytic rate as molar velocity (min\(^{-1}\)) at pH | Relative to that with Cu–D1H as catalyst at pH | \( E_{0}^{\circ} \) (mV vs. SHE) at pH\(^b\) | \( E_{0}^{\circ} \) (mV vs. SHE) reported at pH\(^c\) |
|---------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| A\(\beta\)16         | 6.6  7.4  8.7                                 | 6.6  7.4  8.7                                 | 6.2  7.4  9.4                                | ~6.5  7.4  ~8.5                                |
|                     | 0.28  0.38  0.37                              | 0.40  0.49  0.14                              | +242  +200  +40.9                             | +255\(^{55}\)  +90\(^{42}\)  –153\(^{3}\)  Low\(^c\) |
| D1A                 | 0.09  0.08  0.17                              | 0.13  0.10  0.06                              | +277  +242  +40.9                             | +280\(^{13}\)  +180\(^{7}\)  +300\(^{44}\)  +277\(^{45}\) |
| D1E                 | 0.09  0.10  0.12                              | 0.14  0.13  0.04                              | +283  +218  +35.0                             |                                                              |
| D1N                 | 0.15  0.14  0.15                              | 0.22  0.17  0.06                              | +247  +206  +40.9                             |                                                              |
| A2P                 | 0.32  0.66  1.12                              | 0.46  0.84  0.42                              | +247  +206  +70.4                             |                                                              |
| D1H                 | 0.69  0.78  2.70                              | 1.00  1.00  1.00                              | +183  +118  +46.8                             |                                                              |
| D1H/H14A            | 0.79  0.90  3.29                              | 1.16  1.15  1.22                              | +194  +118  +64.5                             |                                                              |
| Ac-A\(\beta\)16      |                                               |                                               | +277                                          | +283\(^{5}\)  –130\(^{5}\)                     |

\(^a\) Conditions: [Asc]\(_{0}\) = 50 \mu M; [Cu–peptide]\(_{0}\) = 1–20 \mu M with a one-fold excess apo-peptide; buffers (50 mM) of Mops (pH 6.6 and 7.4) and Ches (pH 8.7). The background oxidation rate without the added Cu has been removed, see the Experimental section for details. \(^b\) Calculated from eqn (8) using \( E_{0}^{\circ} = +133 \text{ mV} \) for the redox couple \( \text{Cu}^{2+}/\text{Cu}^{+} \) (note: the \( K_{D} \) values at pH 6.6 and 8.7 were not determined and thus the calculations used data at pH 6.2 and 9.4 instead). \(^c\) Determined by cyclic voltammetry, except a recent theoretical calculation predicted +280 mV for component I and –370 to –810 mV for component II.\(^{13}\)
potential of the copper centre must be lower than that of the oxidant but higher than that of the reductant. At pH 7.4, the formal reduction potentials of ascorbate and dioxygen are $\sim +50$ mV$^{58}$ and $\sim +300$ mV$^{57}$ (vs. SHE), respectively.

The formal reduction potentials of the Cu–Aβ complexes may be estimated via the Nernst relationship of eqn (8) from the respective Cu(i) and Cu(II) affinities of each Aβ16 peptide (Table 6). The reduction potentials are generally more negative for component II than for component I and decrease with increased pH. This is consistent with the more dramatic stabilization effect for Cu(n) coordination at higher pH, especially with the dominant coordination effect of the deprotonated amide nitrogen to the Cu(n) centre in component II.$^{5,43}$ The calculated reduction potentials in this work from the respective dissociation constants generally match those determined experimentally by cyclic voltammetry or predicted from theoretical calculations (Table 6). However, it must be noted that the dissociation constants of the Cu–Aβ complexes are quite large (indicating relatively weak binding). In addition, the estimated values vary considerably with experimental conditions, as do the reduction potentials determined by cyclic voltammetry (Table 6). The redox processes are frequently not well-defined due to the different coordination environments for Cu(i) and Cu(n). Consequently, it is difficult to define the reduction potentials consistently and accurately as can be seen from the data in Table 6. Nevertheless, the statistical data seen there at pH 7.4 suggest that the reduction potentials of most Cu–Aβ complexes studied in this work fall within the potential window for catalytic oxidation of ascorbate by dioxygen (Table 6) and therefore should have no significant thermodynamic impact on their efficiency as catalysts for the aerial oxidation of ascorbate. In fact, there is no clear correlation between the calculated reduction potentials and the observed catalytic rates at the three pH values tested (Table 6).

It is apparent that the difference in the redox activity of the Cu centre in each Aβ16 peptide must be attributed primarily to differences in reaction kinetics that are closely related to the binding modes for both Cu(i) and Cu(II). As an efficient catalytic centre, the metal ion must be able to shuttle between different oxidation states with minimal reorganization energy,$^{44}$ but this condition is often not met for Cu that prefers different ligands for its two biologically accessible oxidation states. Blue copper proteins such as plastocyanin and azurin are pre-organized for electron transfer$^{44}$ and the catalytic activity of these metal binding ligands and binding modes change dynamically with the Cu oxidation state and with reaction conditions. Monomeric Aβ peptides are flexible and unstructured. Both metal binding ligands and binding modes change dynamically with the Cu oxidation state and with reaction conditions. A range of chemical and spectroscopic studies were carried out on copper complexes of several Aβ16 peptides in which the two key residues for Cu(II) binding in the N-terminal region were varied. Key conclusions are as follows:

(i) At lower pH, the Aβ16 peptide binds Cu(i) predominantly as component IA in equilibrium with less stable component IB; at higher pH, these are converted to component II with transition pH$_{K_a}\sim 7.8$ (Fig. 1b and 4b);

(ii) Component I sites involve flexible amino acid sidechains common to the Cu(i) binding ligands, but the metal sites for component II are dictated by a deprotonated backbone amide nitrogen that cannot act as a Cu(II) ligand and is slow to dissociate.$^{5,6,43}$ Consequently, component I is more reactive than component II in both metal substitution and redox catalysis.

(iii) Substitution of Asp1 with His provides an extra imidazole ligand for Cu(i) and also allows the N-terminal His1 to be part of a stable six-membered chelate ring for Cu(n) coordination. Consequently, the Cu(n) centre in Aβ16-D1H is locked into component IA with a dramatic increase in its redox activity.

(iv) Replacement of Ala2 with Pro suppresses component II relative to component I and increases the reactivity of the bound copper.

(v) Substitution of Asp1 with Ala restricts component I to the less stable IB form that is readily converted to component II at physiological pH with decreased catalytic reactivity.

Summary and concluding remarks

Monomeric Aβ peptides are flexible and unstructured. Both metal binding ligands and binding modes change dynamically with the Cu oxidation state and with reaction conditions. A range of chemical and spectroscopic studies were carried out on copper complexes of several Aβ16 peptides in which the two key residues for Cu(n) binding in the N-terminal region were varied. Key conclusions are as follows:

(i) At lower pH, the Aβ16 peptide binds Cu(n) predominantly as component IA in equilibrium with less stable component IB; at higher pH, these are converted to component II with transition pH$_{K_a}\sim 7.8$ (Fig. 1b and 4b);

(ii) Component I sites involve flexible amino acid sidechains common to the Cu(i) binding ligands, but the metal sites for component II are dictated by a deprotonated backbone amide nitrogen that cannot act as a Cu(II) ligand and is slow to dissociate.$^{5,6,43}$ Consequently, component I is more reactive than component II in both metal substitution and redox catalysis.

(iii) Substitution of Asp1 with His provides an extra imidazole ligand for Cu(i) and also allows the N-terminal His1 to be part of a stable six-membered chelate ring for Cu(n) coordination. Consequently, the Cu(n) centre in Aβ16-D1H is locked into component IA with a dramatic increase in its redox activity.

(iv) Replacement of Ala2 with Pro suppresses component II relative to component I and increases the reactivity of the bound copper.

(v) Substitution of Asp1 with Ala restricts component I to the less stable IB form that is readily converted to component II at physiological pH with decreased catalytic reactivity.
This study provides insights into determinants that regulate the reactivity of copper toward Aβ peptides and may guide development towards a better understanding of the factors contributing to oxidative stress present within Alzheimer’s disease.

**Abbreviations**

| Abbreviation | Full Form |
|--------------|-----------|
| Asc          | Ascorbate |
| Bca          | Bicinchoninic anion |
| Bcs          | Bathocuproine disulfonate |
| Ches         | N-Cyclohexyl-2-aminoethanesulfonic acid |
| DP2          | Dansyl peptide 2 (DAE(KDNS)RHDH) |
| DP3          | Dansyl peptide 3 (HP(KDNS)DHDH) |
| DP4          | Dansyl peptide 4 (H(KDNS)HH) |
| eqn          | Equation |
| equiv.       | Equivalent |
| ESI-MS       | Electrospray ionization mass spectrometry |
| Fs           | Ferene S |
| Fz           | Ferrozine |
| Hepes        | 4-(2-Hydroxyethyl)-1-piperazinethanesulfonic acid |
| His          | Histidine |
| Mes          | 2-(N-Morpholino)ethanesulfonic acid |
| Mops         | 3-(N-Morpholino)propanesulfonic acid |

**Acknowledgements**

This work was supported by funding from the Australian Research Council Grant DP130100728. We thank Prof. Ashley Bush for comments and discussions and Mrs Sioe See Volaric for providing technical support for recording EPR spectra.

**References**

1. A. I. Bush, The metallobiology of Alzheimer’s disease, *Trends Neurosci.*, 2003, 26, 207–214.
2. K. P. Kepp, Bioinorganic Chemistry of Alzheimer’s Disease, *Chem. Rev.*, 2012, 112, 5193–5239.
3. C. Hureau, Coordination of redox active metal ions to the amyloid precursor protein and to amyloid-β peptides involved in Alzheimer disease. Part 1: an overview, *Coord. Chem. Rev.*, 2012, 256, 2164–2174.
4. C. Hureau and P. Dorlet, Coordination of redox active metal ions to the amyloid precursor protein and to amyloid-β peptides involved in Alzheimer disease. Part 2: dependence of Cu(II) binding sites with Aβ sequences, *Coord. Chem. Rev.*, 2012, 256, 2175–2187.
5. L. G. Trujano-Ortiz, F. J. González and L. Quintanar, Redox Cycling of Copper–Amyloid β 1–16 Peptide Complexes Is Highly Dependent on the Coordination Mode, *Inorg. Chem.*, 2015, 54, 4–6.
6. T. Branch, P. Girvan, M. Barahona and L. Ying, Introduction of a Fluorescent Probe to Amyloid-β to Reveal Kinetic Insights into Its Interactions with Copper(II), *Angew. Chem., Int. Ed.*, 2015, 54, 1227–1230.
7. C. Hureau, V. Ballard, Y. Coppel, P. L. Solari, E. Fonda and P. Faller, Importance of dynamical processes in the coordination chemistry and redox conversion of copper amyloid-beta complexes, *J. Biol. Inorg. Chem.*, 2009, 14, 995–1000.
8. T. R. Young, A. Kirchner, A. G. Wedd and Z. Xiao, An Integrated Study of the Affinities of the Aβ16 Peptide for Cu(i) and Cu(II): Implications for the Catalytic Production of Reactive Oxygen Species, *Metallomics*, 2014, 6, 505–517.
9. C. Hureau, Y. Coppel, P. Dorlet, P. L. Solari, S. Sayen, E. Guillou, L. Sabater and P. Faller, Deprotonation of the Asp1-Ala2 peptide bond induces modification of the dynamic copper(II) environment in the amyloid-beta peptide near physiological pH, *Angew. Chem., Int. Ed.*, 2009, 48, 9522–9525.
10. S. C. Drew, C. J. Noble, C. L. Masters, G. R. Hanson and K. J. Barnham, Pleomorphic copper coordination by Alzheimer's disease amyloid-beta peptide, *J. Am. Chem. Soc.*, 2009, 131, 1195–1207.
11. C. A. Damante, K. Osz, Z. Nagy, G. Pappalardo, G. Grasso, G. Impellizzeri, E. Rizzarelli and I. Sovago, The metal loading ability of beta-amyloid N-terminus: a combined potentiometric and spectroscopic study of copper(II) complexes with beta-amyloid(1-16), its short or mutated peptide fragments, and its polyethylene glycol (PEG)-ylated analogue, *Inorg. Chem.*, 2008, 47, 9669–9683.
12. P. Dorlet, S. Gambarelli, P. Faller and C. Hureau, Pulse EPR Spectroscopy Reveals the Coordination Sphere of Copper[II] Ions in the 1–16 Amyloid-β Peptide: A Key Role of the First Two N-Terminus Residues, *Angew. Chem., Int. Ed.*, 2009, 48, 9273–9276.
13. S. C. Drew, C. L. Masters and K. J. Barnham, Alanine-2 carbonyl is an oxygen ligand in Cu²⁺ coordination of Alzheimer’s disease amyloid-beta peptide – relevance to N-terminally truncated forms, *J. Am. Chem. Soc.*, 2009, 131, 8760–8761.
14. C. L. Masters, G. Multhaup, G. Simms, J. Pottgiesser, R. N. Martins and K. Beyreuther, Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer’s disease contain the same protein as the amyloid of plaque cores and blood vessels, *EMBO J.*, 1985, 4, 2757–2763.
15. C. L. Masters, G. Simms, N. A. Weinman, G. Multhaup, B. L. McDonald and K. Beyreuther, Amyloid plaque core protein in Alzheimer disease and Down syndrome, *Proc. Natl. Acad. Sci. U. S. A.*, 1985, 82, 4245–4249.
16. J. W. Karr, H. Akintoye, L. J. Kaupp and V. A. Szalai, N-Terminal deletions modify the Cu²⁺ binding site in amyloid-beta, *Biochemistry*, 2005, 44, 5478–5487.
17. J. W. Karr and V. A. Szalai, Role of aspartate-1 in Cu(II) binding to the amyloid-beta peptide of Alzheimer’s disease, *J. Am. Chem. Soc.*, 2007, 129, 3796–3797.
18. C. Ghosh and S. G. Dey, Ligand-field and ligand-binding analysis of the active site of copper-bound Abeta associated with Alzheimer’s disease, *Inorg. Chem.*, 2013, 52, 1318–1327.
19. B. Alies, H. Eury, C. Bijani, L. Rechignat, P. Faller and C. Hureau, pH-Dependent Cu(II) coordination to amyloid-beta peptide: impact of sequence alterations, including the H6R and D7N familial mutations, *Inorg. Chem.*, 2011, 50, 11192–11201.
20 C. Harford and B. Sarkar, Amino Terminal Cu(II)- and Ni(II)-Binding Motif of Proteins and Peptides: Metal Binding, DNA Cleavage, and Other Properties, *Acc. Chem. Res.*, 1997, **30**, 123–130.

21 T. R. Young, C. J. K. Wijekoon, B. Spyrou, P. S. Donnelly, A. G. Wedd and Z. Xiao, A Set of Robust Fluorescent Peptide Probes for Quantification of Cu(II) Binding Affinities in the Micromolar to Femtomolar Range, *Metallomics*, 2015, **7**, 567–578.

22 M. Mital, N. E. Weynfeld, T. Frączyk, M. Z. Wloch, U. E. Wawrzyński, A. Bonna, C. Tumpach, K. J. Barnham, C. L. Haigh, W. Bal and S. C. Drew, A Functional Role for Aβ in Metal Homeostasis? N-Truncation and High-Affinity Copper Binding, *Angew. Chem., Int. Ed.*, 2015, **54**, 10460–10464.

23 N. E. Weynfeld, E. Stefaniak, K. Stachucy, A. Drozd, D. Plonka, S. C. Drew, A. Krężel and W. Bal, Resistance of Cu(II) to Copper Capture by Metallothionein-3 Supports a Function for the Aβ4–42 Peptide as a Synaptic Cull Scavenger, *Angew. Chem.*, 2016, **55**, 8235–8238.

24 K. Y. Djoko, Z. Xiao, D. L. Huffman and A. G. Wedd, Conserved Mechanism of Copper Binding and Transfer. A Comparison of the Copper-Resistance Proteins PcoC from Escherichia coli and CopC from Pseudomonas syringae, *Inorg. Chem.*, 2007, **46**, 4560–4568.

25 W. M. Kok, J. M. Cottam, G. D. Ciccotosto, L. A. Miles, J. A. Karas, D. B. Scanlon, B. R. Roberts, M. W. Parker, R. Cappai, K. J. Barnham and C. A. Hutton, Synthetic dityrosine-linked [small beta]-amyloid dimers form stable, soluble, neurotoxic oligomers, *Chem. Soc.*, 2013, **4**, 4449–4454.

26 Z. Xiao, L. Gottschlich, R. van der Meulen, S. R. Udagedara and A. G. Wedd, Evaluation of quantitative probes for weaker Cu(i) binding sites completes a set of four capable of detecting Cu(i) affinities from nanomolar to attomolar, *Metallomics*, 2013, **5**, 501–513.

27 Z. Xiao, J. Brose, S. Schimo, S. M. Ackland, S. La Fontaine and A. G. Wedd, Unification of the copper(i) binding affinities of the metallo-chaperones Atx1, Atox1 and related proteins: detection probes and affinity standards, *J. Biol. Chem.*, 2011, **286**, 11047–11055.

28 M. I. Karayannis, D. N. Samios and C. H. P. Gousetis, A study of the molar absorptivity of ascorbic acid at different wavelengths and pH values, *Anal. Chim. Acta.*, 1977, **93**, 275–279.

29 G. Milazzo and S. Caroli, *Tables of Standard Electrode Potentials*, Wiley, New York, 1978.

30 C. Reinhard, S. S. Hebert and B. De Strooper, The amyloid-beta precursor protein: integrating structure with biological function, *EMBO J.*, 2005, **24**, 3996–4006.

31 A. I. Bush, C. L. Masters and R. E. Tanzi, Copper, beta-amyloid, and Alzheimer’s disease: tapping a sensitive connection, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 11193–11194.

32 M. W. Beck, S. B. Oh, R. A. Kerr, H. J. Lee, S. H. Kim, S. Kim, M. Jang, B. T. Ruotolo, J.-Y. Lee and M. H. Lim, A rationally designed small molecule for identifying an in vivo link between metal-amyloid-β complexes and the pathogenesis of Alzheimer's disease, *Chem. Sci.*, 2015, **6**, 1879–1886.

33 C. J. K. Wijekoon, T. R. Young, A. G. Wedd and Z. Xiao, The CopC Protein from Pseudomonas fluorescens SBW25 Features A Conserved Novel High Affinity Cu(II) Binding Site, *Inorg. Chem.*, 2015, **54**, 2950–2959.

34 L. Zhang, M. Koay, M. J. Maher, Z. Xiao and A. G. Wedd, Intermolecular transfer of copper ions from the CopC protein of Pseudomonas syringae. Crystal structures of fully loaded Cu(i)Cu(II) forms, *J. Am. Chem. Soc.*, 2006, **128**, 5834–5850.

35 T. J. Lawton, G. E. Kenney, J. D. Hurley and A. C. Rosenzweig, The CopC Family: Structural and Bioinformatic Insights into a Diverse Group of Periplasmic Copper Binding Proteins, *Biochemistry*, 2016, **55**, 2278–2290.

36 C. Kállay, K. Várnagy, G. Micera, D. Sanna and I. Sóvágó, Copper(II) complexes of oligopeptides containing aspartyl and glutamyl residues. Potentiometric and spectroscopic studies, *J. Inorg. Biochem.*, 2005, **99**, 1514–1525.

37 I. Sovago and K. Osz, Metal ion selectivity of oligopeptides, *Dalton Trans.*, 2006, 3841–3854, DOI: 10.1039/B607515K.

38 X. Huang, M. P. Cuajungco, C. S. Atwood, M. A. Hartshorn, J. A. Taedall, G. R. Hanson, K. C. Stokes, M. Leopold, G. Multhaup, L. E. Goldstein, R. C. Scarpa, A. J. Saunders, J. Lim, R. D. Moir, C. Glabe, E. F. Bowden, C. L. Masters, D. P. Fairlie, R. E. Tanzi and A. I. Bush, Cu(II) potentiation of Alzheimer Aβ neurotoxicity: correlation with cell-free hydrogen peroxide production and metal reduction, *J. Biol. Chem.*, 1999, **274**, 37111–37116.

39 R. A. Himes, G. Y. Park, G. S. Siluvai, N. J. Blackburn and K. D. Karlin, Structural Studies of Copper(i) Complexes of Amyloid-β Peptide Fragments: Formation of Two-Coordinate Bis(histidine) Complexes, *Angew. Chem., Int. Ed.*, 2008, **47**, 9084–9087.

40 J. Shearer and V. A. Szalai, The Amyloid-β Peptide of Alzheimer's Disease Binds Cu in a Linear Bis-His Coordination Environment: Insight into a Possible Neuroprotective Mechanism for the Amyloid-β Peptide, *J. Am. Chem. Soc.*, 2008, **130**, 17826–17835.

41 S. Furlan, C. Hureau, P. Faller and G. La Penna, Modeling the Cu+ binding in the 1-16 region of the amyloid-beta peptide involved in Alzheimer’s disease, *J. Phys. Chem. B*, 2010, **114**, 15119–15133.

42 L. Guilloréau, S. Combaltber, A. Sournia-Saquet, H. Mazarguil and P. Faller, Redox chemistry of copper-amyloid-beta: the generation of hydroxyl radical in the presence of ascorbate is linked to redox-potentials and aggregation state, *Chem-BioChem*, 2007, **8**, 1317–1325.

43 J. Ali-Torres, A. Mirats, J.-D. Maréchal, L. Rodríguez-Santiago and M. Sodupe, 3D Structures and Redox Potentials of Cu2+-Aβ(1–16) Complexes at Different pH: A Computational Study, *J. Phys. Chem. B*, 2014, **118**, 4840–4850.

44 V. Ballard, C. Hureau and J.-M. Savéant, Electrochemical and homogeneous electron transfers to the Alzheimer amyloid-β copper complex follow a preorganization mechanism, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 17113–17118.

45 D. Jiang, L. Men, J. Wang, Y. Zhang, S. Chickenyen, Y. Wang and F. Zhou, Redox reactions of copper complexes formed with different β-amyloid peptides and their neuropathological relevance, *Biochemistry*, 2007, **46**, 9270–9282.
X. Zhu, B. Su, X. Wang, M. A. Smith and G. Perry, Causes of oxidative stress in Alzheimer disease, *Cell. Mol. Life Sci.*, 2007, **64**, 2202–2210.

C. Opazo, X. Huang, R. A. Cherny, R. D. Moir, A. E. Roher, A. R. White, R. Cappai, C. L. Masters, R. E. Tanzi, N. C. Inestrosa and A. I. Bush, Metalloenzyme-like activity of Alzheimer’s disease beta-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H₂O₂, *J. Biol. Chem.*, 2002, **277**, 40302–40308.

X. Huang, C. S. Atwood, M. A. Hartshorn, G. Multhaup, L. E. Goldstein, R. C. Scarpa, M. P. Cuajungco, D. N. Gray, J. Lim, R. D. Moir, R. E. Tanzi and A. I. Bush, The A beta peptide of Alzheimer’s disease directly produces hydrogen peroxide through metal ion reduction, *Biochemistry*, 1999, **38**, 7609–7616.

C. Cheignon, P. Faller, D. Testemale, C. Hureau and F. Collin, Metal-catalyzed oxidation of A[small beta] and the resulting reorganization of Cu binding sites promote ROS production, *Metallomics*, 2016, **8**, 1081–1089.

C. S. Atwood, G. Perry, H. Y. Kato, W. D. Jones, K.-Q. Ling, X. Huang, R. D. Moir, D. Wang, L. M. Sayre, M. A. Smith, S. G. Chen and A. I. Bush, Copper Mediates Dityrosine Cross-Linking of Alzheimer’s Amyloid-β, *Biochemistry*, 2004, **43**, 560–568.

R. A. Grünewald, Ascorbic acid in the brain, *Brain Res. Rev.*, 1993, **18**, 123–133.

M. E. Rice, Ascorbate regulation and its neuroprotective role in the brain, *Trends Neurosci.*, 2000, **23**, 209–216.

G. Eskici and P. H. Axelsen, Copper and Oxidative Stress in the Pathogenesis of Alzheimer’s Disease, *Biochemistry*, 2012, **51**, 6289–6311.

K. Reybier, S. Ayala, B. Alix, H. V. Rodriguez, S. Bustos Rodriguez, G. La Penna, F. Collin, C. M. Gomes, C. Hureau and P. Faller, Free Superoxide is an Intermediate in the Production of H₂O₂ by Copper(i)-Aβ Peptide and O₂, *Angew. Chem., Int. Ed.*, 2016, **55**, 1085–1089.

B. Alix, I. Sasaki, O. Proux, S. Sayen, E. Guillot, P. Faller and C. Hureau, Zn impacts Cu coordination to amyloid-[small beta], the Alzheimer’s peptide, but not the ROS production and the associated cell toxicity, *Chem. Commun.*, 2013, **49**, 1214–1216.

B. E. Conway, *Electrochemical Data*, Greenwood Press, New York, 1969.

D. L. Nelson and M. M. Cox, *Lehninger Principles of Biochemistry*, W. H. Freeman, New York, 2004.

R. J. P. Williams, Metallo-enzyme catalysis: the entatic state, *J. Mol. Catal.*, 1985, **30**, 1–26.

S. L. Leong, T. R. Young, K. J. Barnham, A. G. Wedd, M. G. Hinds, Z. Xiao and R. Cappai, Quantification of Copper Binding to Amyloid Precursor Protein Domain 2 and its Caenorhabditis Elegans Ortholog. Implications for Biological Function, *Metallomics*, 2014, **6**, 105–116.