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Insight into Potential Cu(II)-Binding Motifs in the Four Pseudorepeats of Tau Protein

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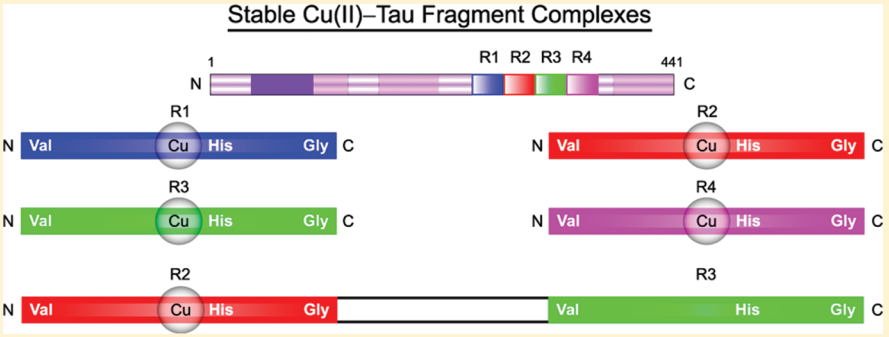
Supporting Information

ABSTRACT:



Tau protein and Cu(II) are believed to be associated with the pathogenesis of Alzheimer's disease. However, little is known about atomic-level interactions between tau protein and Cu(II). Herein, we suggest, on the basis of electron spin resonance (ESR) data, that the four pseudorepeats of tau protein in the microtubule-binding region play an important role in Cu(II) binding. We use a number of tau protein fragments in order to examine Cu(II)-binding site(s) and binding affinities. Continuous-wave (CW) ESR experiments on the four highly conserved octadecapeptides, each of which is a segment of one of the four pseudorepeats, reveal that the equimolar Cu(II) complexes of the four octadecapeptides are similar to one another in terms of the coordination environment and binding affinity. The spectra obtained with pulsed ESR techniques such as electron spin−echo envelope modulation and hyperfine sublevel correlation provide direct evidence that a histidine residue and a backbone amide group coordinate to Cu(II) in each Cu(II)−octadecapeptide complex. The results of CW and pulsed ESR experiments on some chemically modified peptides indicate that the cysteine residues in the second and third pseudorepeats are unlikely to be involved in Cu(II) binding. On the other hand, similar experiments on tau fragments of the second pseudorepeat with different lengths lead to the conclusion that the affinity for Cu(II) decreases as the octadecapeptide is either truncated or elongated. The high Cu(II)-binding affinity of the octadecapeptide is presumably due to the N-terminal amino group stabilizing the Cu(II)−octadecapeptide complex. Finally, the ESR data for a longer tau fragment that contains two octadecapeptides suggest that the Cu(II) binding site(s) of even longer fragments of tau protein is similar to that of a single octadecapeptide.

INTRODUCTION

Alzheimer's disease is the most common form of dementia. The disease is characterized by the presence of two anomalous hallmarks in the brains of patients: extracellular senile plaques and intracellular neurofibrillary tangles.[1,2] It has been reported that the senile plaques are made up of insoluble aggregates of amyloid-β (Aβ) peptide and the neurofibrillary tangles are composed of tau protein aggregates.[3,4]

While the aggregation of the two polypeptides is linked to Alzheimer's disease, some transition metals such as copper, zinc, and iron are also believed to be involved in the neurodegenerative process of the disease.[5−7] Research has shown dyshomeostasis in the brain level of copper, zinc, and iron and anomaly in their metabolism in the case of Alzheimer's disease.[8] Interestingly, some studies have revealed that copper and zinc are colocalized with the Aβ deposits in the senile plaques and their local concentrations are significantly elevated in Alzheimer's disease.[9,10] In addition, Cu(II)- or Zn(II)-induced Aβ aggregations have been reported.[11−13] Accordingly, a number of researchers are focused on elucidating the coordination chemistry of Cu(II) and Zn(II) with Aβ. In particular, our group has proposed that the morphology of Aβ aggregates changes as a function of the Cu(II)-to-peptide ratio.[14,15] By exhaustively examining Cu(II) coordination, our group has recently identified key coordination motifs that might play a role in such changes in morphology.[16] Also, several other groups have reported evidence that atomic-level interactions between Cu(II) and some specific residues of the peptide are correlated with the

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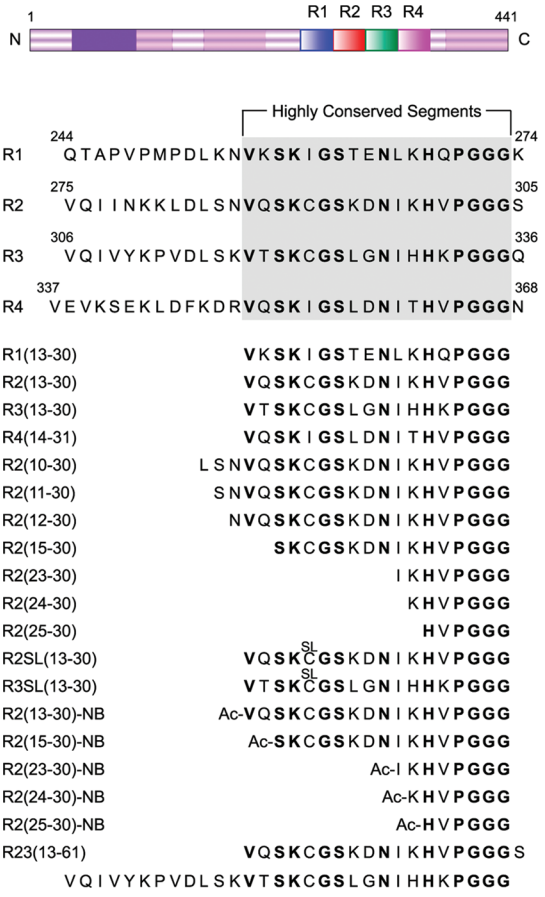

Figure 1. Amino acid sequences of the four pseudorepeats in the longest tau isoform. Each pseudorepeat consists of 31−32 amino acid residues and contains a highly conserved octadecapeptide, which shows >60% homology. The octadecapeptides are shaded in gray, and the conserved residues are indicated in boldface. Also, the tau fragments used in our experiments are listed.

aggregation of the peptide and the toxic nature of Alzheimer's disease.[17−19]

The association of transition metal ions with the neurofibrillary tangles or their main constituent, tau protein, has only recently become relevant. Sayre et al.[20] have strongly suggested that Cu(II), Fe(II), and Fe(III) are involved in the redox reactions occurring in the neurofibrillary tangles as well as in the senile plaques. Involvement of Cu(II) in the neurofibrillary tangles, however, has had less attention paid to it because the intracellular free copper level in neurons is low.[21] Nevertheless, tau in the brain has been found to contain copper and zinc.[22] Also, the dyshomeostasis of transition metal ions in Alzheimer's disease might lead to a local high concentration of copper.[23] In this context, the interaction between Cu(II) and tau protein is of great interest.

Tau is a large protein that consists of up to 441 amino acid residues. In its normal state, it interacts with tubulins to promote their assembly into microtubules and stabilizes the formed microtubules.[24,25] The microtubule-binding region of tau contains three or four pseudorepeats, each of which has 31−32 amino acid residues including a highly homologous 18 amino-acid-sequence that begins with a valine residue and ends with a PGGG sequence; the four pseudorepeats are named R1, R2, R3, and R4, respectively, as illustrated in Figure 1.[26,27] Intriguingly, the highly conserved octadecapeptides in the pseudorepeats are also involved in Cu(II) coordination. Ma et al.[28,29] have shown that the octadecapeptide in R2 or R3 coordinates to Cu(II) and facilitates the peptide aggregation. Similarly, it has been reported that the octadecapeptide in R1 coordinates to Cu(II).[30] Also, it has been revealed that the octadecapeptide in R2 in the presence of Cu(II) can generate hydrogen peroxide as a result of a redox reaction.[31] Soragni et al.[23] have suggested, however, that a longer tau fragment containing all of the four pseudorepeats has only one Cu(II)-binding site and the binding site is present in R2 and/or R3. More recently, Mo et al.[32] have reported that the two cysteine residues and some of the histidine residues in R2 and R3 are involved in the coordination of Zn(II). A more precise picture of Cu(II) coordination is, however, yet to be established.

In this article, we assess the Cu(II) coordination of each of the octadecapeptides in R1, R2, R3, and R4 and elucidate the probable Cu(II)-coordination site(s). In order to obtain information about the Cu(II)-binding site(s) in the octadecapeptides, we have prepared cleaved and/or modified tau fragments. Then, continuous wave (CW) and pulsed electron spin resonance (ESR) experiments, such as electron spin−echo envelope modulation (ESEEM) and hyperfine sublevel correlation (HYSCORE), have been performed on the synthesized peptides mixed with Cu(II). We have found that a histidine residue and a backbone amide group are directly involved in the Cu(II) coordination in each of the four octadecapeptides. The Cu(II) coordination by a backbone amide presumably helps stabilize the Cu(II)−octadecapeptide complex. In addition, our data are consistent with the results of other groups in that each of the four octadecapeptides has at least one Cu(II)-binding site.[28−30]

We also suggest a Cu(II)-coordination site of longer tau fragments containing the four pseudorepeats based on analysis of our ESR results. In order to estimate the Cu(II)-binding site, we have synthesized a 49-amino-acid peptide that contains the highly conserved octadecapeptides in R2 and R3. Most of our data are consistent with the work of Soragni et al.[23] in that there is only one Cu(II)-binding site in the 49-amino-acid peptide. In addition to the consistency, our results provide more precise information about the amino acid residues that are involved in the Cu(II) coordination.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Cu(II)−Peptide Complex Preparation. A nitroxide spin-labeling agent, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTS), was purchased from Toronto Research Chemical (North York, Ontario, Canada). Tau protein fragments as well as Aβ(1−16), DAEFRHDSGYEVHHQK, were synthesized at the Peptide Synthesis Facility of the University of Pittsburgh via the conventional solid-phase fluorenylmethoxycarbonyl chemistry.[33,34] The tau fragments include four octadecapeptides, VKSKIGSTENLKHQPGGG, VQSKCGSKDNIKHVPGGG, VTSKCGSLGNIHHKPGGG, VQSKIGSLDNITHVPGGG, shorter sequences that contain some of the octadecapeptides, and a 49-amino acid peptide that contains the highly conserved octadecapeptides in R2 and R3, VQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGG. In addition, some peptides were modified by either elongation to the N-terminus, acetylation at the N-terminus, or MTS spin-labeling at the cysteine residue(s). The four octadecapeptides, corresponding to Tau(256−273), Tau(287−304), Tau(318−335), and Tau(350−367) in the longest tau isoform, were

termed R1(13−30), R2(13−30), R3(13−30), and R4(14−31), respectively, for the sake of easy connotation of the relative position in each pseudorepeat. Similarly, the 49-amino acid peptide, that is, Tau(287−335), was termed R23(13−61). In addition, peptides with a total of 6−21 amino acid residues, all of which contain the HVPGGG sequence of R2, are also among the synthesized tau fragments: Tau(284−304), Tau(285−304), Tau(286−304), Tau(289−304), Tau(297−304), Tau(298−304), and Tau(299−304) were termed R2(10−30), R2(11−30), R2(12−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30), respectively. Also, some of the synthesized peptides were acetylated at the N-terminus: the acetylated versions of R2(13−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30) were termed R2(13−30)-NB, R2(15−30)-NB, R2(23−30)-NB, R2(24−30)-NB, and R2(25−30)-NB, respectively. Besides, some of the peptides were spin-labeled at the cysteine residue: the MTS spin-labeled versions of R2(13−30) and R3(13−30) were termed R2SL(13−30) and R3SL(13−30), respectively. Each of the synthesized peptides was characterized by high performance liquid chromatography and mass spectrometry. Isotopically enriched [^{63}Cu]Cl_2 was purchased from Cambridge Isotope Laboratory (Andover, MA). *N*-Ethylmorpholine (NEM) was purchased from Sigma−Aldrich (St. Louis, MO). A 100 mM NEM buffer with a pH of 7.4 was prepared by mixing NEM and hydrochloric acid in 50% glycerol. Then, 2.5 mM solutions of tau protein fragments were prepared in the 100 mM NEM buffer. Separately, a 10 mM Cu(II) stock solution was prepared in the same buffer. Mixtures with various Cu(II)-to-peptide molar ratios such as 0.125:1, 0.25:1, 0.5:1, 1:1, 2:1, 4:1, and 8:1 were prepared with a final concentration of 1.25 mM in the peptide.

Electron Spin Resonance Spectroscopy. Cu(II)−peptide mixtures either dissolved or suspended in 100 mM NEM buffer containing 50% glycerol were used for ESR experiments. A 200 μL aliquot of each Cu(II)−peptide mixture solution was transferred into a quartz tube with an inner diameter of 3 mm. All ESR experiments were performed on a Bruker ElexSys E580 FT/CW X-band spectrometer equipped with a Bruker ER 4118X-MD5 dielectric ring resonator. The temperature was adjusted with an Oxford ITC503 temperature controller and an Oxford CF935 dynamic continuous-flow cryostat connected to an Oxford LLT650 low-loss transfer tube.

Continuous-wave ESR experiments were carried out on the sample solutions. All ESR signals were collected at 80 K with a microwave frequency of approximately 9.69 GHz. The magnetic field was generally swept from 2600 to 3600 G for a total of 1024 data points. Other instrumental parameters include a time constant of 40.96 ms, a conversion time of 81.92 ms, a modulation amplitude of 4 G, a modulation frequency of 100 kHz, and a microwave power of 0.1993 mW. The experimentally obtained spectra were compared with the corresponding simulated spectra in Symphonia and WINEPR provided by Bruker.

Three-pulse ESEEM experiments were performed on most of the sample solutions at 20 K with a conventional stimulated-echo pulse sequence of $\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau -$ echo. The first pulse separation, τ, was set at either 144 or 192 ns, and the second pulse separation, T, was varied from 288 ns with a step size of 16 ns for a total of 1024 points. The pulse length was 16 ns, and the magnetic field strength was fixed at either around 3365 G, where the echo intensity was a maximum, or around 3150 G, which corresponded to the $g_{\|}$ position. In addition, a four-step phase cycle was employed to eliminate unwanted signals.[35,36] The real parts of the collected raw data were baseline-corrected and fast Fourier-transformed. Then, the final spectra were obtained as the magnitude of the Fourier transforms.

Four-pulse HYSCORE experiments were performed on some of the sample solutions at 20 K with a pulse sequence of $\pi/2 - \tau - \pi/2 - t_1 - \pi - t_2 - \pi/2 - \tau -$ echo. The first pulse separation, τ, was set at 144 ns, and both the second pulse separation, t_1, and the third pulse separation, t_2, were varied from 144 ns with a step size of 16 ns for a total of 512 points. The pulse lengths were 16 and 32 ns for $\pi/2$ and π pulses, respectively, and the magnetic field strength was fixed at approximately 3365 G, where the echo intensity was a maximum. In addition, a four-step phase cycle was employed to eliminate unwanted signals. The real parts of the collected two-dimensional data were baseline-corrected and apodized with a Hamming window in both dimensions. Then, the processed data were zero-filled to 1024 points in both dimensions before being fast Fourier-transformed. The final spectra were obtained as the contour plots of the magnitude of the two-dimensional Fourier transforms.

ESEEM Data Analysis. Spectral simulations were performed to extract useful information from some of the experimentally obtained ESEEM spectra. For a system where Cu(II) is coupled to ^{14}N, three characteristic peaks between 0 and 3 MHz are typically observed in the ESEEM spectrum.[37−41] The three ESEEM frequencies, ν_0, ν_-, and ν_+ for the ^{14}N (I = 1) NQI transitions are given by[42]

$$\nu_0 = \frac{e^2qQ\eta}{2h};\ \nu_- = \frac{e^2qQ(3-\eta)}{4h};\ \nu_+ = \frac{e^2qQ(3+\eta)}{4h} \quad (1)$$

where e is the elementary charge, q is the z-component of the electric field gradient across the nucleus, Q is the ^{14}N nuclear quadrupole moment, η is the asymmetry parameter, and h is Planck's constant.

The ESEEM spectrum also contains a broad peak that is attributed to a double quantum transition.[37−41] The theoretical double quantum transition frequency is given by[43]

$$\nu_{DQ} = 2\sqrt{\left(\nu_I + \frac{A}{2}\right)^2 + \left(\frac{B}{2}\right)^2 + \left(\frac{e^2qQ}{4h}\right)^2(3+\eta^2)} \quad (2)$$

where ν_{DQ} is the double quantum transition frequency, ν_I is the Larmor frequency of ^{14}N, and A and B are the secular and the pseudosecular part of the hyperfine interaction, respectively.

RESULTS AND DISCUSSION

The longest isoform of tau, which consists of 441 amino acid residues, contains four pseudorepeats. Each pseudorepeat consists of 31−32 amino acid residues and has a highly conserved octadecapeptide that begins with a valine residue and ends with a PGGG sequence. The sequences of the pseudorepeats, R1, R2, R3, and R4, are shown in Figure 1. Interestingly, the highly conserved octadecapeptides of R1, R2, and R3 have been found to bind Cu(II).[28−30]

A series of ESR experiments were conducted on synthesized tau protein fragments, most of which contain some of the highly conserved segments of the four pseudorepeats, R1, R2, R3, and R4. The highly conserved octadecapeptides of R1, R2, R3, and R4, corresponding to Tau(256−273), Tau(287−304), Tau(318−335),

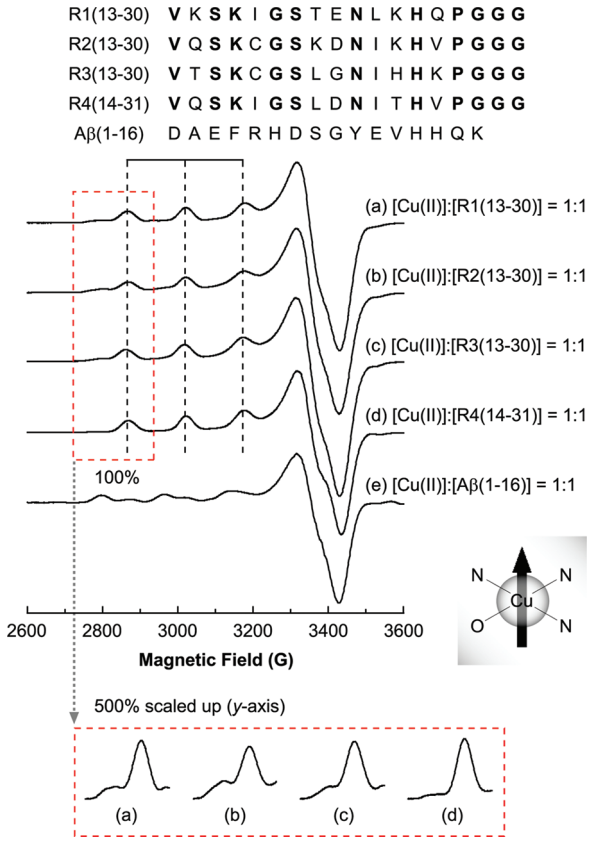

Figure 2. CW-ESR spectra of R1(13−30), R2(13−30), R3(13−30), R4(14−31), and Aβ(1−16) mixed with an equimolar amount of Cu(II). The amino acid sequences of the four octadecapeptides and Aβ(1−16) are illustrated with conserved residues indicated in boldface. The similarity in *g* values, *A* values, as well as intensity suggests that their binding site(s) and binding affinities are comparable.

and Tau(350−367) in the longest tau isoform, were termed R1(13−30), R2(13−30), R3(13−30), and R4(14−31), respectively. Other fragments with a total of 6−21 amino acid residues, all of which contain the HVPGGG sequence of R2, were named in a similar manner: Tau(284−304), Tau(285−304), Tau(286−304), Tau(289−304), Tau(297−304), Tau(298−304), and Tau(299−304) were termed R2(10−30), R2(11−30), R2(12−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30), respectively. Also, The N-acetylated versions of R2(13−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30) were termed R2(13−30)-NB, R2(15−30)-NB, R2(23−30)-NB, R2(24−30)-NB, and R2(25−30)-NB, respectively. The 49-amino acid peptide that contains the highly conserved octadecapeptides in R2 and R3 was named R23(13−61). The nitroxide spin-labeled versions of R2(13−30) and R3(13−30) were named R2SL(13−30) and R3SL(13−30), respectively.

Similarity in ESR Parameters between the Four Octadecapeptides, R1(13−30), R2(13−30), R3(13−30), and R4(14−31): Similar Cu(II)-Binding Site(s) and Cu(II)-Binding Affinities. The four octadecapeptides, each of which is the highly conserved segment in a pseudorepeat, were compared in terms of Cu(II) coordination by means of several ESR methods.

First, CW-ESR experiments were carried out on the octadecapeptides and Aβ(1−16), each of which was mixed with an equimolar amount of Cu(II). Figure 2 shows the CW-ESR spectra of the Cu(II) complexes of the octadecapeptides. The ESR spectra of the Cu(II) complexes of the octadecapeptides consist of two components. Both components have similar *g* values, *A* values, and linewidths for all the octadecapeptides. The $g_{\|}$ value and $A_{\|}$ value of the dominant component (component 1) are 2.23 ± 0.005 and 157 ± 1 G, respectively. The ESR parameters, which are determined by spectral simulations, are consistent with a square-planar Cu(II)-coordination geometry with three nitrogen donors and one oxygen donor on the equatorial plane.[44−48] Also, each of the four spectra has a second component (component 2), which is relatively less significant. The second component is clearly visible as a shoulder upon magnification of a portion of the spectra, as shown in the inset of Figure 2. The $g_{\|}$ value and $A_{\|}$ value of the minor component, 2.25 ± 0.005 and 183 ± 1 G, respectively, are consistent with a square-planar geometry with either three nitrogen donors and one oxygen donor or four nitrogen donors.[44,47]

The simulation results also show that the minor component accounts for 5−20% of each Cu(II)−peptide complex. The comparison between experimental and simulated spectra is provided in Supporting Information, Figure S1 and Tables S1 and S2. In addition, the CW-ESR spectra of the octadecapeptides mixed with different molar equivalents of Cu(II) exhibit almost identical ESR parameters at all the Cu(II)-to-peptide ratios. In particular, the ratio between the two components remains almost unchanged. The CW-ESR spectra of the octadecapeptides mixed with Cu(II) at various Cu(II)-to-peptide ratios are presented in Supporting Information, Figure S2.

It is known that the ESR signals from aqueous Cu(II) complexes are minimized in NEM buffer at a pH of 7.4.[15,45,49] In NEM buffer, the Cu(II)−peptide complex contributes to the ESR signal intensity, while a fraction of Cu(II) that is not coordinated by the peptide does not. If the binding affinity of a peptide is very low, only a small fraction of Cu(II) contributes to the ESR intensity, which leads to a relatively low double integral of the ESR spectrum. Thus, comparison of the double integrals of the first derivative ESR spectra provides information about the binding affinities of the octadecapeptides. The double integrals of the spectra are similar to one another within a difference of 8% for the Cu(II) complexes of R1, R2, R3, R4, and Aβ(1−16). Considering that CW-ESR instrumental parameters including the microwave power and modulation amplitude are identical through all CW-ESR experiments, it is concluded that each octadecapeptide has a Cu(II)-binding affinity comparable to that of Aβ(1−16). The double integrals are provided in Supporting Information, Table S2.

Next, pulsed ESR experiments such as ESEEM and HYSCORE were performed on the equimolar Cu(II) complexes as used for the CW-ESR experiments mentioned above. Figure 3 illustrates the three-pulse ESEEM spectra of the four Cu(II)−peptide complexes. Each of the spectra has three peaks at or around 0.55, 0.98, and 1.53 MHz. The sum of the lower two frequencies coincides with the highest one within the resolution error range of ±0.03 MHz. Also, the three peaks exhibit little field dependence. Detailed information about the ESEEM spectra obtained at different magnetic fields is provided in Supporting Information, Figure S3. Both of these properties indicate that they are mainly due to the nuclear quadrupole interaction (NQI).[42,43,50]

By comparison of the experimentally obtained frequencies with those of eq 1, the nuclear quadrupole parameters, e^2qQ/h and η, are determined to be 1.67 ± 0.03 MHz and 0.66 ± 0.02, respectively. In addition to these three peaks, each spectrum also has a peak around 4.1 MHz, which is assigned to the double

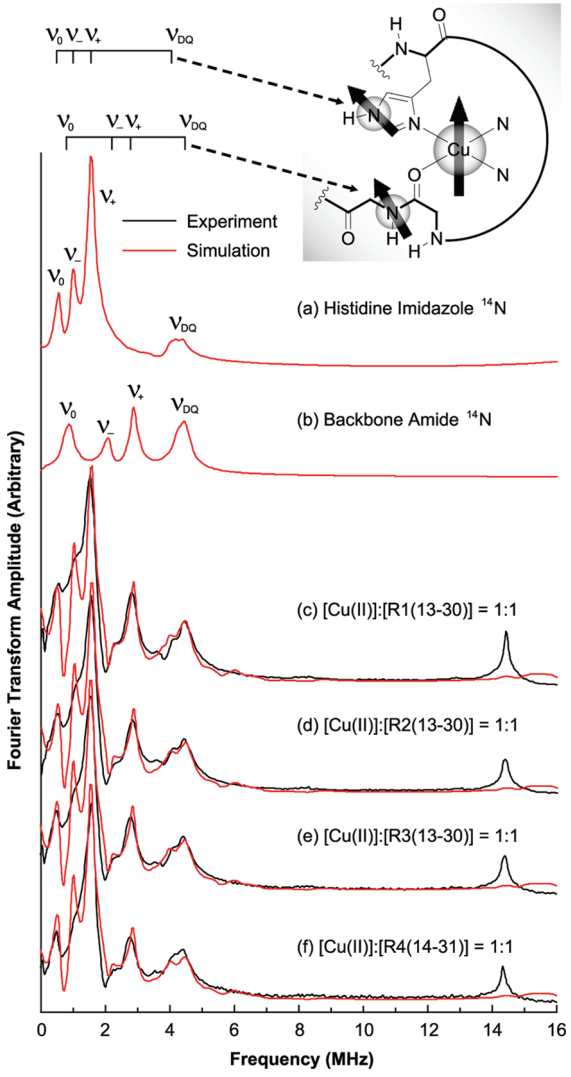

Figure 3. Experimental and simulated three-pulse ESEEM spectra of R1(13−30), R2(13−30), R3(13−30), and R4(14−31) mixed with an equimolar amount of Cu(II). The ESEEM frequencies at or around 0.55, 0.98, 1.53, and 4.1 MHz indicate that Cu(II) is coordinated by histidine. Also, the ESEEM frequencies at or around 0.75, 2.08, 2.80, and 4.4 MHz indicate that amide backbone is involved in the Cu(II) coordination. Accordingly, two ^{14}N nuclei, remote nitrogen of imidazole and backbone amide nitrogen, are considered in the simulations. The hyperfine interaction parameters obtained with the simulations indicate that the Cu(II) is more strongly bound to the imidazole than to the backbone amide.

quantum transition. All of these values including the NQI and double quantum transition frequencies and the two calculated parameters are comparable to those for Cu(II) complexes of histidine imidazole in proteins that were determined by McCracken et al.[37−40,51] On the basis of the analysis of the NQI and double quantum frequencies, we have concluded that histidine imidazole coordinates to Cu(II) in all of the four complexes.

Besides the four peaks assigned to the Cu(II)−imidazole coordination, each spectrum also exhibits more peaks at or around ∼0.75, 2.08, 2.80, and 4.4 MHz. Detailed information about the frequencies observed at two different magnetic fields is provided in Supporting Information, Figure S3. These values are comparable to those for the Cu(II) coordinated by a backbone amide that were reported by Burns et al.[46] Their research has proven that the amide nitrogen of the third glycine residue in the PHGGGW sequence gives rise to three ^{14}N nuclear quadrupole transition frequencies at around ∼0.80, 2.00, and 2.80 MHz. Also, they have assigned a broad peak around 4.4 MHz to the double quantum transition. The similarity between their data and our results suggests that a backbone amide in the octadecapeptide coordinates to Cu(II). With the experimentally obtained frequencies, the nuclear quadrupole parameters, e^2qQ/h and η, are determined to be 3.25 ± 0.03 MHz and 0.46 ± 0.02, respectively.

Nevertheless, which backbone amide coordinates to Cu(II) is still unclear. We initially suspected that one of the three consecutive glycine residues in the octadecapeptides is responsible for the characteristic ^{14}N-ESEEM peaks at around ∼0.80, 2.00, and 2.80 MHz. However, the ESEEM spectrum remains almost unchanged when any of the three glycine residues is labeled with ^{15}N (data not shown), which signifies that none of the three glycine residues coordinate to Cu(II). Recently, Drew et al.[52] have reported that the carbonyl oxygen of Ala2 in Aβ(1−16) directly coordinates to Cu(II) and the amide nitrogen of Glu3 is responsible for the characteristic ^{14}N-ESEEM peak at around 2.8 MHz. Considering the fact that glycine and alanine are the two least bulky amino acids, one can suggest that Cu(II) is more likely to be coordinated by the carbonyl oxygen of a less bulky residue than that of a bulkier one. Thus, we conjecture that the carbonyl oxygen of the fourth glycine residue or one of the two serine residues in the octadecapeptide coordinates to Cu(II).

Furthermore, spectral simulations provide information about the hyperfine interaction between the electron spin and the nuclear spin of ^{14}N. Figure 3 also shows the best-fit simulated spectra of the four Cu(II)−peptide complexes, while the ESR parameters are shown in Supporting Information, Table S3. The atoms that strongly coordinate to Cu(II) in the complexes of the four octadecapeptides are perpendicular to the $g_{\|}$ axis and show little orientation selectivity in the ESEEM spectra when the external magnetic field is almost perpendicular to the axis.[37,40] Thus, an axial hyperfine tensor is assumed for the system and parametrized by two hyperfine constants, A_{iso} and T_{dip}, which represent the isotropic part and anisotropic part, respectively.

Our simulation results reveal that both A_{iso} and T_{dip} are larger for the remote ^{14}N nucleus, which implies that the coordination between Cu(II) and the imidazole nitrogen is stronger than that between Cu(II) and the carbonyl oxygen in the backbone. Also, the angle between the z-axis of the nuclear quadrupole tensor and the interspin vector has been found to be approximately 90° for all of the complexes, which indicates that the interspin vector is in the plane of the π system containing the Cu(II)-coordinating atom and the ESEEM active atom.[40] The definition of angles that determine the orientation of the external magnetic field or the electron−nuclear interspin vector with respect to the principal axis system of the nuclear quadrupole tensor is provided in Supporting Information, Figure S4. The line width of the peaks below 2 MHz is narrower in the simulated spectra than in the experimental spectra. One possible explanation for the difference is the contribution of an ESEEM-active ^{14}N nucleus of component 2 in the complexes to the ESEEM signals in the experiments. Also, it is plausible that some of the ESEEM parameters such as A_{iso} and T_{dip}, and the angle between the nuclear quadrupole axis and the interspin vector, are ranges of values rather than single values.

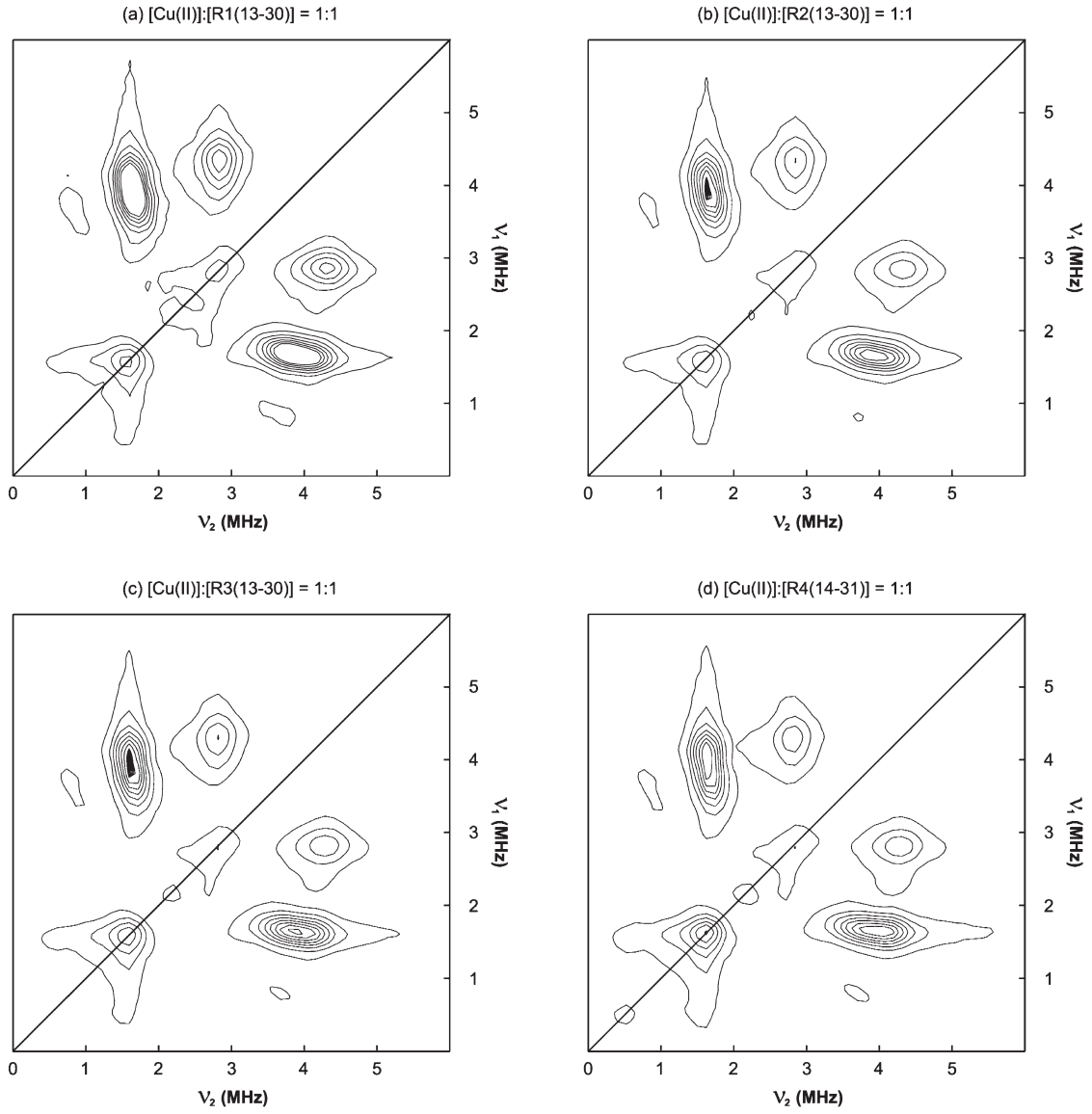

Figure 4. HYSCORE spectra of R1(13−30), R2(13−30), R3(13−30), and R4(14−31) mixed with an equimolar amount of Cu(II). The cross-peak around (1.6 MHz, 4.0 MHz) is ascribed to the Cu(II) ion that is coordinated by histidine. The cross-peak around (2.8 MHz, 4.3 MHz) is unique to the Cu(II)−peptide complex in which amide backbone is involved in the Cu(II) coordination.

Also, our simulation result shows that the Cu(II)−R3(13−30) complex containing two histidine residues has ESEEM parameters similar to those of the other Cu(II)−octadecapeptide complexes in each of which only one histidine residue exists. The similarity in the ESEEM parameters indicates that the two histidine residues in R3 are unlikely to simultaneously coordinate to Cu(II). Jiang et al.[40] have shown that the intensity of the peak due to the double quantum transition relative to that of the peaks due to the nuclear quadrupole transitions increases with the number of coordinating histidine residues. Thus, the peak due to the double quantum transition would be more intense in the spectrum of the Cu(II)−R3(13−30) complex if the two histidine residues in R3 simultaneously coordinated to Cu(II). However, it is still unclear which histidine residue in R3 coordinates to Cu(II). It is suspected that both histidine residues contribute to the Cu(II) coordination even though they do not simultaneously coordinate to Cu(II). Several research groups have already reported that His13 and His14 in the Cu(II)−Aβ(1−16) complex can interchangeably bind to Cu(II).[53−55] Considering that the two histidine residues in R3 are also adjacent to each other, it is probable that they interchangeably coordinate to Cu(II).

The identity of some peaks is further confirmed by the four-pulse HYSCORE experiments. The HYSCORE spectra of the four Cu(II)−peptide complexes are illustrated in Figure 4. Each of the spectra contains a cross-peak around (1.6 MHz, 4.0 MHz). This peak appears owing to the correlation between the ^{14}N NQI and double quantum transition for the remote nitrogen of the Cu(II)-coordinated histidine imidazole.[40,46] Also, each spectrum displays another cross-peak around (2.8 MHz, 4.3 MHz). This peak is attributed to the correlation between the ^{14}N NQI and double quantum transition for the nearest amide nitrogen of the Cu(II)-coordinated backbone carbonyl oxygen.[46,52]

Taken together, CW and pulsed ESR experiments and simulations indicate that the four octadecapeptides, R1(13−30), R2(13−30), R3(13−30), and R4(14−31), have almost identical Cu(II)-binding site(s) and similar binding affinities for Cu(II).

Especially, the ESEEM and HYSCORE results show that a histidine imidazole ring and a backbone amide coordinate to Cu(II) in the equimolar Cu(II) complexes of the four octadecapeptides. We suspect that the high homology of the four octadecapeptides accounts for the similar binding site(s) and similar binding affinities.

Roles of Histidine Residues, Backbone Amide Groups, and N-Terminal Amino Groups in the Cu(II)-Coordination Environment. In order to obtain more information about the Cu(II)-binding site(s), we performed CW-ESR experiments on the Cu(II) complexes of shorter tau protein fragments in the R2 pseudorepeat whose Cu(II)-binding property has already been reported by Ma et al.[29] Separately, Burns et al.[45−48] revealed that a repeated hexapeptide containing one histidine residue and three consecutive glycine residues, PHGGGW, has a good binding affinity for Cu(II) and serves as a minimal binding sequence in the prion protein. Since the CW-ESR and ESEEM parameters of the octadecapeptides are almost identical to those of the hexapeptide in the prion protein, we initially suspected that, in tau protein, a hexapeptide containing one histidine residue and three consecutive glycine residues, HVPGGG, might serve as a minimal binding sequence.

Figure 5 shows the CW-ESR spectra of the equimolar mixtures of Cu(II) and one of tau protein fragments in the R2 pseudorepeat. Each of the fragments contains a total of 6−21 amino acid residues including the HVPGGG sequence. Unfortunately, the CW-ESR parameters of the Cu(II)−HVPGGG complex are significantly different from those of the Cu(II) complex of the corresponding octadecapeptide, VQSKCGSKDNIKHVPGGG. Also, the spectra of the Cu(II) complexes of R2(10−30), R2(11−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30) are different from one another in terms of ESR parameters and intensity. Interestingly, however, the spectra of the Cu(II) complexes of the nonadecapeptide and octadecapeptide, that is, R2(12−30) and R2(13−30), have similar ESR parameters, which indicates that only the nonadecapeptide and octadecapeptide have similar Cu(II)-binding site(s). In addition, the three longest fragments used for the experiments, R2(10−30), R2(11−30), and R2(12−30), exhibit lower Cu(II)-binding affinities than R2(13−30), that is, the octadecapeptide. Each of the shorter fragments including R2(15−30), R2(23−30), R2(24−30), and R2(25−30) also shows a lower Cu(II)-binding affinity than the octadecapeptide. The $g_{\|}$ and $A_{\|}$ values of the spectra of the Cu(II)−R2(15−30) and Cu(II)−R2(23−30) complexes correspond to four nitrogen donors on the equatorial plane, which indicates that, unlike Cu(II)−R2(13−30), no backbone carbonyl oxygen is directly involved in the Cu(II) coordination. Thus, the relatively higher Cu(II)-binding affinity of R2(13−30) may be attributed to the Cu(II) coordination by a backbone amide. The ESR parameters such as $g_{\|}$ and $A_{\|}$ values and double integrals of the CW-ESR spectra are shown in Supporting Information, Table S4. Taken together, the CW-ESR results indicate that while the octadecapeptide, R2(13−30), has the highest binding affinity for Cu(II), the mere existence of the octadecapeptide sequence, VQSKCGSKDNIKHVPGGG, in a peptide does not suffice for the Cu(II) coordination with a high binding affinity.

More intriguingly, the corresponding ESEEM and HYSCORE spectra of the Cu(II) complexes of the tau fragments show that the Cu(II) coordination by a backbone amide is most evident in the octadecapeptide, R2(13−30). Since the octadecapeptide appears to have a higher Cu(II)-binding affinity than any other shorter or longer fragments used in the experiments, the Cu(II) coordination by a backbone amide is likely to play an important role in stabilizing the complex. The pulsed ESR data also reveal that a histidine residue directly coordinates to Cu(II) in any fragments that contain the hexapeptide HVPGGG sequence. In fact, some peaks or cross-peaks characteristic of Cu(II)−imidazole coordination appear in the ESEEM or HYSCORE spectra of the Cu(II) complexes of the tau fragments in the R2 pseudorepeat including R2(11−30), R2(12−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30). The ESEEM and HYSCORE spectra of the Cu(II) complexes of several fragments in the R2 pseudorepeat are provided in Supporting Information, Figures S5 and S6. The Cu(II) coordination by the histidine residue signifies that the histidine residue (particularly the imidazole ring) serves as an anchor for Cu(II) irrespective of the existence of other residues.

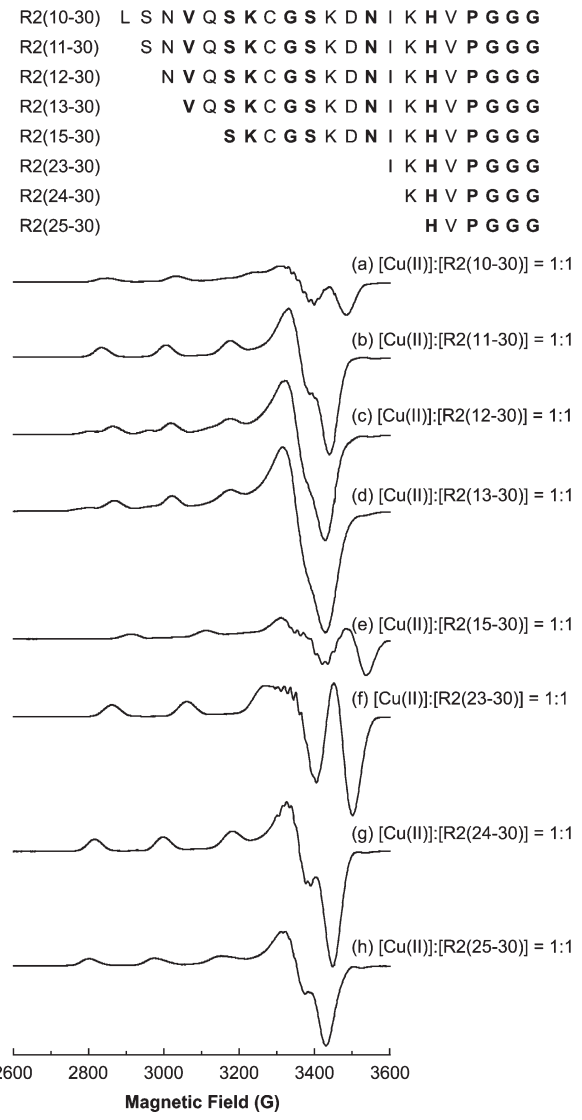

Figure 5. CW-ESR spectra of R2(10−30), R2(11−30), R2(12−30), R2(13−30), R2(15−30), R2(23−30), R2(24−30), and R2(25−30) mixed with an equimolar amount of Cu(II). The amino acid sequences of the eight tau fragments are illustrated with conserved residues indicated in boldface. Most spectra have different g values, A values, as well as intensity, which suggests that their binding site(s) and binding affinities are different.

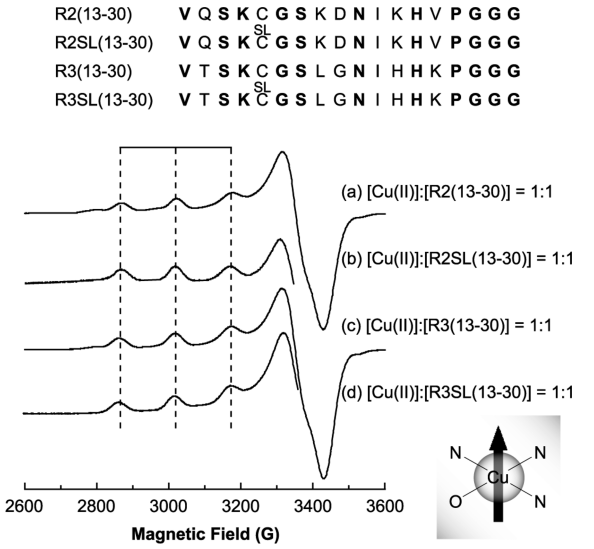

Figure 6. CW-ESR spectra of R2(13−30), R2SL(13−30), R3(13−30), and R3SL(13−30) mixed with an equimolar amount of Cu(II). The amino acid sequences of the four peptides are illustrated with conserved residues indicated in boldface. The similarity in *g* values, *A* values, as well as intensity suggests that their binding site(s) and binding affinities are comparable.

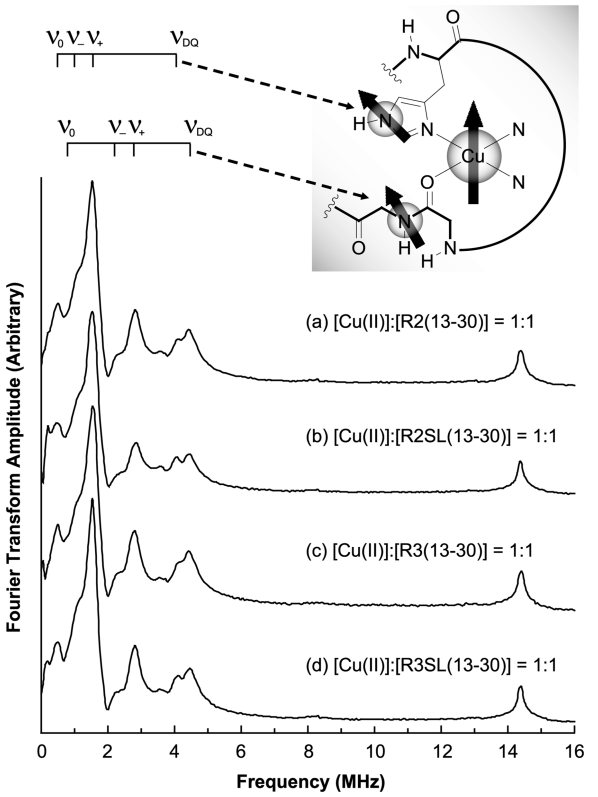

Figure 7. Three-pulse ESEEM spectra of R2(13−30), R2SL(13−30), R3(13−30), and R3SL(13−30) mixed with an equimolar amount of Cu(II). The ESEEM frequencies at or around 0.55, 0.98, 1.53, and 4.1 MHz indicate that Cu(II) is coordinated by histidine. Also, the ESEEM frequencies at or around 2.08, 2.80, and 4.4 MHz indicate that amide backbone is involved in the Cu(II) coordination.

Generally, histidine and cysteine residues in a peptide are considered to have a higher affinity for Cu(II) than the other amino acid residues.[56−59] Ma et al.[29] have suggested the possibility that the histidine and cysteine residues in R2(13−30) coordinate to Cu(II) based on their NMR results. On the other hand, they have also argued that the two histidine residues in R3(13−30) coordinate to Cu(II) and that, unlike the case of R2(13−30), the cysteine residues are not directly involved in R3(13−30).[28] While some of the ESR parameters such as $g_{\|}$ values, $A_{\|}$ values, and intensities might be similar between complexes with different Cu(II) coordination environments, a complex with a sulfur donor is very unlikely to show a CW-ESR spectrum nearly identical to that of a complex with no sulfur. Thus, if the sulfhydryl group coordinated to Cu(II) in R2(13−30) but not in R3(13−30), the CW-ESR spectra of the two Cu(II) complexes would be noticeably different. However, as shown in Figure 2, it is clear that the CW-ESR spectrum of the equimolar mixture of Cu(II) and R2(13−30) is similar to that of Cu(II) and R3(13−30). In order to resolve the discrepancy, we carried out CW-ESR, ESEEM, and HYSCORE experiments on the Cu(II) complexes of R2SL-(13−30) and R3SL(13−30), each of which has a nitroxide spin-label at the cysteine residue.

Figure 6 shows that the CW-ESR spectra of the Cu(II) complexes of the spin-labeled peptides are similar to their nonlabeled counterparts except for the region where the nitroxide lines appear, which is not shown in the spectra. The similarity signifies that the free sulfhydryl group in the cysteine residue is not involved in the Cu(II) coordination because the spin-labeled octadecapeptide has no free sulfhydryl group as a disulfide bond is formed. Under some conditions, the sulfhydryl group in the R2 or R3 pseudorepeat might be oxidized to form a dimer linked by a disulfide bond. To assess the formation of a disulfide bond in the Cu(II)−R2(13−30) and Cu(II)−R3-(13−30) complexes used for our ESR experiments, mass spectrometry and electrophoresis were conducted (see Supporting Information). While the mass spectra of the Cu(II)−R2-(13−30) and Cu(II)−R3(13−30) complexes suggest the formation of dimers, there is no unambiguous evidence of disulfide bonds. Sodium dodecyl sulfate polyacrylamide gel electrophoresis results show similar results with and without tris(2-carboxyethyl)phosphine, a reducing agent. The mass spectrometry and electrophoresis results, taken together, indicate that the dimers of either R2(13−30) or R3(13−30) may not be stabilized by a disulfide bond. Our results are consistent with those of Rosenberg et al.[60] who have proposed the formation of dimeric tau protein with no disulfide bond. The mass spectra and the electrophoretogram are provided in Supporting Information, Figures S7 and S8, respectively.

Figures 7 and 8 illustrate the ESEEM and HYSCORE spectra of the Cu(II) complexes of the spin-labeled octadecapeptides, respectively. Each of the ESEEM spectra has four peaks at or around 0.55, 0.98, 1.53, and 4.1 MHz, which are due to the involvement of the imidazole ring in the Cu(II) coordination. Similarly, in the corresponding HYSCORE spectra, a cross-peak around (1.6 MHz, 4.0 MHz) is evident in all cases. Also, the four peaks assigned to the Cu(II)−amide coordination, that is, the peaks at or around 0.80, 2.08, 2.80, and 4.4 MHz, and the corresponding cross-peak around (2.8 MHz, 4.3 MHz) appear in each ESEEM and HYSCORE spectrum. Therefore, it is inferred that the cysteine side-chain of the octadecapeptide does not coordinate Cu(II). Furthermore, the similarity in ESR parameters between the four octadecapeptides, R1(13−30), R2(13−30), R3(13−30), and R4(14−31), implies

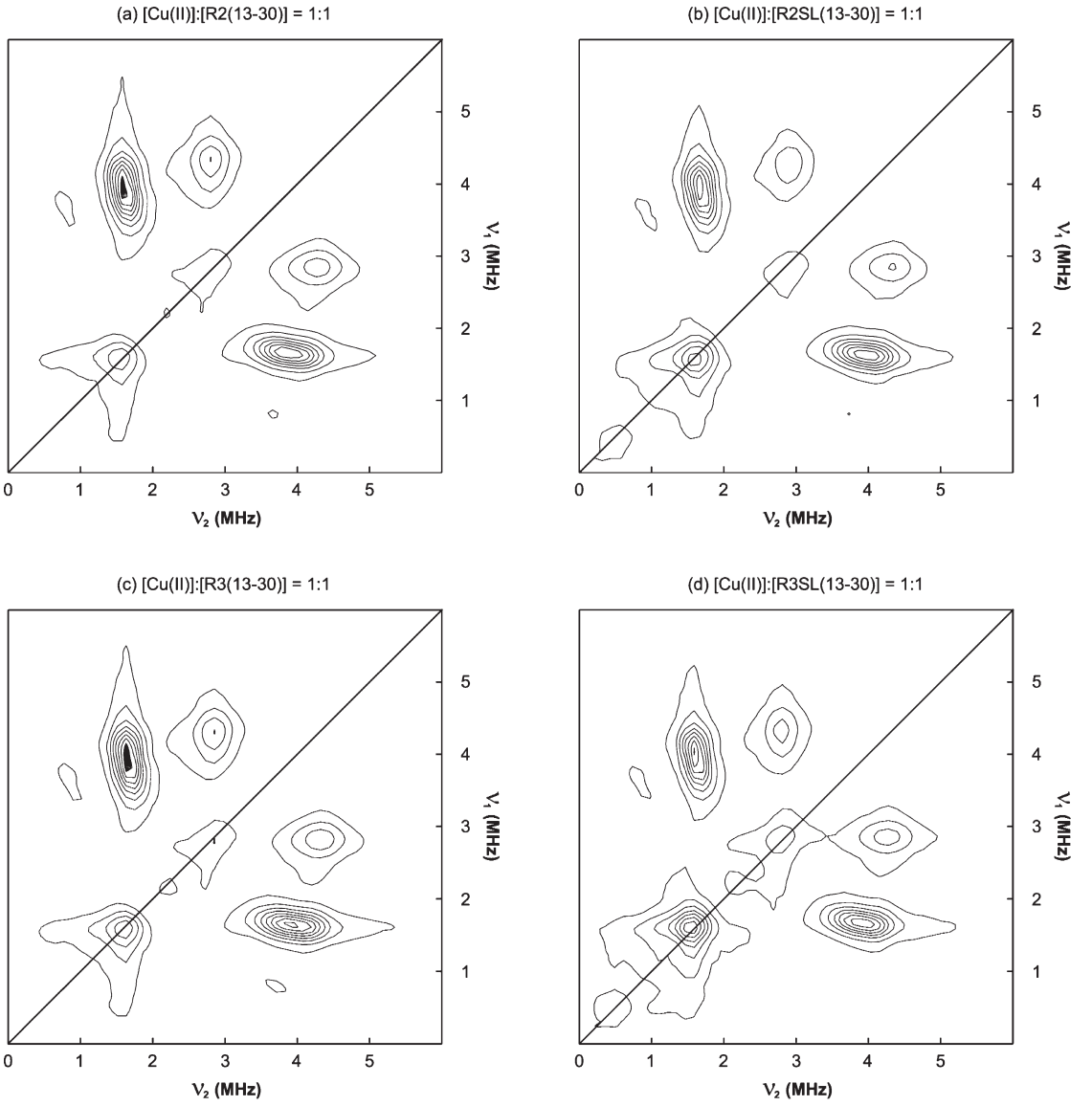

Figure 8. HYSCORE spectra of R2(13−30), R2SL(13−30), R3(13−30), and R3SL(13−30) mixed with an equimolar amount of Cu(II). The cross-peak around (1.6 MHz, 4.0 MHz) is ascribed to the Cu(II) ion that is coordinated by histidine. The cross-peak around (2.8 MHz, 4.3 MHz) is unique to the Cu(II)−peptide complex in which amide backbone is involved in the Cu(II) coordination.

that the cysteine residue of R2 or R3 does not coordinate to Cu(II). If the sulfhydryl group of R2 and R3 coordinated to Cu(II), the ESR spectra of the Cu(II)−R2(13−30) and Cu(II)−R3(13−30) complexes would be significantly different from those of the Cu(II)−R1(13−30) and Cu(II)−R4(14−31), in which there is no cysteine residue.

Aside from histidine and cysteine, Ma et al.[28,29] have also proposed that the N-terminus coordinates to Cu(II) in the octadecapeptide. Generally, it is probable that the N-terminal amino group can directly coordinate to Cu(II) or stabilize the Cu(II)−peptide complex structure.[45,61,62] In order to obtain information about the role of the N-terminus in the tau fragments, we performed CW-ESR experiments on the acetylated versions of several tau protein fragments in the R2 pseudorepeat. Each of the fragments contains a total of 6−18 amino acid residues including the HVPGGG sequence. Figure 9 shows that the CW-ESR spectra of the Cu(II) complexes of the acetylated tau fragments are similar to one another but different from their nonacetylated counterparts in terms of ESR parameters such as *g* values, *A* values, and linewidths. However, comparison of the double integrals reveals that the binding affinities of the acetylated tau fragments are much lower than their nonacetylated counterparts. The double integrals of the CW-ESR spectra are provided in Supporting Information, Table S4. The difference in binding affinity indicates that the N-terminal amino group plays a role in the Cu(II) coordination. On the basis of the fact that the N-terminal amino group can be protonated at a pH of 7.4, we suggest that the amino group directly coordinates to Cu(II) or stabilizes the complex structure by electrostatic interactions. Also, it is suspected that the conformation of R2(13−30) and R2(12−30), and presumably the corresponding octadecapeptides and nonadecapeptides in the other three pseudorepeats, helps the N-terminus stabilize the Cu(II)−amide coordination because the Cu(II) coordination by a backbone amide is evident only in the octadecapeptides and nonadecapeptides as shown in Supporting Information, Figures S5 and S6.

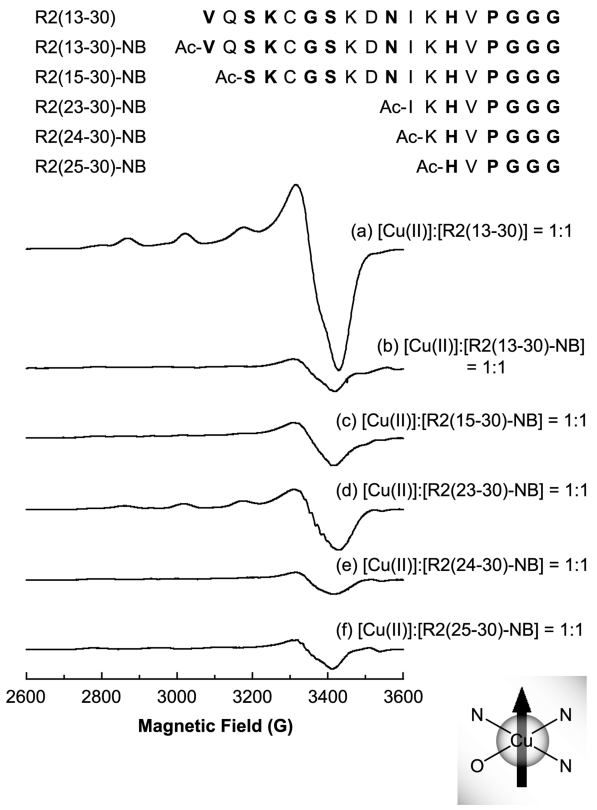

Figure 9. CW-ESR spectra of R2(13−30), R2(13−30)-NB, R2(15−30)-NB, R2(23−30)-NB, R2(24−30)-NB, and R2(25−30)-NB mixed with an equimolar amount of Cu(II). The amino acid sequences of the acetylated peptides are illustrated with conserved residues indicated in boldface. Most of the spectra of the tau fragments with the acetylated N-terminus have similar *g* values and *A* values. However, the intensities of the acetylated versions are significantly lower than their nonacetylated counterparts.

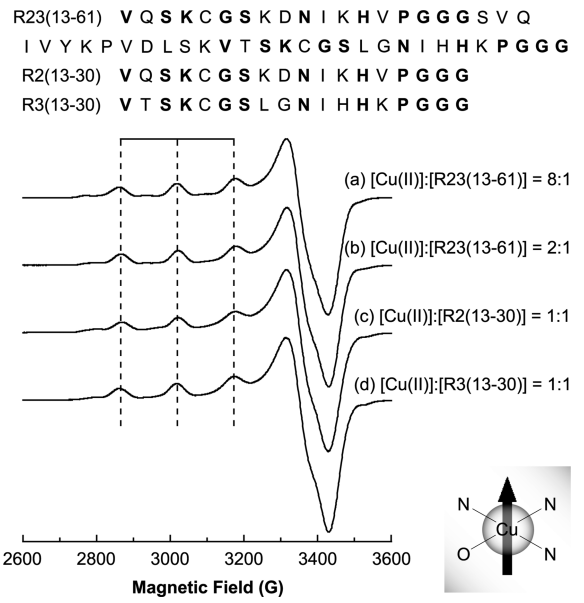

Figure 10. CW-ESR spectra of R23(13−61), R2(13−30), and R3(13−30) mixed with Cu(II). The amino acid sequences of the three peptides are illustrated with conserved residues indicated in boldface. All the spectra have almost identical ESR parameters such as *g* values and *A* values. Also, the double-integrated intensities of the spectra are not significantly different from one another in spite of different Cu(II)-to-peptide ratios.

While the Cu(II) coordination by a backbone carbonyl oxygen is evident from the ESEEM and HYSCORE spectra, a backbone amide nitrogen might be another directly coordinating atom. Burns et al.[48] have already shown that the second glycine residue of the PHGGGW sequence in the prion protein provides one nitrogen donor and one oxygen donor: the backbone amide nitrogen and the backbone carbonyl oxygen. Since the Cu(II) coordination by both the amide nitrogen and the carbonyl oxygen from the same residue forms a five-membered ring, conferring stability to the protein structure. Similarly, Dorlet et al.[54] have revealed that a five-membered ring can also be formed by the Cu(II) coordination by one nitrogen donor and one oxygen donor from an aspartate or an alanine residue. Therefore, it is possible that the residue of which the backbone carbonyl oxygen coordinates to Cu(II) also provides a nitrogen donor, that is, the backbone amide nitrogen, in the Cu(II)−octadecapeptide complexes.

All in all, it is concluded that the Cu(II)−octadecapeptide complexes have a specific conformation that is favorable for the Cu(II) coordination, and the N-terminus plays a role in the conformation. However, the identity of some amino acid residues that coordinate to Cu(II) is yet to be elucidated.

Cu(II)-Binding Site(s) in the Cu(II) Complexes of Longer Tau Fragments. In order to compare the Cu(II) coordination to the octadecapeptides and a longer tau fragment containing two octadecapeptides, R2 and R3, we also performed CW-ESR experiments on R23(13−61) mixed with Cu(II) at Cu(II)-to-peptide molar ratios of 2:1 and 8:1 and compared the spectra with those of the equimolar mixtures of Cu(II) and either R2(13−30) or R3(13−30). Figure 10 shows that the CW-ESR spectra of the mixtures of Cu(II) and R23(13−61) are similar to those of the equimolar mixtures in terms of ESR parameters including *g* values, *A* values, and linewidths. The similarity signifies that the longer peptide has a Cu(II)-binding site similar or identical to that of R2(13−30), R3(13−30), or probably the other two octadecapeptides. Interestingly, the intensities of the four spectra are almost the same even though two or eight equivalents of Cu(II) ions are added to the longer peptide, R23(13−61). Given the fixed CW-ESR instrumental parameters including the microwave power and modulation amplitude, the lack of significant difference in intensity suggests that the number of Cu(II)-binding sites in R23(13−61) is essentially the same as that present in either R2(13−30) or R3(13−30). Even if there is a second Cu(II)-binding site in R23(13−61), the binding affinity is in the millimolar range at best. On the assumption that the octadecapeptides have only one Cu(II)-binding site, our results support those of Soragni et al.,[23] who have found that even a longer tau fragment containing all of the four pseudorepeats coordinates to Cu(II) with a stoichiometry of 1.

Interestingly, in the case of the prion protein, which has five or more Cu(II)-binding sites,[63] the binding affinity decreases with the Cu(II)-to-peptide ratio. One explanation for the decrease in the binding affinity is negative cooperativity owing to either conformation changes or increase in the positive charge.[64] In a similar fashion, the relatively small number of Cu(II)-binding sites in the full-length tau protein might be partially due to negative cooperativity.

Also, pulsed ESR experiments such as ESEEM and HYSCORE were performed on the Cu(II) complex of R23(13−61).

The spectra reveal that at least one histidine imidazole and one backbone amide coordinate to Cu(II) in the Cu(II) complex of R23(13−61) as in the Cu(II) complex of the octadecapeptides. The ESEEM and HYSCORE spectra of the Cu(II)−R23(13−61) complex are shown in Supporting Information, Figure S9. We suspect that the histidine residue of R2 is likely to coordinate to Cu(II) in the Cu(II)−R23(13−61) complex because the histidine residue is more proximal to the N-terminus than the other histidine residues. The N-terminal amino group may help stabilize the structure of the complex in a fashion similar to that proposed in the case of the stable Cu(II)−octadecapeptide complexes. Accordingly, it is plausible that a longer tau fragment including all of the four pseudorepeats has only one binding site with a similar coordination environment where histidine imidazole and backbone amide are involved. Also, it is anticipated that the structure is stabilized by the N-terminal amino group or similar functional groups including the amino group of a lysine residue. However, if no amino group is available for stabilizing the Cu(II) complex of the full-length tau protein, the Cu(II)-binding site might be different from that expected on the basis of the data of the Cu(II) complexes of tau fragments. One possibility is that, in the full-length tau protein, residues from more than one pseudorepeat are involved in the Cu(II) coordination. Indeed, the results of Soragni et al.[23] suggest that residues from both R2 and R3 pseudorepeats are close to the Cu(II)-binding site in the full-length tau protein.

Finally, it is noteworthy that a histidine residue directly coordinates to Cu(II) in any fragments that contain the hexapeptide HVPGGG sequence. On the other hand, a backbone amide coordinates to Cu(II) only in some cases. As previously mentioned, the Cu(II)−amide coordination is observed in a number of stable Cu(II) complexes of tau fragments. Therefore, in the full-length tau protein, at least one histidine residue and some functional groups that may stabilize a Cu(II)−amide coordination are likely to be involved in the Cu(II) coordination. While amino group(s) may be indirectly involved, possible effects of sulfhydryl group(s) on the Cu(II) coordination cannot be ruled out. Even though the roles of the cysteine residues in the Cu(II) complexes of tau fragments are not clearly seen in our data, the potential involvement of the cysteine residues is suggested by the work of other researchers.[23,29]

SUMMARY

In this article, we find out that each octadecapeptide in the four pseudorepeats of tau protein coordinates to Cu(II) with a binding affinity similar to that of Aβ(1−16) peptide and provide information about the probable Cu(II) binding site(s) by employing several ESR techniques. Our CW-ESR results reveal that the four octadecapeptides have similar binding site(s). Also, our ESEEM and HYSCORE results show that a nitrogen atom of the histidine imidazole and a carbonyl oxygen of a backbone amide coordinate to Cu(II). The data from further ESR experiments on some modified tau fragments suggest that the existence of a free N-terminus along with a specific conformation is also essential to the high Cu(II)-binding affinity. In addition, we propose, on the basis of our ESR results on the 49-amino acid peptide that contains two of the four octadecapeptides, that a longer peptide with the four pseudorepeats may have Cu(II)-binding site(s) in which the functional groups involved in the Cu(II) coordination to the octadecapeptides play a crucial role. Our findings are meaningful in that they furnish an atomic-level insight into the Cu(II) coordination of tau protein, an intrinsically disordered protein.

ASSOCIATED CONTENT

Supporting Information. CW-ESR simulation (Figure S1 and Tables S1 and S2), CW-ESR spectra of the octadecapeptides mixed with Cu(II) at different ratios (Figure S2), detailed information about three-pulse ESEEM spectra of the equimolar Cu(II)−R1(13−30) complex (Figure S3), detailed information about three-pulse ESEEM simulation (Figure S4 and Table S3), three-pulse ESEEM and HYSCORE spectra of the Cu(II) complexes of tau fragments in R2 (Figures S5 and S6), comparison of CW-ESR spectra of the Cu(II) complexes of tau fragments in R2 (Table S4), mass spectra of the equimolar Cu(II)−R2(13−30) and Cu(II)−R3(13−30) complexes (Figure S7), electrophoretogram of the equimolar Cu(II)−R2(13−30) and Cu(II)−R3-(13−30) complexes (Figure S8), and three-pulse ESEEM and HYSCORE spectra of the Cu(II)−R23(13−61) complex (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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