Capturing a Reactive State of Amyloid Aggregates

NMR-BASED CHARACTERIZATION OF COPPER-BOUND ALZHEIMER DISEASE AMYLOID β-FIBRILS IN A REDOX CYCLE

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Background: Association of redox-active Cu²⁺ with aggregated Aβ in amyloid plaques has been linked with oxidative stress and oxidative stress in AD.

Results: Cu²⁺/Cu⁺-bound Aβ fibrils undergo a redox cycle reaction with ascorbate and oxygen to produce H₂O₂.

Conclusion: Cu²⁺/Cu⁺ ions bound to histidines of Aβ fibril offer enzyme-like reaction centers.

Significance: The first site-specific structural evidence is presented on Cu⁺-bound Aβ fibrils that generate ROS.

The interaction of redox-active copper ions with misfolded amyloid β (Aβ) is linked to production of reactive oxygen species (ROS), which has been associated with oxidative stress and neuronal damages in Alzheimer disease. Despite intensive studies, it is still not conclusive how the interaction of Cu⁺/Cu²⁺ with Aβ aggregates leads to ROS production even at the in vitro level. In this study, we examined the interaction between Cu⁺/Cu²⁺ and Aβ fibrils by solid-state NMR (SSNMR) and other spectroscopic methods. Our photometric studies confirmed the production of ∼60 μM hydrogen peroxide (H₂O₂) from a solution of 20 μM Cu²⁺-ions in complex with Aβ(1–40) in fibrils ([Cu²⁺]/[Aβ] = 0.4) within 2 h of incubation after addition of biological reducing agent ascorbate at the physiological concentration (∼1 mM). Furthermore, SSNMR ¹H T₁ measurements demonstrated that during ROS production the conversion of paramagnetic Cu²⁺ into diamagnetic Cu⁺ occurs while the reactive Cu⁺ ions remain bound to the amyloid fibrils. The results also suggest that O₂ is required for rapid recycling of Cu⁺ bound to Aβ back to Cu²⁺, which allows for continuous production of H₂O₂. Both ¹³C and ¹⁵N SSNMR results show that Cu⁺ coordinates to Aβ(1–40) fibrils primarily through the side chain Nα of both His-13 and His-14, suggesting major rearrangements from the Cu²⁺ coordination via Nα in the redox cycle. ¹³C SSNMR chemical shift analysis suggests that the overall Aβ conformations are largely unaffected by Cu⁺ binding. These results present crucial site-specific evidence of how the full-length Aβ in amyloid fibrils offers catalytic Cu⁺ centers.

Progressive accumulation of amyloid plaques in the brain is a major pathogenic event that characterizes Alzheimer disease (AD)² (1, 2), which is a multisymptom neural disorder. The primary components of the plaque deposits are amyloid fibrils of 40- and 42-residue amyloid-β (Aβ) peptides (3). The AD plaques are reported to contain high concentrations of redox-active metals such as Cu²⁺ (~0.4 mM) and Fe³⁺ (~1 mM) (4), and Aβ has been shown to bind these metal ions with high affinity (4, 5). Accumulation of the redox-active metal ions by these amyloid aggregates has been proposed to promote formation of reactive oxygen species (ROS), which may lead to oxidative damages implicated as a common and major disruption observed in AD brains (6–10). Indeed, previous in vitro experiments indicated that Cu²⁺ ions in submicromolar concentrations along with Aβ peptide are sufficient for the generation of ROS (11–17). Recent studies demonstrated that the redox activity in AD lesions is inhibited by prior exposure of the tissue sections to copper and iron chelators (18–20).

Oxidative stress caused by ROS is believed to be one of the key events in the pathogenesis of AD (14, 21, 22). Thus, the ROS production through the redox reaction of Cu²⁺-bound Aβ has been the subject of intense efforts while the Cu²⁺-Aβ complex has been a potential therapeutic target (23–33). Several reaction mechanisms have been proposed for the generation of ROS by Cu²⁺-Aβ, yet the exact mechanism remains experimentally unknown (15). A compelling hypothesis from recent studies by mass spectroscopy and other techniques is that the Cu²⁺-ion in complex with Aβ fibrils can be reduced to Cu⁺ by biological reducing agents, such as ascorbic acid, cholesterol, or dopamine (12), and that Cu⁺ may play a vital role in the production of ROS with subsequent oxidation of the His or Tyr residue on Aβ (6, 11, 15, 34–37). It was recently proposed that during the production of ROS, the redox-active Cu²⁺ ions bound to Aβ aggregates undergo a redox cycle (i.e. Cu²⁺ ↔ Cu⁺) in the presence of O₂ and biological reductant ascorbate, of which the reported concentration in the brain is in a range of 0.2–10 mM (16). Alternatively, it has been proposed that the oxidizable sulfur group on the side chain of Met-35 of Aβ may participate in the electron transfer redox reaction with Cu²⁺ to produce ROS such as H₂O₂ (34, 36, 38, 39). Indeed, it has been shown that Aβ isolated from plaques contains oxidized Met-35 (39, 40). However, the redox reaction mechanism and associated structural changes involving copper-bound Aβ aggregates in the production of ROS are not well understood due to lack of structural studies correlating the Cu⁺/Cu²⁺-Aβ complex to the genera-
tion of ROS. There have been intense efforts to study the structures of Cu\(^{2+}\)-bound monomeric and aggregated A\(\beta\) by NMR, EPR, x-ray diffraction, and other methods (28, 31–33, 41–44) to better understand the ROS production mechanism in AD at the molecular level. Despite the implicated importance of the less stable Cu\(^{+}\) state, there are only a handful of structural studies for Cu\(^{+}\)-bound A\(\beta\) (45–48). Recent extended x-ray absorption fine structure spectroscopy and x-ray absorption spectroscopy studies showed that the N-terminal A\(\beta\) fragments A\(\beta\)6–14 and A\(\beta\)1–16 bind Cu\(^{+}\) in a linear two-coordination geometry possibly through two adjacent histidine residues (His-13 and His-14) (46). Additional NMR studies for A\(\beta\)1–16 indicated the involvement of all three histidine residues (His-6, His-13, and His-14) in Cu\(^{+}\) binding via dynamic exchange between these ligands (47, 48). These previous studies, however, focused mainly on short fragments of A\(\beta\) because of the challenges in capturing the transient Cu\(^{+}\) species with amyloid aggregates. Although Cu\(^{+}\) binding was examined for an SDS-stabilized oligomeric A\(\beta\)1–42 species in a recent extended x-ray absorption fine structure spectroscopy study, the analysis merely indicated the co-existence of various coordination motifs (41). Thus, very little structural information is currently available for Cu\(^{+}\) binding of physiologically relevant aggregated full-length A\(\beta\) such as A\(\beta\)1–40 or A\(\beta\)1–42.

In this study, we examined the structures of Cu\(^{+}\)/Cu\(^{2+}\)-bound A\(\beta\) fibrils in relation to its redox chemistry that is responsible for ROS generation. We show that the A\(\beta\)1–40 fibrils bound to Cu\(^{2+}\) produce a major ROS, H\(_2\)O\(_2\), under aerobic conditions in the presence of ascorbate. Redox conversions between Cu\(^{2+}\) to Cu\(^{+}\) are clearly demonstrated by SSNMR, which is an increasingly powerful tool for structural analysis of insoluble protein assemblies (33, 49–61) and protein complexes with metal ions (33, 52, 53, 62–65). SSNMR results also suggest that Cu\(^{+}\) remains in complex with A\(\beta\) fibrils through His-13 and His-14 in the redox cycle. Narrow \(^{15}\)N chemical shift line widths found for Cu\(^{+}\)-bound His-13 and His-14 indicate that Cu\(^{+}\) coordination is likely to make the His residues structurally more ordered in a specific tautomeric state. \(^{13}\)C SSNMR analysis suggests that the overall conformations of A\(\beta\) in the hydrophobic core regions in fibrils are not altered by Cu\(^{+}\) binding or redox reaction. Our results demonstrate the first site-specific structural insights into the origin of ROS in AD through studying Cu\(^{+}\) binding to A\(\beta\)1–40 fibrils and its involvement in the redox cycle and resulting ROS production.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—Wang resin, Fmoc-protected amino acids, and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate were purchased from Peptide International (Louisville, KY). N,N-Diisopropylamidine, N-methylpyrrolidone, and dichloromethane were purchased from Applied Biosystems (Foster City, CA). Piperidine, bathocuproine disulfonic acid disodium salt (BCS), ascobic acid, coumarin-3-carboxylic acid, sodium azide, thioflavin-T, and selectively \(^{13}\)C\(_{2}\)-labeled methionine were obtained from Sigma. Sodium hydroxide, potassium phosphate, and sodium phosphate were acquired from Fisher. D\(_2\)O was purchased from Cambridge Isotope Laboratories (Andover, MA). \(^{13}\)C- and \(^{15}\)N-labeled amino acids were purchased from Isotec/Sigma. H\(_2\)O\(_2\) assay kit (K265-200) was purchased from Biovision (Mountain View, CA).

**Synthesis and Purification of A\(\beta\)(1–40) Peptide**—A\(\beta\)(1–40) peptide (NH\(_2\)-DAEFRHDSGYEVHHQKLFRVFEVGGGIGLWVQ severity was synthesized and purified as reported previously (56). Briefly, A\(\beta\)(1–40) was synthesized using solid-phase peptide synthesis with standard Fmoc synthesis and cleavage protocols (54, 56). The crude peptide was purified by HPLC using acetonitrile and water gradient with 0.1% trifluoroacetic acid. \(^{13}\)C and \(^{15}\)N labeling was introduced as described previously by incorporating Fmoc-protected uniformly \(^{13}\)C- and \(^{15}\)N-labeled amino acids at selected residues. The Fmoc protection of the uniformly \(^{13}\)C- and \(^{15}\)N-labeled amino acids was performed at the University of Illinois at Chicago Research Resource Center using the protocol of Fields et al. (66). The purity of the peptide samples was >95% after HPLC purification, based on MALDI-TOF mass analysis performed at the University of Illinois at Chicago Research Center. The purified samples were stored at -20 °C until use. The labeling schemes used for SSNMR experiments are as follows: (a) Val-12, Ala-21, Gly-33, and Met-35 (e\(^{-}\)CH\(_3\)); (b) His-13, Ala-30, Gly-38, and Val-39; (c) His-14, Ile-32, Val-36, and Gly-37; and (d) Val-18, Phe-19, Gly-29, and Ile-31.

**Preparation of A\(\beta\) Amyloid Fibril**—A solution of 1 mM A\(\beta\)(1–40) was prepared by first dissolving the A\(\beta\)(1–40) peptide in 10 mM NaOH by brief vortexing. Then the peptide solution was diluted to a final concentration of 100 \(\mu\)M with 10 mM phosphate buffer containing 0.02% NaN\(_3\), sonicated for 30 s in an ice bath, and filtered through an Amicon ultracentrifugal filter (molecular mass cutoff at 50 kDa) at 3200 \(\times\) g and -5 °C to remove pre-existing aggregates (55, 56). The final pH of the A\(\beta\)(1–40) solution was 7.4. The concentration of A\(\beta\)(1–40) was determined based on the UV-visible absorbance at \(\lambda = 280\) nm and \(\varepsilon = 1280\) \(\text{m}^{-1}\text{cm}^{-1}\) (56). The A\(\beta\)(1–40) fibrils were prepared by incubating the filtered solution at room temperature with constant agitation for 2 weeks. The fibril formation was monitored by ThT fluorescence assay (55).

**Photometric Quantification of Hydrogen Peroxide**—The quantification of H\(_2\)O\(_2\) was performed per the instructions given in the assay kit. A 48-\(\mu\)l aliquot of 98 or 196 \(\mu\)M A\(\beta\)(1–40) fibrils with or without CuCl\(_2\) (\(f_{\text{Cu/A\(\beta\)}} = 0.4\) or 0.0) was added to each well, where \(f_{\text{Cu/A\(\beta\)}}\) denotes a ratio of copper ion concentration with respect to the A\(\beta\) concentration in a monomeric unit. The peroxide detection reagent mixture of 50 \(\mu\)l (46 \(\mu\)l of assay buffer, 2 \(\mu\)l of OxiRed probe solution, 2 \(\mu\)l of horseradish peroxidase (HRP)) was immediately added to each well. Next, 2 \(\mu\)l of 50 mM ascorbate solution at a neutral pH or H\(_2\)O was added, gently mixed, and incubated at room temperature for 10 min before the first measurement. In the presence of HRP, the OxiRed probe reacts with H\(_2\)O\(_2\) to produce a product that can be detected at \(\lambda_{\text{max}} = 570\) nm. The product was quantified by a Dynex microplate reader. The concentration of H\(_2\)O\(_2\) produced from Cu\(^{2+}\)-A\(\beta\)(1–40) fibrils and ascorbate was determined from the H\(_2\)O\(_2\) calibration curve. For control experiments on Cu\(^{2+}\)-bound monomeric A\(\beta\) or A\(\beta\)-free Cu\(^{2+}\) solution, a 48-\(\mu\)l
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Aliquot of 98 μM Aβ monomer solution with 40 μM CuCl₂ or 40 μM CuCl₂ solution was used. A 10 mM phosphate buffer was used to prepare the solutions at pH 7.4. The subsequent experiments were performed following the protocol described above. Three duplicates were performed for each trial, and the errors were estimated from the standard deviations.

Quantification of Reduced Cu²⁺ by BCS—Cu⁺ ions associated with Aβ fibril were estimated by a photometric assay with BCS, a Cu⁺-specific indicator that forms a Cu⁺-BCS complex, which shows a new absorption at 483 nm. The assay was performed as described previously (67). For analysis of the supernatant, the Aβ aggregate was pelleted by centrifugation at 16,000 × g for 10 min, and the resulting supernatant was analyzed for Aβ aggregates by ThT fluorescence measurements (55). The fibrils were completely removed with less than 1% remaining in solution as estimated by the ThT fluorescence. Then the supernatant was analyzed by BCS assay for the amount of Cu⁺ remaining in solution.

Electron Microscopy—A JEOL JEM-1220 transmission electron microscope at an accelerating voltage of 120 kV was used for the morphological analysis of Aβ(1–40) fibrils with Cu²⁺ and ascorbate. A 10-μl aliquot of sample was spotted on a carbon-coated Formvar 200-mesh copper grid (Electron Microscopy Sciences, Hatfield, PA) for 30 s and dried with a tissue paper. The grid was negatively stained with ~10 μl of 2% uranyl acetate solution for 1 min and again dried with a tissue paper. Finally, the grid was air-dried and analyzed at the University of Illinois at Chicago Research Resource Center.

Solid-state NMR—All SSNMR experiments were performed on Varian Infinity Plus and Bruker Avance III spectrometers with a home-built 2.5-mm MAS triple-resonance probe at 9.4 tesla (¹H frequency of 400.2 MHz). The spinning speed was set to 20,000 ± 3 Hz throughout all experiments. Approximately 2.5–4.0 mg of labeled Aβ(1–40) fibril sample was used in each experiment. ¹³C chemical shifts were referenced to neat tetramethylsilane (TMS) using the secondary reference of adamantane ¹³CH₂ signal at 38.48 ppm. In one-dimensional (1D) ¹³C CP-MAS experiments, adiabatic CP transfer was used. During the CP period, the ¹³C radio frequency (RF) field amplitude was linearly swept from 45 to 65 kHz during a contact time of 1.0 ms, while the ¹H RF amplitude was kept constant at 75 kHz. During the detection period, ¹H (TPPM) decoupling of 90 kHz was employed. ²D CP-MAS experiments were performed following the protocol described above. For each ²D SSNMR measurement, a finite-pulse radio-frequency-driven recoupling (fpRFDR) pulse sequence with a mixing time of 1.6 ms and a ¹³C π/2-pulse width of 15 μs were used (69). After the adiabatic CP and the following t₁ evolution period, a real or imaginary component of the magnetization was stored along the z axis. After the mixing period, the signal was collected in the acquisition period of 10 ms. For each t₁ point, 128 scans of the signals were accumulated. A total of 100 complex t₁ points were recorded with a t₁ increment of 38.1 μs. The assignments of ¹³C shifts for these residues are listed in supplemental Table S1.

Amyloid Fibrils Samples for SSNMR Experiments—For SSNMR experiments, the Aβ(1–40) fibrils were recovered by centrifuging the sample at 3200 × g for 1.5 h (30 min at a time) at −5 °C. The supernatant was removed, and the recovered fibrils were frozen in liquid N₂ and lyophilized. The lyophilized sample was then packed into a 2.5-mm MAS rotor for SSNMR experiments. The powder sample in the rotor was rehydrated by adding the supernatant buffer (2 μl per mg of fibrils) and centrifuging down at 2000 × g for 2 min. The rehydrated sample was incubated overnight at 4 °C before data acquisition.

For the Cu²⁺-Aβ fibril sample, 2 μl of a concentrated CuCl₂ solution (300 mM, pH 7.4) was added to 15 ml of the fibril solution (100 μM in monomer equivalence) to give a final mole ratio of 0.4, and the sample was incubated at 4 °C for 24 h with a brief vortexing to mix the sample. Then the Cu²⁺-bound Aβ fibrils were recovered by centrifugation at 3200 × g for 1.5 h (30 min at a time) at −5 °C. The supernatant was discarded, and the resulting fibril pellet was frozen in liquid N₂, lyophilized, and packed into a 2.5-mm MAS rotor for SSNMR analysis and rehydrated as mentioned previously.

For the preparation of the Cu²⁺-Aβ fibril sample incubated with ascorbate, an ascorbate solution at 340 mM (0.7 μl per mg of fibrils) was added to a 2.5-mm MAS rotor packed with Cu²⁺-Aβ(1–40) fibrils by centrifuging at 6000 × g for 15 min. To avoid pH-induced chemical shift changes in the sample, the ascorbic acid solution was neutralized in equimolar NaOH before introducing into the rotor. The sample was then used for SSNMR experiments. For the 1D and ²D experiments in Figs. 5, 7, and 8, the mole ratio of Aβ fibrils to ascorbate is fₕₐₜ/Aβ = 1. The sample temperature was set to 12 °C during the SSNMR experiments. For the ¹H T₁ measurements that require sample exposure to O₂ in the air in Fig. 3, the SSNMR rotor containing the Cu²⁺-Aβ fibrils and ascorbate with the rotor cap removed was placed in a centrifuge tube with a tiny hole on the cap and incubated at room temperature for ~14 h with gentle shaking at 100 rpm on a New Brunswick Scientific Excella E24 incubator shaker. In subsequent ¹H T₁ SSNMR measurements,
the temperature was set to 24 °C. The sample was exposed to air three times.

Solution NMR Experiments for Monomeric Aβ—The 1H solution NMR spectra in Fig. 10 were collected on a Bruker Avance 900-MHz spectrometer equipped with a cryoprobe at the Center of Structural Biology, University of Illinois at Chicago. The sample temperature was set to 10 °C in all the experiments. Three samples were prepared for the regular 1H and 1H T1 NMR measurements for (a) Aβ(1–40) monomers, (b) Cu2+-Aβ(1–40), and (c) Cu2+-Aβ(1–40) with ascorbate. Aβ(1–40) monomer solution at ~90 μM and pH 7.4 was incubated (a) without and (b) with CuCl2 for 15 min. The ascorbate was then added to the mixture and incubated for another 15 min for the data in Cu2+-Aβ(1–40) with ascorbate. The molar ratios were set to fCu/Aβ = 0.4 and fasc/Asc = 1. 1H NMR and 1H T1 measurements were performed for the all the samples. 1H T1 measurements were performed to confirm the formation of Cu2+ from Cu2+ ions. If excess Cu2+ ions are present, nonspecific interactions between Cu2+ and Aβ may result in an extensive loss of backbone amide NH signals because the paramagnetic Cu2+ center can significantly enhance transverse/longitudinal relaxation of 1H signals out to 10 Å. To minimize the excessive relaxation effect through such nonspecific interactions, we herein prepared the sample so that [Aβ(1–40)]/[Cu2+] = 1: 0.4 in a molar ratio.

RESULTS

ROS Production by Cu2+-bound Aβ Fibril—The production of ROS, such as H2O2 from Cu2+-Aβ complexes, was observed when one or more physiological reducing agents are available (11, 12). However, only a limited number of previous studies have reported the interaction between Cu2+ and Aβ fibrils, and their proposed roles in the ROS production are somewhat contradictory (15, 16). Thus, we first tested the generation of H2O2 from the Cu2+-Aβ(1–40) fibril complex in the presence of reducing agent ascorbate. Although our group and others have reported the presence of more cytotoxic diffusible aggregates for Aβ (3, 55, 56, 70–72), we selected Aβ(1–40) fibrils as our initial target because they are more stable and better characterized. In Fig. 1, we monitored the H2O2 concentration by incubating a Cu2+-bound Aβ(1–40) fibril sample (~50 μM Aβ and 20 μM Cu2+ ions) in the presence of 1 mM ascorbate (green bars) and in the absence of ascorbate (red bars) by photometric assay (see under “Materials and Methods”). The data clearly demonstrated the production of ~40 μM H2O2 within an hour under these conditions. However, in the absence of ascorbate, no detectable H2O2 was produced. We previously confirmed 0.4 mol eq of Cu2+ ions are strongly bound to Aβ(1–40) in fibrils (33). Thus, there is no effect from excess or unbound free Cu2+ in the production of H2O2. The ascorbate concentration at 1 mM was chosen based on the estimated physiological ascorbate concentration of 0.2–10 mM in the brain (16, 73). The concentration of Cu2+ is 1 order of magnitude lower than the copper ion concentration found in the AD plaques (0.4 mM) (4). Nevertheless, a submillimolar level of H2O2 was generated within several hours from the Cu2+-Aβ(1–40) fibrils/ascorbate solution. We also confirmed the generation of another destructive ROS, hydroxyl radicals (data not shown). Although it was found that H2O2 is more efficiently generated by an equivalent concentration of free Cu2+ ions without Aβ (78 ± 6 μM at 1 h incubation) or by Cu2+-bound Aβ monomer (78 ± 3 μM at 1 h) in the presence of 1 mM ascorbate, in the physiological environment a high concentration of free Cu2+ ions or monomeric Aβ is not likely to be present. Less efficient H2O2 productions by Cu2+-bound Aβ fibril may be attributed to limited accessibility of ascorbate due to bulky Aβ fibrils. We also confirmed that doubling the Cu2+ and Aβ fibril concentrations ([Aβ] ~100 μM and [Cu2+] = 40 μM) proportionally increased the H2O2 production rate (85 ± 1 μM at 1 h). Thus, it is unquestionable that Cu2+-bound Aβ fibrils are promoting the ROS production under the near-physiological condition, which mimics the conditions for AD with plaque and copper ion accumulation. Taken together, the results have demonstrated H2O2 generation from the Cu2+-bound Aβ aggregates, establishing relevance of this system used for the present structural studies with the ROS production, which was reported in AD.

In Situ NMR Detection of Redox Cycling for Cu+/Cu2+-bound Aβ(1–40) Fibrils—Mechanistically, the existence of redox cycling between Cu2+ and Cu+ is strong evidence for the conversion of molecular oxygen into H2O2 (11, 12, 16, 74, 75). Hence, we next examined the possibility of detecting the redox state of copper ions after addition of ascorbate by SSNMR. In general, it is difficult to examine the redox state of a metal ion when the ion is bound to an insoluble protein such as amyloid fibrils. SSNMR is one of very few methodologies that provide access to detailed molecular structures of amyloid fibrils (76). In addition, NMR relaxation parameters are very sensitive probes of paramagnetic ions such as Cu2+ (77). By utilizing paramagnetic T1 and T2 relaxation enhancements by Cu2+ in 1H and 13C SSNMR, we recently reported that Aβ(1–40) fibrils bind Cu2+ via the side chains of amino acid residues His-13, His-14, Glu-3/11, and CO2 terminus of Val-40 (33). In contrast

![FIGURE 1. Incubation time dependence of H2O2 concentration produced by Aβ(1–40) fibrils alone (blue), Aβ(1–40) fibrils with 20 μM Cu2+ (red), and Aβ(1–40) fibrils with 20 μM Cu2+ in the presence of 1 mM ascorbate (green). The concentration of Aβ fibrils is 47 μM (based on monomer concentration). With these concentrations of Cu2+ and ascorbate available, Aβ(1–40) fibrils can efficiently catalyze the production of ~100 μM H2O2 within the first 4 h of the incubation (green). In the absence of reducing agent ascorbate, both Aβ fibrils with and without Cu2+ generated only trace amounts of H2O2 (blue and red). The H2O2 assay was performed by a photometric method in which the H2O2 generated reacts with horseradish peroxidase (HRP) in the presence of OxRed probe to produce a product with absorption at 570 nm.](image)
to Cu^{2+}, diamagnetic Cu^+ ions yield no paramagnetic relaxation enhancements. Thus, the redox reaction from Cu^{2+} to Cu^+ can be monitored by examining the NMR relaxation parameters (62).

Fig. 2 shows the incubation time dependence of $^{1H}$ longitudinal relaxation time ($T_1$) for Cu^{2+}-bound Aβ(1–40) fibrils. The ascorbate concentration was 1 mol eq with respect to Aβ. An overall paramagnetic relaxation effect was determined by averaging the observed $^{1H}$ $T_1$ values for several $^{13C}$ CP-MAS signals over the spectral region.

The initial $^{1H}$ $T_1$ values showed an $\sim$6-fold increase to 576 ± 13 ms after the incubation time ($t$) of 1.5 h (Fig. 2A). The $T_1$ value at $t = 1.5$ h is comparable with that of Cu^{2+}-free Aβ fibrils. Thus, the data indicate that paramagnetic Cu^{2+} bound to amyloid fibrils is completely reduced to diamagnetic Cu^+ by ascorbate. Interestingly, after 70 h the $T_1$ value gradually decreased to 60% of the highest value (Fig. 2B). It is likely that Cu^+ is oxidized back to the more stable Cu^{2+} when the concentration of ascorbate is gradually decreased. However, we found difficulties in quantitatively reproducing the re-oxidation reaction, and the incubation time required for the $^{1H}$ $T_1$ decrease varied considerably from experiment to experiment.

After realizing the possibility that this re-oxidation reaction of Cu^{2+} may require oxygen from air, we performed a modified experiment shown in Fig. 3 with Aβ(1–40) isotope labeled at Val-18, Phe-19, Gly-29, and Ile-31. First, to speed up the redox reaction, we used 5 mol eq of ascorbate to Aβ; the observed $^{1H}$ $T_1$ rapidly increased from 83 ± 3 to 577 ± 29 ms in 2 h. Second, in the modified experiment, after observing complete conversion of Cu^{2+} to Cu^+ that is reflected to the $^{1H}$ $T_1$ value, we made oxygen accessible to the sample by halting the $^{1H}$ $T_1$ measurement and opening the cap of the MAS rotor containing the Aβ sample. After overnight incubation at 24°C (Fig. 3, *orange arrows*), we resumed the $^{1H}$ $T_1$ measurements. This process was repeated three successive times. As indicated by the *orange arrow* in Fig. 3 at 5–13 h, the $^{1H}$ $T_1$ dropped considerably to 364 ± 23 ms during the first incubation with oxygen; the shorter $T_1$ value by enhanced paramagnetic relaxation suggests that Cu^+ was reoxidized to Cu^{2+} in the presence of oxygen. At this point, it is likely that the excess ascorbate prevented the complete oxidation of Cu^+ ions. Nevertheless, the $^{1H}$ $T_1$ value in Fig. 3 decreased much faster than the $T_1$ value for Fig. 2, which was measured for the sample without the exposure to air. Subsequent cycles of $^{1H}$ $T_1$ monitoring and exposure to air confirmed the rapid redox recycling between Cu^{2+} and Cu^+ in association with the amyloid fibrils. As $^{1H}$ $T_1$ of the amyloid fibrils is affected only when Cu^{2+} is bound to Aβ, the data clearly suggest that Cu^{2+} is bound to Aβ throughout the redox cycle. We also confirmed the reduction from Cu^{2+} to Cu^+ at a physiological ascorbate concentration (1 μM) by a photometric assay using a Cu^+-selective indicator (Fig. 4). For a solution containing a suspended Cu^{2+}-Aβ fibril complex ($f_{Cu/AB} = 0.4$) with an Aβ concentration of 100 μM (in monomer equivalence), it took only 15 min for the Cu^{2+} ions bound to Aβ to be nearly completely reduced to Cu^+ (~98%) (Fig. 4, *red*). The supernatant of the solution after centrifugation at 16,000 × g for 10 min nearly completely removed Cu^{2+} from the solution (Fig. 4, *green*). This suggests that Cu^+ ions are still bound to amyloid fibrils, which were pelleted down by centrifugation. Although previous studies implied Cu^+ association with Aβ, these studies used either fragments of Aβ or Aβ in monomeric forms rather than Aβ aggregates (45, 46, 67, 79). Results from our SSNMR and photometric studies clearly demonstrate that Cu^{2+} and Cu^+ ions undergo redox cycling while bound to Aβ fibrils of full-length Aβ in the presence of ascorbate and oxygen for the first time. This also allows us to identify the Cu^{2+}-bound Aβ fibrils as more reactive amyloid aggregates, which are most likely to be responsible for the ROS production. As will be discussed further below, our SSNMR approach offers a new avenue for in situ detection of binding modes for Cu^{2+} as well as Cu^{2+} ions with the aggregated proteins in the redox cycle.
Redox Reaction and Structural Features of Cu\(^{2+}\)-bound A\(\beta\) Fibrils Probed by \(^{13}\)C SSNMR—Next, we carried out \(^{13}\)C SSNMR experiments to reveal the molecular level structural details and dynamics of Cu\(^{2+}\)-A\(\beta\)(1–40) fibrils during the redox cycle. Fig. 5, A and B, shows \(^{13}\)C CP-MAS spectra of Cu\(^{2+}\)-free and Cu\(^{2+}\)-bound A\(\beta\)(1–40) fibrils prepared with uniform \(^{13}\)C- and \(^{15}\)N-labeled amino acids at Val-12, Ala-21, Gly-33, and with selectively \(^{13}\)C-labeled Met-35 (at the e\(\text{CH}_3\) position). Compared with the spectrum of the Cu\(^{2+}\)-free sample in Fig. 5A, Fig. 5B shows selective loss in signal intensities of \(^{13}\)C\(_{\alpha}\), \(^{13}\)C\(_{\beta}\) and \(^{13}\)C\(_{\gamma}\) of Val-12 (blue arrows), whereas the chemical shifts or signal intensities of Ala-21 and Gly-33 are unaffected (33). The corresponding \(^1H\ T_1\) value of Cu\(^{2+}\)-bound A\(\beta\)(1–40) fibrils is \(55 \pm 3\) ms, which is much shorter than that of A\(\beta\)(1–40) fibrils without Cu\(^{2+}\) (370 \(\pm 18\) ms). Selective quenching of Val-12 signals (Fig. 5B) and \(^1H\ T_1\) reduction are consistent with Cu\(^{2+}\) binding at the side chains of His-13, His-14, and other residues of A\(\beta\) in fibrils (33). We then incubated the Cu\(^{2+}\)-A\(\beta\) fibrils with 1 mol eq of ascorbate to A\(\beta\) at room temperature. After \(t = 1.5\) h, we collected a \(^{13}\)C CP-MAS spectrum for this sample (Fig. 5C). Clearly, the \(^{13}\)C signals of Val-12 reduce to the original intensities (~95%) of A\(\beta\)(1–40) fibrils without Cu\(^{2+}\) (Fig. 5C, red arrows). We attribute this to reduction of paramagnetic Cu\(^{2+}\) bound to A\(\beta\) to diamagnetic Cu\(^{+}\). The \(^1H\ T_1\) value at a 1.5-h incubation with ascorbate (351 \(\pm 14\) ms) is also consistent with reduction of Cu\(^{2+}\) to Cu\(^{+}\). Based on the assumption that paramagnetic relaxation enhancement in \(^1H\ T_1\) is proportional to Cu\(^{2+}\) concentration, then ~95% of Cu\(^{2+}\) was converted to Cu\(^{+}\). Although it is possible to explain this observation by the removal of Cu\(^{2+}\) from A\(\beta\) due to chelation by ascorbate, the \(^{13}\)C SSNMR data suggest that this is not the case as will be discussed below. The NMR data suggest that copper ions are subjected to redox cycling between Cu\(^{2+}\) and Cu\(^{+}\) while remaining coordinated to the A\(\beta\) fibrils.

In addition to biological reductants such as ascorbate, the Met-35 residue of A\(\beta\) has been proposed to play a significant role in the redox chemistry of copper ions in this system as well (34). The methyl-\(^{13}\)C\(_{\epsilon}\) of Met-35 has been assigned to a signal at
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FIGURE 6. Transmission electron microscopy images of Aβ(1–40) fibrils (100 μM monomer concentration) (A); Aβ(1–40) fibrils incubated 40 μM Cu²⁺ for 24 h at 4 °C (B), and Cu²⁺-Aβ(1–40) fibrils incubated with 1 mM ascorbate for 24 h at room temperature (C). The scale bar represents 100 nm in length. Both Cu²⁺ binding and redox reaction upon addition of ascorbate do not alter the overall morphology of the Aβ fibrils, which showed diameters of ~10 nm and typical lengths of 1 μm or greater.

~15 ppm (33), but the signal overlapping with methyl-13C signals of Val-12 and Ala-21 (Fig. 5, A – C) does not allow for unambiguous determination of the paramagnetic influence from Cu²⁺. A minor peak at 40 ppm (indicated by # in Fig. 5) has also been assigned to the methyl-13C of oxidized methionine (80). The fact that the intensity of this signal at 40 ppm did not change for Cu²⁺-bound fibrils suggests that no additional oxidation of Met-35 had occurred during the redox cycle (Fig. 5C). This small population of oxidized methionine probably resulted from the initial dissolution of Aβ peptide in a dilute sodium hydroxide solution (10 mM) or during the incubation period (34, 80). A control experiment revealed significant oxidation of L-methionine after incubation with Cu²⁺ and ascorbate (data not shown) (81). This indicates that the unique structural arrangements of Aβ fibrils may limit solvent accessibility of the Met-35 side chain (33) in the aggregated Aβ sample, thus preventing its involvement in the redox reaction.

Interestingly, the 13C shifts of Val-12, Ala-21, Ala-30, Ile-32, Gly-33, Val-36, Gly-37, Gly-38, and Val-39 for the Cu²⁺-bound Aβ fibrils are essentially unaffected by Cu²⁺ binding (see Fig. 5, A and C, and supplemental Table S1). 13C chemical shifts are sensitive probes to conformational changes, and the results suggest that the overall Aβ structures in fibrils are largely unaffected by the reduction of Cu²⁺ to Cu⁺. Further analysis of these samples by 2D 13C/13C fpRFDR (69) experiment showed well resolved, nearly identical chemical shift cross-peaks (±0.2 ppm) (Fig. 5, D – F). Transmission electron microscopy indicated no morphological differences among these samples (Fig. 6). The lack of change in chemical shifts with indistinguishable transmission electron microscopy images suggests no significant alterations or degradation to the overall structure in the hydrophobic core of the fibril, although the local binding modes and conformations of the residues coordinated to ions may show structural differences. The results prove that amyloid fibrils of Aβ offer a robust architecture for the ROS production, which not only accumulates copper ions but also promotes copper-based reactions in a catalytic manner.

Histidine Coordination to Cu⁺ in Aβ(1–40) Fibrils Probed by 1D 15N and 2D 13C/15N SSNMR—To better understand the redox chemistry of copper-bound Aβ fibrils, we investigate the coordination modality of Cu⁺ on Aβ fibrils by SSNMR with focus on the coordination to His-13 and His-14 residues. As mentioned earlier, we reported that Aβ(1–40) fibrils bind Cu²⁺ via the side chains of His-13, His-14, and the carboxyl terminus of Val-40 and carboxyl side chains of Glu, based on SSNMR and molecular dynamic simulation studies (33). Previous NMR studies using a short Aβ fragment Aβ(1–16) have proposed Cu⁺ binding at the histidine residues (i.e. His-6, His-13, and His-14) in the N-terminal region of Aβ (47, 48). However, no studies have demonstrated the interactions between Cu⁺ and Aβ fibrils. Herein, we conducted SSNMR analyses of copper-bound and -free Aβ(1–40) fibrils that are 13C/15N-enriched for His-13 and His-14. His-6 was not included in the analysis because the N-terminal region around His-6 of Aβ(1–40) fibrils typically does not yield strong SSNMR signals presumably due to structural dynamics. Table 1 summarizes our chemical shift assignments for His-13 and His-14 from this study in comparison with those for published 13C and 15N shifts for histidine residues in various tautomeric states and Cu⁺ and Zn²⁺ coordination states. We explain the interactions between Cu⁺ and Aβ suggested from the data below.

The imidazole side chain of histidine has two potential copper-binding sites, Nδ and Nε. To determine which nitrogen is coordinated to Cu⁺, we performed the 1D 15N CP-MAS (Fig. 7) and 2D 13C/15N correlation experiments (Fig. 8) on the same set of copper-free (A and D), Cu²⁺-bound (B and E), and Cu⁺-bound 13C- and 15N-labeled (C and F) Aβ fibrils for His-13 (A – C) and His-14 (D – F). The 15N chemical shifts of His side chains are highly dependent on the tautomeric state of the imidazole ring, which in turn depends on the pH of the sample (62, 82, 83). At the neutral pH used in this study, the deprotonated 15Nδ in the τ-tautomer and deprotonated 15Nε in the π-tautomer both resonate at ~250 ppm, whereas protonated species 15NδH and 15NεH resonate at 160–190 ppm (see Table 1, b) (82, 84). For copper-free Aβ(1–40) fibrils (Fig. 7, A and D), the 15N CP-MAS spectra show signals for His-13 at 228 and 172 ppm (Fig. 7A) and those for His-14 at 250 and 164 ppm (Fig. 7D) (82, 83). Based on the 2D 13C/15N correlation spectra (Fig. 8, A and D), the 15N signals at 172 and 228 ppm for the His-13 sample are assigned to 15NδH and 15Nε, respectively, whereas the signals at 164 and 250 ppm for His-14 are to 15NδH and 15Nε, respectively. The 13C chemical shifts of the imidazole ring
are assigned by 2D $^{13}$C/$^{15}$N correlation spectra (Fig. 9, A and D). The lower signal intensity for $^{15}$N is attributed to a lower CP efficiency of the nonprotonated species. Additional minor $^{15}$N peaks are observed at 178 and 198 ppm for the His-14 sample (* in Fig. 7D), which are assigned, respectively, to $^{15}$N-H and $^{15}$N-H for a bi-protonated His species as discussed below. The data indicate heterogeneous coordination environments for His-14 and possibly reflect hydrogen bonding from a neighboring residue.

Upon Cu$^{2+}$ binding, the $^{13}$C signals for both labeled samples at ~164/172 ppm in the $^{15}$N CP-MAS are quenched by ~40%, whereas the $^{13}$C signals at ~228/250 ppm are unaffected (Fig. 7, B and E); this observation suggests that Cu$^{2+}$ is coordinated to $^{15}$N-H for both His-13 and His-14 (33). When Cu$^{2+}$ is reduced to Cu$^{+}$ by ascorbate, the $^{13}$C signals at ~170 ppm are fully recovered, and new peaks at ~210 ppm emerge (Fig. 7, C and F), which we assigned to Cu$^{+}$-bound $^{15}$N-H based on correlations to $C_\epsilon$ and $C_\gamma$ of the imidazole ring.

In the 2D $^{13}$C/$^{15}$N correlation spectra of copper-free Aβ fibrils (Fig. 8, A and D), the $^{13}$C signal at 171.5 ppm for His-13 shows strong cross-peaks with directly connected $C_\alpha$ and $C_\epsilon$, whereas the $^{15}$N signal at 227.6 ppm displays cross-peaks only to $C_\gamma$ and $C_\epsilon$. Thus, $^{13}$C (171.5 ppm) and $^{15}$N (227.6 ppm) are unambiguously assigned for His-13. Similarly, for His-14, we

| TABLE 1 |
| --- |
| Comparison of $^{13}$C and $^{15}$N chemical shifts observed for copper-free and Cu$^{2+}$-bound His-13 and His-14 side chains of Aβ(1–40) fibrils herein with published data on $^{13}$C, $^{15}$N chemical shifts of Cu$^{2+}$- and Zn$^{2+}$-bound histidine residues in proteins and copper-free histidine in different tautomeric states. |

Part f shows the difference of $^{13}$C and $^{15}$N shifts. The red number denotes the difference that is greater than 17 ppm.

| Residues | Fraction | $^{13}$C (ppm) | $^{15}$N (ppm) | Difference ($^{13}$C–$^{15}$N) (ppm) |
| --- | --- | --- | --- | --- |
| His-13 (Cu$^{2+}$-bound) | N=H | 167 | 171 | 4.0 |
| His-14 (Cu$^{2+}$-bound) | N=H | 169 | 171 | 2.0 |

* Data not known.

** FIGURE 8.** 2D $^{13}$C/$^{15}$N chemical shift correlation spectra of Aβ(1–40) fibrils that are $^{13}$C- and $^{15}$N-labeled at His-13 (A–C) and His-14 (D–F) alone (A and D), with Cu$^{2+}$ (B and E), and with Cu$^{+}$ (C and F) with signal assignments for major tautomeric forms of His side chains. The cross-peaks corresponding to minor tautomeric forms of $N_\delta$ and $N_\epsilon$ are shown in blue dashed circles. The disappearance of the $N_\delta$ cross-peaks for the Cu$^{2+}$-bound Aβ fibrils is due to the fact that the $^{13}$N polarization is transferred to the paramagnetically quenched $^{13}$C signals (B and E). The appearance of a new signal in Cα $\omega_1$, $\omega_2$ ~212, 136 ppm and in Fα $\omega_3$, $\omega_4$ ~208, 136 ppm after the incubation with ascorbate indicates Cu$^{+}$ binding to the $N_\delta$ (based on correlations to $C_\epsilon$ and $C_\gamma$) of the imidazole ring.
FIGURE 9. Comparison of 2D $^{13}$C/$^{13}$C fpRFDR (top and middle panels) and 2D $^{13}$C/$^{15}$N spectra (bottom panel) of His-13 (A–C) and His-14 (D–F) side chains of 100 μM Aβ(1–40) fibrils alone (A and D), Aβ fibril sample with 0.4 mole equivalent of Cu$^{2+}$ (B and E), and Aβ fibril sample with both Cu$^{2+}$ and 1 mole equivalent of ascorbate (C and F). The assignments for C$_p$–C$_γ$ and C$_γ$–C$_γ$ correlations are shown along the dashed lines for Aβ(1–40) fibrils alone and Cu$^{2+}$–Aβ(1–40) fibrils in A, D, and C, F. For His-14 in D, the assignments for both $γ$-tautomer (orange dashed line) and biprotonated form (blue dashed line) are shown with color-coded arrows. The cross-peaks corresponding to other minor tautomeric forms of N$_γ$ and N$_ε$ are shown in blue dashed circles. The disappearance of the N$_γ$ cross-peaks for the Cu$^{2+}$-bound Aβ fibrils is due to the fact that the $^{15}$N polarization is transferred to the paramagnetically quenched $^{13}$C signals (B and E). Binding of Cu$^{2+}$ at N$_ε$ of the imidazole ring is suggested by the appearance of new signals correlating $^{15}$N$_ε$ to $^{13}$C$_γ$, at ($ω_1$, $ω_2$) = (212 and 136 ppm) in C and at (208 and 139 ppm) in F after incubation with ascorbate. The 2D $^{13}$C/$^{13}$C correlation data were collected by the fpRFDR sequence (69) as discussed in Fig. 5, D–F. The 2D $^{15}$N/$^{13}$C correlation data are reproduced from Fig. 8 for comparison.
could identify correlations of $^{15}$N$_H$ at $\sim$164 ppm with $^{13}$C$_e$ at 136 ppm and $^{13}$C$_b$ at 114 ppm as well as the correlation of $^{15}$N$_b$ at $\sim$250 ppm with $^{13}$C$_c$ at 136 ppm. The spectrum for His-14 in Fig. 8D shows another sets of cross-peaks (blue dashed circles) involving $^{13}$N signals indicated by the asterisks in Fig. 7D. These signals are attributed to the bipolement form (82, 83) of His-14 based on the $^{13}$C and $^{15}$N chemical shifts (see also Fig. 9).

Most of these resonances are quenched by Cu$^{2+}$ binding to His-13 and His-14 to Aβ(1–40) fibrils and are in agreement with previous studies (Fig. 8, B and E) (33). Bi-protonated species for His-14 is negligible in Fig. 8E and suggests the replacement of a side chain NH proton with Cu$^{2+}$.

Next, we examine the 2D $^{15}$N/$^{13}$C correlation spectra of Cu$^{+}$-bound Aβ fibrils following incubation with ascorbate (Fig. 8, C and F). In Fig. 8C, the spectrum for His-13 shows a notable cross-peak connecting the $^{15}$N$_b$ resonance at 212 ppm to $^{13}$C$_b$ at 136 ppm (red dotted square), whereas the $^{15}$N$_b$ signal at 170 ppm correlates to $^{13}$C$_b$ at 116 ppm and $^{13}$C$_c$ at 137 ppm. The $^{15}$N$_c$ resonance at 212 ppm is distinctively shifted from $\sim$228 ppm and most likely assigned to N$_b$ bound to Cu$^{2+}$ (N$_b$-Cu$^{2+}$; see Table 1). As the pH was adjusted carefully (see under “Materials and Methods”), the observed spectral changes are not attributed to the pH change. An additional minor cross-peak (blue circle) can be assigned to $^{15}$N$_b$H/$^{13}$C$_b$ or $^{15}$N$_b$H/$^{13}$C$_c$ when Cu$^{+}$ is coordinated to N$_b$. Comparable shifts have been observed for His-50 and His-128 of reduced copper-zinc superoxide dismutase (Table 1, b). We also observe similar patterns for His-14 to those above for His-13. The spectrum in Fig. 8F indicates correlations of $^{15}$N$_b$-Cu$^{+}$ at 208 ppm to $^{13}$C$_c$ at 139 ppm and $^{13}$C$_e$ at 136 ppm (red dotted square) for His-14. There are also correlations of $^{15}$N$_H$ at 169 ppm to $^{13}$C$_b$ at 115 ppm and $^{13}$C$_c$ at 136 ppm. The $^{15}$N$_b$-Cu$^{+}$ signals for His-14 sample show distinctively narrower peaks at 208 ppm unlike the broader multiple resonances observed for the copper-free sample in Fig. 8D. Similarly, the $^{13}$C line widths for the His-13 and His-14 residues in the 2D $^{13}$C/$^{13}$C spectra (Fig. 9, C and F) are narrower for the Cu$^{+}$-bound form than those for the copper-free form. These results suggest that Cu$^{+}$ coordination to Aβ fibrils is likely to make the His residues more structurally ordered. The result shows an interesting contrast with $^{1}$H solution state NMR data for mononmeric Cu$^{2+}$-bound Aβ(1–40), which display exchange broadening (Fig. 10) probably due to the transient nature of Cu$^{+}$ binding to monomeric Aβ.

The observed $^{15}$N chemical shifts of $\sim$210 ppm for $^{15}$N$_b$-Cu$^{+}$ are comparable with the reported $^{15}$N$_b$ shift of 220–227 ppm for His-82 and His-125 in ba3 oxidase subunit II, for which N$_b$ sites of the histidine residues are coordinated to Cu$^{2+}$ (see Table 1, c) (85, 86). Furthermore, the $^{13}$C$_b$ chemical shift is known to be very sensitive to the protonation or Cu$^{2+}$-coordination state of the neighboring N$_b$. The $^{13}$C$_b$ shifts of His for the protonated and nonprotonated N$_b$ typically show $\sim$115 and $\sim$125 ppm, respectively (83, 84). The $^{13}$C$_b$ shifts of Cu$^{2+}$-Aβ(1–40) fibrils observed at $\sim$115 ppm (Fig. 9, C and F) indicate protonated N$_b$, which is consistent with Cu$^{2+}$ coordination to N$_b$, rather than N$_c$ unlike Cu$^{2+}$. A recent statistical analysis of the $^{13}$C$_b$ and $^{13}$C$_c$ chemical shifts of histidine coordinated to Zn$^{2+}$ (84) predicted that when the chemical shift difference between $^{13}$C$_b$ and $^{13}$C$_c$ ($\Delta_{bc}$) is $>17$ ppm, Zn$^{2+}$ coordination is likely to occur via the N$_b$ (84). This empirical relationship of $^{13}$C chemical shift data is also applicable to His coordinated to Cu$^{2+}$ for ba3 oxidase subunit II and superoxide dismutase (see Table 1) (85–87). Accordingly, the $\Delta_{bc}$ values of 21–22 ppm observed for His-13 and His-14 of Cu$^{2+}$-bound Aβ(1–40) fibrils (Table 1) suggest that Cu$^{2+}$ is most likely coordinated to the $^{15}$N$_b$ of His. Thus, the results indicate significant rearrangements of the copper-coordination structure through the redox conversion between Cu$^{2+}$-Aβ and Cu$^{2+}$-Aβ. Weak cross-peaks observed at $\omega_N = \sim$170 ppm and $\omega_C = \sim$125 ppm in Fig. 8 (C and F) suggest the presence of minor species for which Cu$^{2+}$ may be coordinated to N$_c$. Such heterogeneous coordination modes have also been indicated in our previous study on the Cu$^{2+}$-Aβ fibril complex, although additional data are needed to confirm assignments for the minor species. Taken together, the SSNMR results provide strong evidence that Cu$^{2+}$ ions preferentially coordinate to Aβ fibrils via the N$_b$ of both His-13 and His-14 during the course of Cu$^{2+}$/Cu$^{2+}$ redox cycling, which has been associated with the generation of ROS in AD.

**DISCUSSION**

Redox-active transition metals such as Cu$^{2+}$ and Fe$^{3+}$ have been implicated in the cause of extensive oxidative damages observed in the brains of AD patients (7, 9, 12, 14, 88). Researchers have exhaustively studied the interactions between these metal ions and AD-associated Aβ peptides (6, 7, 9, 11, 12, 31, 52, 88–90). Nonetheless, given that these metal ions are isolated together with aggregated plaques/fibrils of Aβ, a critical question remains unanswered. What is the role of these

**FIGURE 10.** $^{1}$H solution-state NMR spectra (aromatic region) of 92 μM Aβ(1–40) monomer (A), with 37 μM Cu$^{2+}$ (B), and with both Cu$^{2+}$ and 1 mole equivalent of ascorbate (C). The aromatic side chain $^{1}$H signals of His (asterisks in A) and Tyr (A) residues of Aβ(1–40) peptide are assigned based on a previous report (30). As expected, the Cu$^{2+}$ ions broaden signals and in some peaks beyond detection in B. The amino acid residues affected by Cu$^{2+}$ include Phe, His, and Tyr. As reported previously by Hou and Zagorski (30), it is likely that Cu$^{2+}$ is bound to Aβ via the side chains of histidine (i.e. at His-6, His-13, and His-14) and some of the acidic amino acid residues on the N terminus. Reduction of Cu$^{2+}$ to Cu$^{+}$ by ascorbate recovers a majority of the signals, but some remained broadened, especially the side chain protons of His residues, indicating the interactions between Cu$^{+}$ and histidine amino acids of the Aβ(1–40) peptide. The broadening is likely to be attributed to exchange broadening due to transient Cu$^{2+}$ binding. These results suggest Aβ binding of Cu$^{2+}$ at the histidine residues. Thus, the histidine residues of monomeric Aβ(1–40) are involved in binding of both Cu$^{2+}$ and Cu$^{+}$.
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Metal ions complexed to aggregated Aβ fibrils in regard to the oxidative stress in AD? In this study, we have shown the ability of Cu²⁺/Cu²⁺ to form complexes with Aβ(1–40) fibrils and generate harmful ROS such as H₂O₂ and hydroxyl radicals as monitored by photometric assay and SSNMR. This study clearly illustrates the unique redox properties and structure of Cu²⁺-bound Aβ fibrils, which are likely to have relevance to the pathogenesis of AD. Quantitative analysis based on photometric assay suggests generation of submillimolar concentrations of H₂O₂ by a 50-µM Aβ(1–40) fibril sample with a 0.4 mol eq of Cu²⁺ in the presence of ascorbate. It was reported that the exposure to 50–250 µM H₂O₂ for 24 h terminated ~50% of SH-SY5Y cells, which are a model cell system widely used for studying neural cell death (91, 92). Furthermore, by utilizing various SSNMR experiments, we have shown that the electron transfer between Cu, ascorbate, and oxygen does occur and that both Cu²⁺ and Cu⁺ remain bound to the fibrils during the redox reaction.

A generalized mechanism of the redox reaction entails initial reduction of Cu²⁺ to Cu⁺ by ascorbate, resulting in H₂O₂ formation; this is followed by an electron transfer from Cu⁺ to O₂ to oxidize back to Cu²⁺. The redox cycle indicated by our study is as shown in Reactions 1 and 2,

\[ \text{Cu}^{2+} + \text{Aβ} + \text{Asc} \rightarrow \text{Cu}^{+} - \text{Aβ} + \text{Oxd.Asc} + \text{H}^+ \]  
**REACTION 1**

\[ \text{Cu}^{-} - \text{Aβ} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightarrow \text{Cu}^{2+} - \text{Aβ} + \frac{1}{2}\text{H}_2\text{O}_2 \]  
**REACTION 2**

where Oxd.Asc denotes oxidized ascorbate. In the process, each O₂ can accept two electrons to generate H₂O₂. The presence of excess ascorbate and O₂ with Cu²⁺-Aβ can lead to a significant amount of locally accumulated H₂O₂ and may result in oxidative damage of neurons. The requirements of cellular reductant and molecular oxygen for the ROS production imply that the diffusible Aβ aggregates, rather than immobile fibrils, may be a more efficient agent for catalyzing such reaction in terms of accessibility to cellular reducing agent(s). Such diffusible aggregates of Aβ have been reported to be more toxic to neural cells (56), although it is possible that the amyloid fibril of Aβ plays a major role in the ROS production at a late stage of AD where amyloid plaque is abundant. Furthermore, based on the SSNMR data, we propose that the Met-35 residue on Aβ is not involved in the redox cycle and that both His-13 and His-14 of Aβ(1–40) fibrils can bind to Cu²⁺ and Cu⁺ in different coordination modes through N₆ and N₆ respectively. As shown previously in our study on Cu²⁺-bound Aβ fibril (33), coordination of Cu⁺ ions to other potential ligands such as the C-terminal carboxyl group of Val-40 or Ala-42 may depend on conformations of Aβ in fibrils. Further structural studies will be required for elucidating fuller details of the coordination of Cu⁺ ions to Aβ fibril, which may influence the ROS production rates. Nevertheless, our previous study showed that Cu²⁺ ions are likely to bind to His-13 and His-14 and other residues around the N terminus for different types of Aβ(1–40) fibrils prepared by agitated and quiescent conditions (33). In summary, the results in this report indicate that Aβ(1–40) fibrils become a strong catalyst that attracts copper ions and introduce cyclic redox reactions involving Cu²⁺/Cu⁺ ions in a continuous manner. Such reactions may contribute to the oxidative stress and a cascade of the downstream events in AD. Although additional studies are still needed, this study has presented the first detailed structural insight into the reactive Cu⁺ complex with Aβ fibrils with site specificity, which has not been achieved in many previous studies on copper ion-bound Aβ systems.

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