Copper Amyloid Peptide

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34 GHz Pulsed ENDOR Characterization of the Copper Coordination of an Amyloid β Peptide Relevant to Alzheimer's Disease**

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Alzheimer's disease (AD), a neurodegenerative disorder, afflicts more than 26 million people worldwide.[1] However, no drugs or therapeutics have been developed to date to treat AD. The presence of accumulated amyloid plaques is the pathological hallmark of AD, and high concentrations of copper ions are found within the plaques.[2] Furthermore, there is growing evidence that copper ions play an important role in AD pathogenesis by an oxidative stress pathway.[3] The coordination of copper(II) to amyloid-β peptide (Aβ) has been shown to affect the key feature of the peptide structure, and thus, the structure controls the catalytic production of reactive oxygen species.[4] Because the coordination environment has a critical effect on copper reactivity, the elucidation of the structural details of the Cu^{II} coordination sphere is essential in understanding the molecular mechanisms of amyloid fibrillization. Moreover, in-depth knowledge of the coordination environment of Cu^{II} in amyloid peptide at the molecular level is particularly important for the rational design of therapeutic agents for AD. Thus, exploration of the coordination environment of Cu^{II} has been a main theme in this research area.[5–7] However, the unambiguous identification of the Cu^{II} coordination mode still remains to be achieved.

Herein, we present the first use of 34 GHz pulsed ENDOR (electron nuclear double-resonance) spectroscopy to characterize the Cu^{II} ion coordination mode of Cu^{II}-Aβ(1–16), with a comparison of its deletion of aspartic acid (D1), Cu^{II}-Aβ(2–16) at physiological pH, namely 7.4. Previous studies used site-specific isotope labeling to assign the coupling between Cu^{II} and amino acid residues by using ESEEM and HYSCORE.[5d,6c,7a] Pulsed ENDOR spectroscopy is a powerful technique used to detect nuclei directly coupled to an active-site metal center.[8] Although the use of 9 GHz X-band pulsed ENDOR to probe the Cu^{II} site in amyloid peptides has been reported,[7a] this suffers from the overlap of signals from strongly coupled nitrogen atoms, that is, the hyperfine splitting of nitrogen at 20–30 MHz with the proton ENDOR signals, which are centered at the proton Larmor frequency of 15 MHz. This problem can be eliminated through the use of 34 GHz Q-band ENDOR spectroscopy, because the proton Larmor frequency is four times greater at 34 GHz compared with that in the X-band at 9 GHz. Thus, we performed a series of 34 GHz ENDOR experiments to probe nuclei (^1H, ^2H, ^{14}N, and ^{17}O) coupled to the Cu^{II} in amyloid peptide. Our 34 GHz ENDOR results provide independent direct spectroscopic evidence of key features that determine the structural basis of Cu^{II}-Aβ.

Figure 1 a shows the 34 GHz ^1H ENDOR spectra taken at a field near $g_{\perp}$ for Cu-Aβ(1–16) prepared in H_2O and D_2O buffers. The ^1H ENDOR spectrum of Cu-Aβ(1–16) in H_2O contains features with a number of doublets centered at the

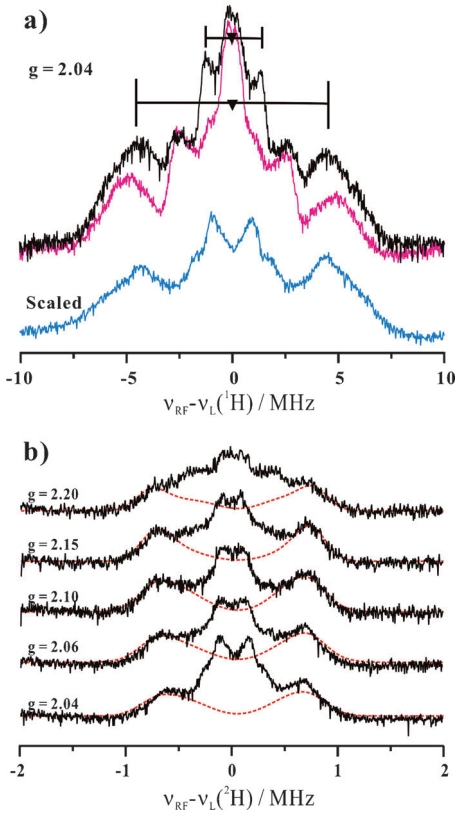

Figure 1. a) ^1H Davies ENDOR spectra of Cu^{II}-Aβ(1–16) in H_2O (black) and in D_2O (red). ^2H Mims ENDOR of Cu^{II}-Aβ(1–16) in D_2O (blue). Experimental conditions: microwave frequency 33.8 GHz; $T = 8$ K; Davies ENDOR π width 64 ns, π/2 width 32 ns, $\tau = 400$ ns, RF pulse width 20 μs; Mims ENDOR π/2 width 32 ns, $\tau = 300$ ns, RF pulse width 40 μs. b) 2D field-dependent ^2H Mims ENDOR (black) and corresponding simulations (red). Experimental conditions: π/2 width 32 ns, $\tau = 400$ ns, RF pulse width = 40 μs. Simulation parameters: $g = [2.24, 2.04, 2.04]$, $A = [2.30, -1.61, -1.61]$ MHz, Euler angles = $[\beta,\alpha] = [70,30]$, $P = [0.045, 0.045, -0.09]$ MHz.

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proton Larmor frequency, $\nu_L(^1H)$, and each split shows hyperfine couplings that range in magnitude to a maximum hyperfine value of approximately 10 MHz. When the sample was exchanged into D_2O buffer solution, the ENDOR intensity peaks of the strongly coupled proton with a hyperfine coupling value of approximately 10 MHz, and the weakly coupled proton with a hyperfine coupling value of approximately 3 MHz decreased, which indicates that these intensity peaks arise from the exchangeable protons. This observation is unambiguously confirmed by the appearance of the ^2H ENDOR signal upon D_2O exchange of the buffer solution, and the splittings in the spectrum match the ^1H ENDOR doublets scaled by a magnetogyric ratio (γ_H/γ_D) of 6.5. Quadrupole splitting expected to be observed for ^2H ($I=1$) is not resolved.

First, we focus on the origin of the strongly coupled exchangeable proton(s). To aid analysis and gain more structural information, field-dependent ^1H ENDOR as well as ^2H ENDOR spectra were collected across the EPR envelope. The experimental and simulated ^1H ENDOR spectra are shown in the Supporting Information, Figure S1. Analysis of a 2D field-frequency pattern composed of spectra collected across the EPR envelope of Cu^{II}-Aβ yields a nearly axial hyperfine coupling tensor for the exchangeable proton of $A=[15.0,\ -10.5,\ -10.5]$ MHz, which corresponds to an isotropic hyperfine coupling of $A_{iso}=-2$ MHz and an anisotropic hyperfine coupling of $T_{dip}=8.5$ MHz. This result indicates that the hyperfine coupling to the strongly coupled solvent-exchangeable proton is mainly dipolar, and a distance of 2.1 Å between the Cu^{II} center and this proton was obtained using the point-dipole approximation with a dipolar contribution $T_{dip}=8.5$ MHz. Furthermore, the Cu^{II}-H vector with Euler angles of $\theta=70°$ and $\varphi=30°$ in the g-tensor frame indicates that the coupled proton is positioned near the equatorial plane.

Similarly, the 2D field-frequency pattern of the ^2H ENDOR shown in Figure 1b corresponds to that of the ^1H ENDOR pattern. The ^2H ENDOR spectra are simpler because only the exchangeable deuterium can be observed. A simulation of the ^2H ENDOR spectra was performed using the same hyperfine parameters used for the ^1H ENDOR simulation but was simply scaled by a factor, that is, the magnetogyric ratio of 6.5. The simulated spectra reproduced the experimental data well, which confirms that the proton/deuterium is indeed exchangeable.

The strongly coupled hyperfine coupling of $T_{dip}=$ 8.5 MHz is in the expected range for the interaction of Cu^{II} with the protons of equatorially bound water molecules.[9] If this is the case, spectral changes in CW-EPR can be observed when ^{17}O-labeled water is used.[10] Thus, we measured the CW-EPR of Cu^{II}-Aβ incubated in $H_2{}^{17}O$; however, no changes were detected upon $H_2{}^{17}O$ exchange of the buffer solution (Supporting Information, Figure S2). Furthermore, the ^{17}O ENDOR spectrum exhibits only weak coupling around the ^{17}O Larmor frequency (see below). Thus, the presence of an equatorial water ligand can be ruled out.

Another possibility is that aspartic acid (D1) may be directly coordinated to Cu^{II} by the NH_2 terminus as an equatorial ligand, which has been speculated by authors of other spectroscopic studies.[6,7,11] If this is the case, the proton ENDOR could give rise to this class of strongly coupled proton and can exhibit exchangeable proton(s). Thus, we compared the ^1H ENDOR of Cu-Aβ(1–16) with that of Cu-Aβ(2–16).

Figure 2a shows comparisons of the ^1H Davies ENDOR of Cu^{II} coordinated to Aβ(1–16) and Aβ(2–16) taken at near $g_\parallel$ and $g_\perp$. Interestingly, the ^1H ENDOR of the deletion of

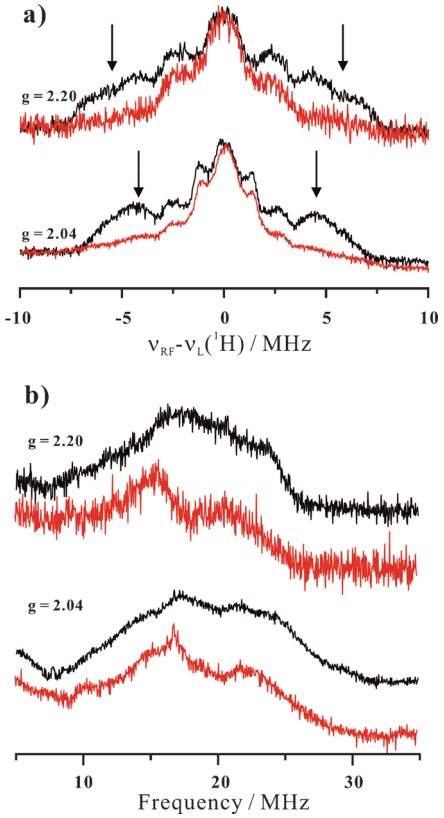

Figure 2. a) ^1H Davies ENDOR spectra of Cu^{II}-Aβ(1–16) (black) and Cu^{II}-Aβ(2–16) (red). b) ^{14}N Davies ENDOR spectra of Cu^{II}-Aβ(1–16) (black) and Cu^{II}-Aβ(2–16) (red). Experimental conditions: microwave frequency 33.8 GHz; $T=8$ K; π pulse width 64 ns; π/2 pulse width 32 ns; $\tau=400$ ns, RF pulse width 20 μs.

aspartic acid (D1), Cu^{II}-Aβ(2–16) shows the disappearance of the strongly coupled proton signal with the hyperfine coupling of approximately 10 MHz observed in the ^1H ENDOR of Cu^{II}-Aβ(1–16). This result supports the hypothesis that the strongly coupled exchangeable proton may arise from the NH_2 terminus D1. This assignment was further verified by the detection of the strongly coupled nitrogen ENDOR signals. The strongly coupled nitrogen nuclei can be well observed by Davies ENDOR.

Figure 2b shows the ^{14}N Davies ENDOR spectrum of Cu-Aβ(1–16) obtained in two different magnetic fields. The ^{14}N Davies ENDOR spectrum exhibits rather unresolved features that range from approximately 10 MHz to 30 MHz, which are typical for strongly coupled ^{14}N ligands directly bound to Cu^{II}.[12] These signals arise solely from ^{14}N ENDOR without any proton signals. These broad ^{14}N peaks in the

regime of strongly coupled signals are centered at half of the hyperfine value and split by twice the Larmor frequency; they therefore correspond to hyperfine couplings of 30–50 MHz. These ^{14}N couplings are similar to those reported for other copper proteins with histidyl nitrogen atoms as ligands[12] and are consistent with copper sites that contain three nitrogen ligands.[13]

Interestingly, the ^{14}N Davies ENDOR spectrum of the Cu^{II}-Aβ(2–16) is significantly different from that of Aβ(1–16), as shown in Figure 2b. The deletion of the coordinated nitrogen can presumably give rise to different spectra. Indeed, ^{14}N ENDOR suggests that D1 removal does disrupt the Cu^{II} equatorial coordination geometry. Thus, the ENDOR results support the notion that the different ^{14}N ENDOR signal is due to the alternation of the nitrogen coordination of the Cu^{II} in Aβ(2–16). Changes in both the ^{1}H and ^{14}N ENDOR spectra thus offer new and strong evidence that the NH_2 terminus of aspartic acid (D1) is bound to the Cu^{II} site of Aβ(1–16) as an equatorial nitrogen ligand.

Next, we investigated the source of the weakly coupled exchangeable proton with the hyperfine coupling of approximately 3 MHz observed in the ^{1}H ENDOR spectrum of the Cu^{II}-Aβ(1–16) (Figure 1a). This ^{1}H coupling is similar to couplings reported for other copper proteins with an axial water ligand.[9,14] In the case of water axially bound to Cu^{II}, the ^{1}H hyperfine coupling of water should be smaller, in the range of 3–4 MHz, and exhibit dipolar character. Whether this weakly coupled exchangeable proton arises from an axial water ligand was further investigated by ^{17}O ENDOR because the direct detection of oxygen using ^{17}O labeling is indicative of water-derived ligands.

Figure 3 shows the ^{17}O Mims ENDOR spectra of Cu^{II}-Aβ(1–16) prepared in $H_2{}^{17}O$ and $H_2{}^{16}O$. The ^{17}O ENDOR spectrum of the sample prepared in $H_2{}^{17}O$ exhibits a broad peak with a splitting of 1.5 MHz centered at the ^{17}O Larmor frequency, whereas that of $H_2{}^{16}O$ exhibits a flat line. This finding clearly indicates that the peak around the ^{17}O Larmor frequency arises from the ^{17}O of water, which is weakly coupled to the Cu^{II} site. The ^{17}O hyperfine coupling of the axial water ligand is expected to be significantly smaller than that of the equatorial ligand because the coupling between axial-ligand atoms and the unpaired electron in the $d_{x^2-y^2}$ orbital of Cu is weak;[10,15] thus, the ^{17}O ENDOR signal from the axial water ligand is supposed to be situated close to the ^{17}O Larmor frequency. To estimate the ^{17}O hyperfine coupling of the axially coordinated water, ^{17}O Mims ENDOR measurements were performed on $[Cu^{II}(H_2O)_6]^{2+}$ as a reference sample (Supporting Information, Figure S5). The ^{17}O Mims ENDOR of $[Cu^{II}(H_2O)_6]^{2+}$ shows two doublets centered at the Larmor frequency of ^{17}O with a hyperfine coupling of about 0.6 MHz at near $g_{\|}$. These ENDOR peaks are further split into a quartet with a hyperfine coupling of about 1.6 MHz and resolved quadrupole splittings at near $g_{\perp}$. The magnitude of the hyperfine couplings of ^{17}O in Cu^{II}-Aβ(1–16) is comparable to that for axially bound water to Cu^{II}, although the ENDOR features are less-well-resolved. Thus, we favor assigning this signal to an axially bound water rather than to non-coordinating waters in the vicinity of the Cu^{II} site based on our ^{1}H and ^{17}O ENDOR results. Additionally, no strongly coupled ^{17}O signal was observed, which confirms that there is no equatorially bound water.

To support our spectroscopic results, DFT calculations were performed by comparing two models: one in which an axial water is present and one in which it is absent. The DFT computational study can be exploited to determine which model is energetically favorable and thus can allow a comparison of the stability of the complexes. We started with a Cu^{II} complex model, which has a square-planar geometry. This model contains three nitrogen atoms and one oxygen atom coordinated to Cu^{II}: one nitrogen atom from the NH_2 terminus, two nitrogen atoms from two histidines, and the carbonyl of D1 as an oxygen donor. This model was chosen based on our ENDOR results and because it is one of the most plausible models at physiological pH.[6,7]

The DFT-optimized structure of the active site of Cu-Aβ is shown in Figure 4. An interesting feature of the difference between the two models is that the planarity of the equatorial plane of the complex increases upon the axial binding of water to the Cu site (Supporting Information, Figure S6). Furthermore, a preliminary DFT estimation of the relative

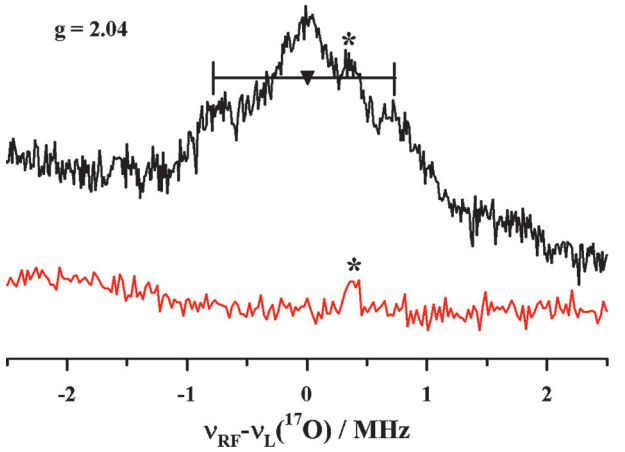

Figure 3. ^{17}O Mims ENDOR spectra of Cu^{II}-Aβ(1–16) in $H_2{}^{17}O$ (black) and $H_2{}^{16}O$ (red). Experimental conditions: microwave frequency 33.8 GHz; $T = 8$ K; $\pi/2$ width 32 ns; $\tau = 400$ ns; RF pulse width 20 μs. * indicates an RF-amplifier harmonic from ^{1}H resonances.

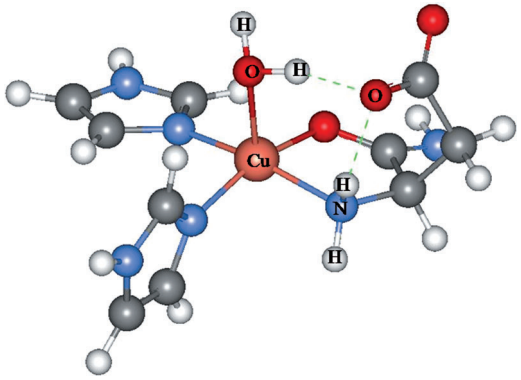

Figure 4. The DFT-optimized structure of the active site of the Cu-Aβ complex.

stability of the two models indicates that the complex with an axial water molecule coordinated to Cu^{II} is more stable by approximately 9.4 kcal mol^{-1}. This result is due to the hydrogen bonding of an axial water ligand with a carboxylate of aspartic acid (D1), as indicated by a dashed line in Figure 4. Thus, the DFT study also gives preference to a water axially bound to Cu^{II}, which is consistent with our ENDOR results.

This model is similar to the prion protein octarepeat region in which a tryptophan residue is hydrogen-bonded to an axially bound water on Cu^{II}.[14,16] As observed in prion proteins, hydrogen bonding may stabilize the native binding site and thereby create stable contacts; thus, such hydrogen bonding interactions may be critical for the copper binding site of copper peptide complexes.

In summary, we employed 34 GHz pulsed ENDOR spectroscopy to characterize the coordination environment of Cu^{II} in amyloid peptide. The ^{1}H ENDOR spectrum revealed the presence of exchangeable protons, which was further confirmed by the ^{2}H ENDOR spectrum. The strongly coupled exchangeable proton was assigned to the proton of the NH_2 terminus of D1, which should be positioned near the equatorial plane based on our ^{1}H ENDOR results. Furthermore, the ^{14}N ENDOR spectrum of Cu-Aβ(1–16) differs markedly from that of its deletion of aspartic acid D1, Cu-Aβ(2–16), which indicates that the nitrogen atom directly coordinated to Cu^{II} is altered. Both ^{1}H/^{2}H ENDOR and ^{14}N ENDOR provide direct evidence that the NH_2 terminus of D1 is an equatorial ligand of Cu^{II}. Furthermore, the ^{17}O ENDOR together with the ^{1}H ENDOR suggest that water is an axial ligand of Cu^{II}. This result may indicate the importance of hydrogen bonding in the stabilization of the Cu^{II}-Aβ coordination geometry. Overall, our 34 GHz ENDOR results provide independent direct spectroscopic evidence of key features that determine the structural basis of Cu-Aβ. This structural basis will serve as a guide in the elucidation of the mechanistic details of amyloid fibrillization at the molecular level and thus provide a framework for the development of therapeutic agents for Alzheimer's disease.

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