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# Alanine-2 Carbonyl is an Oxygen Ligand in $Cu^{2+}$ Coordination of Alzheimer's Disease Amyloid-$\beta$ Peptide − Relevance to N-Terminally Truncated Forms

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by deposition of extracellular amyloid plaques, the major component being the amyloid-$\beta$ peptide (A$\beta$).[1] Metal ions such as copper are found in high concentrations within plaques in the AD brain.[2] The mechanisms that lead to plaque formation and disease are not fully understood, although metal-induced A$\beta$ oligomerization is believed to play a key role, possibly via an oxidative stress pathway.[3] To understand the role that copper might play in this process, a detailed knowledge of the fundamental $Cu^{2+}$/A$\beta$ interactions is essential. Here we identify an equatorial oxygen ligand from A$\beta$ and postulate a role for $Cu^{2+}$-promoted amide hydrolysis in generating truncated A$\beta$3−40/42 species found in AD plaques.

Electron Paramagnetic Resonance (EPR) spectroscopy is a powerful technique for investigating protein interactions with paramagnetic $Cu^{2+}$. Using conventional continuous-wave (CW) EPR at X-band frequencies, studies of $Cu^{2+}$ interactions with monomeric A$\beta$ have revealed two main species in the physiological pH range, referred to as "component I" (low pH) and "component II" (high pH). The identity of the ligands associated with these $Cu^{2+}$ coordination modes has been controversial. We were recently able to resolve and analyze the superhyperfine interactions between $Cu^{2+}$ and A$\beta$; this enabled us to employ isotopic labeling to probe the local $Cu^{2+}$ environment, a superior method to introducing mutations or other modifying groups because the peptide retains its native structure. Using a library of A$\beta$16 analogues with site-specific $^{15}$N-labeling of Asp1, His6, His13, His14, we demonstrated that the component I signal comprises two independent coordination modes, {$N_a^{D1}$, O, $N_\varepsilon^{H6}$, $N_\varepsilon^{H13}$} (component Ia) and {$N_a^{D1}$, O, $N_\varepsilon^{H6}$, $N_\varepsilon^{H14}$} (component Ib), while component II is defined by {O, $N_\varepsilon^{H6}$, $N_\varepsilon^{H13}$, $N_\varepsilon^{H14}$} coordination.[4]

The identity of the oxygen ligand(s) involved in these coordination modes remained elusive, although $^{17}$O labeling ruled out equatorial coordination by both water (in A$\beta$ fibrils)[5] and the phenolate of Tyr10.[4] A backbone carbonyl oxygen ligand has been proposed for the component II coordination mode.[6] Hyperfine sublevel correlation (HYSCORE) spectroscopy recently identified hyperfine coupling with a noncoordinating backbone amide three bonds from $Cu^{2+}$,[4] consistent with oxygen coordination by the backbone carbonyl group of the preceding residue.[7] Here, we used additional $^{15}$N and $^{13}$C labeling in conjunction with HYSCORE spectroscopy to unambiguously identify the backbone carbonyl oxygen of Alanine-2 as the oxygen ligand in the component II coordination mode. Moreover, using selective $^{13}$C-labeling of Asp1, Glu3, Asp7, and Glu11, we found no evidence to support the equatorial coordination of a $\beta$-$COO^-$ group in the physiological pH range.

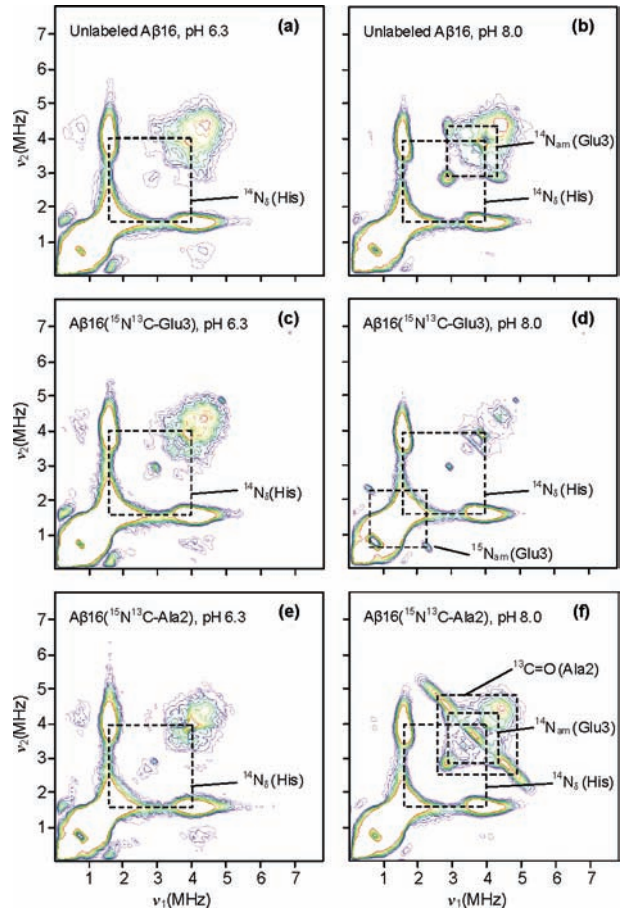


***Figure 1.*** X-band (9.71 GHz) HYSCORE spectra ($\tau$ = 144 ns) of $Cu^{2+}$/A$\beta$16 analogues (0.9 equiv of $^{65}CuCl_2$), obtained at 3370 G (near $g_\perp$). At pH 8.0, the shift of the amide cross-peaks upon $^{15}$N-labeling of Glu3 and the appearance of $^{13}$C correlation ridges upon $^{13}$C-labeling of Ala2 identify the carbonyl of Ala2 as the oxygen ligand in component II.

Figure 1 compares the HYSCORE spectra of the native $Cu^{2+}$/A$\beta$ complex with $Cu^{2+}$/A$\beta$16($^{15}$N$^{13}$C-Glu3) and $Cu^{2+}$/A$\beta$16($^{15}$N$^{13}$C-Ala2) analogues. At pH 8.0, cross-peaks near (2.9, 4.3) and (4.3, 2.9) MHz (Figure 1b) are consistent with the double-quantum transitions of a noncoordinating backbone amide three bonds from $Cu^{2+}$, with $a_{iso}(^{14}N_{am}) \approx 1$ MHz and $4K \approx 3$ MHz (eq 2 in the Supporting Information).[4,7,8] When the A$\beta$16($^{15}$N$^{13}$C-Glu3) analogue was studied under the same conditions, the $^{14}$N cross-peaks were replaced with

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features centered upon the $^{15}N$ Larmor frequency ($\nu_{15N}$ = 1.45 MHz at 3370 G) with a splitting of ~1.7 MHz (Figure 1d). These observations suggested that the amide of Glu3 is three bonds away from $Cu^{2+}$, thereby implicating the backbone carbonyl of Ala2 as an oxygen ligand (Figure 2). This assignment was unambiguously confirmed by the appearance of $^{13}C$ correlation ridges centered on the $^{13}C$ Larmor frequency ($\nu_{13C}$ = 3.61 MHz at 3370 G) in an A$\beta$16($^{15}N^{13}C$-Ala2) analogue, with a splitting of ~2.4 MHz and a width of ~0.9 MHz (Figure 1f). At 3085 G, in the $g_{||}$ region of the spectrum (Figure S1 in the Supporting Information), the $^{15}N_{am}$ cross-peaks from Glu3 are split by ~1.5 MHz, while the $^{13}C$ ridges of Ala2 have a splitting of ~3.4 MHz and a width of ~0.9 MHz (Figure S3 in the Supporting Information). From these splittings, $|a_{iso}(^{15}N_{am})| \approx 1.6$ MHz and $|a_{iso}(^{13}C{=}O)| \approx 3$ MHz can be estimated. Isotropic hyperfine couplings of this magnitude can only be explained by a through-bond pathway resulting from equatorial coordination of the carbonyl oxygen of Ala2 (Figure 2).[7,9,10] At pH 6.3, where component II coordination is not favored,[4,11] the $^{13}C$ correlation ridges are not present (Figure 1e) showing that the Ala2 carbonyl is an oxygen ligand exclusive to component II.

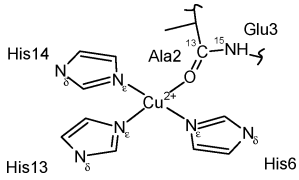


***Figure 2.*** Proposed structure of the $\{O_{C=O}^{A2}, N_{\varepsilon}^{H6}, N_{\varepsilon}^{H13}, N_{\varepsilon}^{H14}\}$ coordination mode corresponding to component II, deduced by selective $^{15}N$-labeling of Glu3 and $^{13}C$-labeling of Ala2 (indicated).

To investigate the identity of the oxygen ligand(s) in the component Ia and Ib coordination modes, we further examined a number of other $^{13}C$-labeled peptide analogues. Previous HYSCORE studies of $Cu^{2+}$/A$\beta$16($^{15}N^{13}C$-Asp1) detected coupling of $|a_{iso}(^{13}C)| \approx 3$ MHz at pH 6.3, consistent with $\beta$-$COO^-$ coordination;[4,10] however, the possibility that the observed signal resulted from spin density on the $^{13}C_{\alpha}$ nucleus of Asp1 due to the coordination of the amino terminus could not be ruled out. We therefore synthesized an A$\beta$16($^{13}C$(4)-Asp1) analogue containing a selective $^{13}C$ label on the $\beta$-$COO^-$ side group. In this instance, no $^{13}C$ correlation ridges were observed at pH 6.3 (Figure S2 in the Supporting Information), showing that the $\beta$-$COO^-$ of Asp1 is not an equatorial oxygen ligand. The origin of the $^{13}C$ correlation ridges in the A$\beta$16($^{15}N^{13}C$-Asp1) analogue can therefore be ascribed to either the spin density on the $^{13}C_{\alpha}$ carbon due to coordination of the amino terminus or/and the spin density on the $^{13}C(1)$ carbon due to oxygen coordination of its backbone carbonyl. Interestingly, at pH 8.0 and 3085 G, a $^{13}C$ peak near the diagonal with $|a_{iso}(^{13}C)| < 1$ is present (Figure S1 in the Supporting Information). Such a feature would be consistent with the $\beta$-$COO^-$ of Asp1 being an axial ligand in component II.

In addition to Asp1, we also screened for equatorial coordination of other $\beta$-$COO^-$ side groups. No $^{13}C$ correlation ridges associated with $|a_{iso}(^{13}C)| > 1$ MHz were observed for A$\beta$16($^{15}N^{13}C$-Glu3), A$\beta$16($^{15}N^{13}C$-Asp7), or A$\beta$16($^{15}N^{13}C$-Glu11) at pH 6.3 or 8.0 (Figure S1 in the Supporting Information), suggesting that no $\beta$-$COO^-$ side chains provide equatorial oxygen ligands in the physiological pH range. These results are consistent with those of Karr and Szalai, who reported no change in the principle $g_{||}$ and $A_{||}$(Cu) parameters using CW-EPR following point mutation of Asp1, Glu3, Asp7, and Glu11.[11] No features implicating coordination of a $^{31}P$ buffer molecule in components Ia/b appeared in any HYSCORE spectra ($\nu_{31P}$ = 5.81 MHz at 3370 G). In the hydrophobic environment of A$\beta$40 fibrils, pH 7.2 buffer enriched with $^{17}O$ (35−40%) failed to produce any broadening of the $Cu^{2+}$ hyperfine resonances expected from an equatorial $H_2(^{17}O)$ ligand.[5] We can also rule out an equatorial water ligand in the more hydrophilic environment of soluble A$\beta$16, as deduced by the absence of characteristic $^1H$ correlation ridges centered upon the $^1H$ Larmor frequency in our HYSCORE spectra (Figure S4 in the Supporting Information). There is some evidence from our data to suggest that coordination of the backbone carbonyl of Asp1 may occur at pH 6.3 (Figure S5 in the Supporting Information); however, atom-specific $^{13}C$-labeling of the carbonyl of Asp1 is required to investigate this possibility.

Identification of the oxygen ligand in the component II coordination mode (Figure 2) represents a significant step toward elucidating the role of $Cu^{2+}$/A$\beta$ interactions in the production of reactive oxygen species.[12] Moreover, carbonyl coordination of $Cu^{2+}$ by Ala2 may explain the presence of N-terminally truncated A$\beta$3−40/42 and the cyclized pyroglutamate A$\beta$3(pE)−40/42 species in both diffuse and cored AD plaques,[13−15] which are proposed to be crucial for the initiation of the disease.[16] Polarization of the carbonyl moiety by $Cu^{2+}$ can promote amide hydrolysis and cleavage of the peptide bond,[17,18] in this instance between Ala2 and Glu3 (Figure 2), leaving a truncated N3 species. Our future efforts will be directed toward identifying the remaining oxygen ligand(s) in components Ia and Ib coordination and theoretical modeling of $Cu^{2+}$-promoted amide hydrolysis.

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**Supporting Information Available:** Experimental methods, echo-detected EPR spectra of $Cu^{2+}$/A$\beta$16, HYSCORE spectra of A$\beta$16, A$\beta$16($^{13}C$(4)-Asp1), A$\beta$16($^{15}N^{13}C$-Ala2), A$\beta$16($^{15}N^{13}C$-Glu3), A$\beta$16($^{15}N^{13}C$-Asp7), and A$\beta$16($^{15}N^{13}C$-Glu11) at 3085 and 3370 G. This material is available free of charge via the Internet at http://pubs.acs.org.

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