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Peptide Interactions

Pulse EPR Spectroscopy Reveals the Coordination Sphere of Copper(II) Ions in the 1–16 Amyloid-β Peptide: A Key Role of the First Two N-Terminus Residues**

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Alzheimer's disease (AD) is characterized by the deposit of extracellular amyloid plaques, which consist predominantly of the aggregated peptide amyloid-β (Aβ). The aggregation mechanism is not very well understood, but it is proposed that aggregated forms of Aβ are neurotoxic because of the production of reactive oxygen species (ROS). Copper ions have been found to accumulate in amyloid plaques. Moreover, Cu ions have been proposed to be linked to the aggregation of the Aβ peptide and/or to the increased generation of ROS.[1,2] In this context, Cu^{II} coordination to Aβ becomes a key feature, because it will directly affect the structure of the peptide and, hence, the aggregation behavior. The catalytic ROS production of the Cu^{II} ions coordinated by Aβ will also be governed by the binding mode. Therefore, this system has been extensively studied. However, the unambiguous identification of the Cu^{II} ligands has remained difficult and no real consensus has emerged in the literature yet.[3] It has been shown that all of the ligands of the high affinity Cu^{II} site are contained in the Aβ16 N-terminal domain (DAEFRHDSGYEVHHQK);[4] hence, this soluble fragment has been widely used as a model for the Cu^{II} binding site in the native Aβ40/42 peptides. In addition, pH-dependence studies of Cu^{II} coordination to Aβ revealed that two complexes, distinguishable by conventional 9 GHz EPR spectroscopy,[5–7] are present near physiological pH value;[8] these complexes are often called components I (lower pH values) and II (higher pH values). This rendered the analysis of the results difficult and impaired the unambiguous identification of the Cu^{II} ligands. Earlier works focused on spectroscopic characterizations of native and chemically modified Aβ peptides (mutations, NH_2-terminus acetylation, histidine methylation, peptide truncation, etc.) and the ligands were deduced from the changes observed.[4–7,9] Ambiguities remained nonetheless, because those spectroscopic changes can also be induced by indirect effects. Recent studies used specific isotopic labeling to detect direct interactions with the native Aβ sequence by using pulse EPR techniques such as ESEEM and HYSCORE.[10–12] Shin and Saxena proposed three histidines bound at pH 7.4,[10] whereas Drew et al. proposed the binding of two histidines and aspartate 1 (D1) by its N terminus and side-chain carboxylate group at pH 6.3–6.9 and of three histidines and the carbonyl group of alanine 2 (A2) at pH 8.[11,12]

In the present work, we used a wide range of EPR methods, including continuous wave (cw), ESEEM, 4-pulse HYSCORE, and, for the first time with these complexes, pulse ENDOR and 6-pulse HYSCORE, combined with specific isotopic labeling to study Cu^{II}–Aβ16. The use of these multiple EPR techniques has the advantage of detecting couplings of atoms directly bound to the Cu^{II} ion (ENDOR, cw), as well as couplings to nuclei further away (HYSCORE, ESEEM). In order to study components I and II separately, we used a similar approach to that in our previous study of prion peptides[13] and worked at selected pH values (6.5 and 9.0) for which the EPR spectra indicated a single predominant species.[14] A set of specifically isotope-labeled Aβ16 peptides comprising uniformly ^{13}C- and ^{15}N-labeled D1, ^{15}N-labeled A2, and uniform ^{15}N labeling for each of the three histidine residues was used.

To study component II, the Davies ENDOR spectra shown in Figure 1 have been obtained at pH 9.0 on unlabeled, labeled-D1, and labeled-A2 samples. ENDOR resonances are observed over the range 10–40 MHz. In order to minimize proton contributions, the samples were prepared in a fully deuterated medium to eliminate exchangeable protons and a hard inversion pulse was used to lower signals from weakly coupled nuclei.[15] The resonances observed therefore arise primarily from nitrogen nuclei in the strong coupling regime for which the ENDOR signal is centered at half the hyperfine-coupling value and split by twice the Larmor frequency. Despite the low resolution of the spectra due to overlapping signals from the various interacting nuclei, it is clear that the spectra recorded for the labeled-D1 and -A2 samples differ from that obtained for the unlabeled sample (Figure 1b and c). When the ^{14}N nucleus is replaced by an ^{15}N nucleus in the labeled samples, part of the signal is shifted towards a higher frequency, which indicates a stronger coupling, as expected

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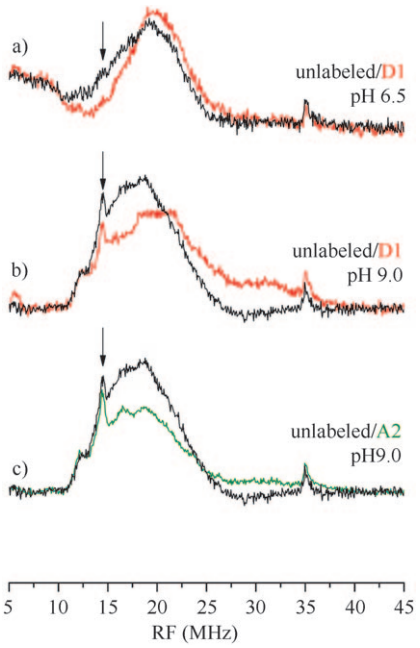

Figure 1. Davies ENDOR spectra recorded at $g_\perp$ for the unlabeled Cu^{II}–Aβ16 (black) and for the labeled-D1 (red, a and b) and the labeled-A2 samples (green, c) in D_2O. Experimental conditions: microwave frequency of 9.76 GHz; $T = 15$ K; magnetic field of 340.7 mT; inversion pulse length of 32 ns. Arrows indicate the ^1H Larmor frequency.

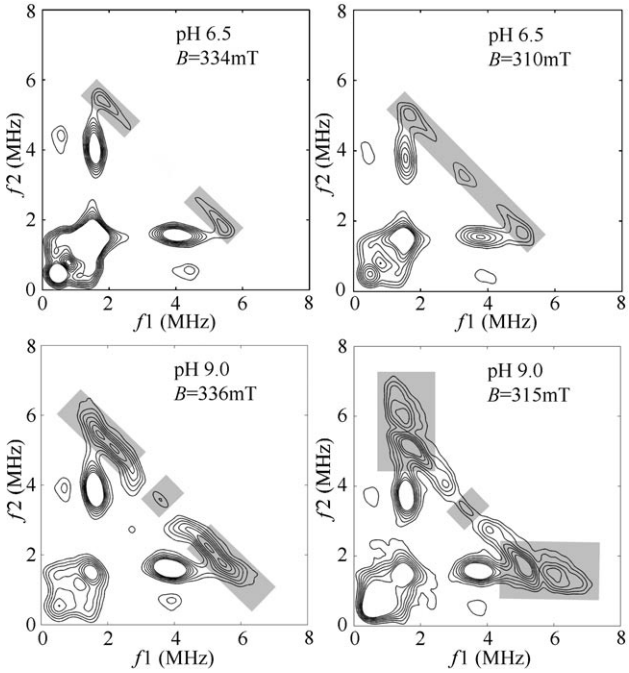

Figure 2. 4-pulse HYSCORE (++) quadrant contour plots for labeled-D1 Cu^{II}–Aβ16 at $g_\perp$ (left panels) and near $g_\parallel$ (right panels). The areas shaded in gray indicate the signals that are not present in the unlabeled samples but are detected in the labeled-D1 samples. Experimental conditions for all spectra: microwave frequency of 9.66 GHz; $T = 4.2$ K. Spectra are the sum of six different spectra recorded with different tau values from 104 to 264 ns by steps of 32 ns. Contour levels should not be compared between the different spectra.

from the nuclear g (g_n) values for both nuclei. This is direct evidence that both the NH_2 terminus and the amide nitrogen atom of A2 are equatorial ligands to the Cu^{II} ion in component II. This result is also supported by changes observed in the EPR spectra.[14]

As we reported before,[16] the 3-pulse ESEEM experiments indicates that only one histidine residue is bound in an equatorial position at pH 9.0, because no combination line is observed compared to what would be expected if several histidine residues were bound simultaneously (Figure S2 in the Supporting Information). The HYSCORE experiments performed on the labeled-histidine peptides lead to the detection of ^{15}N signals in all three cases with no strong advantage of one histidine over the other two.[14] This indicates that all three histidine residues are in equilibrium as the ligand of the Cu^{II} ion in component II. In addition to the histidine signal, another weakly coupled nitrogen nucleus is detected, in both the ESEEM and HYSCORE spectra (Figure 2). In the HYSCORE spectra, a set of cross-peaks is observed for the unlabeled peptide at (4.3, 2.8 MHz), in agreement with the study by Drew et al.[11] The shape and the position of this feature are strikingly comparable to those observed by Burns and co-workers for an amide nitrogen atom of the peptide backbone if the adjacent carbonyl function is coordinated to the Cu^{II} ion in equatorial position.[17] Based on the fact that EPR and ENDOR measurements have shown that the NH_2-terminus nitrogen atom and the amide nitrogen atom of A2 are sequential equatorial ligands of the Cu^{II} ion at pH 9.0 and on the similarity of the peptide coiling around the metal ion in the prion analogue,[17] it follows that the carbonyl group of A2 should be the subsequent ligand of the Cu^{II} ion in component II, with the backbone nitrogen atom of E3 as the origin of the nitrogen signal observed here with pulse EPR spectroscopy. Indeed, this has now been proven with adequate labeling.[12]

When the HYSCORE experiment is performed on the labeled-D1 peptide, all of the features present in the unlabeled peptide are retained and additional ridges centered on the ^{13}C Larmor frequency ($\nu_n(^{13}C) = 3.6$ MHz at 336 mT and 3.4 MHz at 315 mT) are readily observed (Figure 2). In addition, the spectra clearly indicate that two distinct carbon nuclei are detected. Both the NH_2 terminus and the amide group of A2 have been proved to be coordinated to the Cu^{II} ion, so these signals can be attributed to the C_α and C_δ atoms of D1 (Scheme 1). The equatorial ligands of the Cu^{II} ion in component II are therefore the NH_2 terminus, the deprotonated amide and the carbonyl groups of A2, and one histidine. In addition, a ^{13}C signal is also observed at the Larmor frequency, which indicates a weakly coupled nucleus; this is consistent with the binding of the carboxylate side chain of D1 in an axial position.

At pH 6.5, the Davies ENDOR spectrum also demonstrates the binding of the NH_2 terminus as an equatorial ligand (Figure 1 a). The smaller range spanned by the nitrogen resonances compared to that at pH 9.0 indicates that the coupling of the terminus nitrogen atom is smaller in component I, which supports a less-tight binding than that

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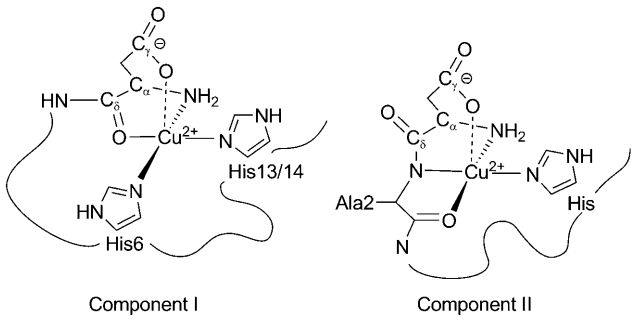

Scheme 1. The Cu^{II}–Aβ16 interaction at the physiological pH value.

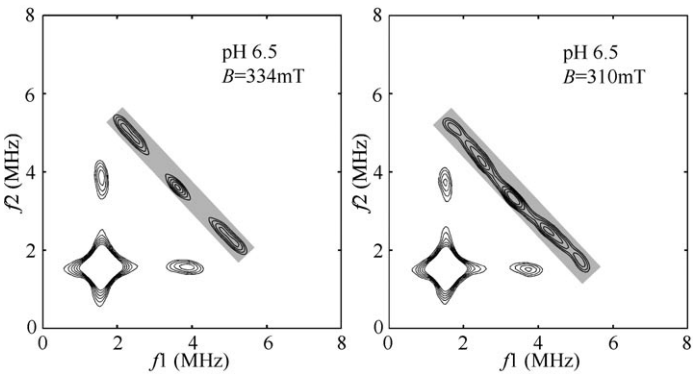

Figure 3. 6-pulse HYSCORE (++) quadrant contour plots for labeled-D1 Cu^{II}–Aβ16 at $g_\perp$ (left panel) and near $g_\parallel$ (right panel). The areas shaded in gray indicate the signals that are not present in the unlabeled samples but are detected in the labeled-D1 samples. Experimental conditions: microwave frequency of 9.66 GHz; $T = 4.2$ K; $\tau_1 = 68$ ns, $\tau_2 = 108$ ns.

in component II. The 3-pulse ESEEM experiments indicate that at least two histidine residues are bound.[14,16] The HYSCORE spectra recorded on the labeled-histidine peptides indicate that all three residues participate in the equatorial coordination sphere, but not simultaneously. Indeed, a comparison of the ^{15}N and ^{14}N cross-peaks indicates that the intensity for H13 and H14 is comparable and about half of that for H6.[14] Together with the 3-pulse ESEEM spectra, these results strongly support the simultaneous binding of two histidine residues, H6 on one position and either H13 or H14 on the second position, in agreement with the study by Drew et al.[11]

In addition to the histidine signals, another set of cross-peaks at (4.3, 2.8 MHz), again strongly similar to the resonances of an amide nitrogen atom, is clearly apparent in the HYSCORE spectra of the labeled-histidine peptides. This signal, which has shallow modulations, is not detected in the unlabeled sample. By contrast, the signals from the histidine nitrogen atom are deeply modulated. The depth of those modulations is further increased in this case with the simultaneous binding of two histidine residues. In 4-pulse HYSCORE experiments, it has been shown that cross-peaks from nuclei with shallow modulations can be suppressed by nuclei with deep modulations.[18] In certain cases, the 6-pulse sequence can be used to avoid this problem, as described recently.[19] We therefore recorded the 6-pulse HYSCORE spectrum on the unlabeled Cu^{II}-Aβ16 complex at pH 6.5 and detected the cross-peaks from the amide ^{14}N nucleus (Figure S9 in the Supporting Information). These cross-peaks are not detected on the labeled-A2 sample and we are therefore attributing them to the nitrogen atom of A2.[14] We note that the low-frequency ^{15}N signal in the labeled-A2 sample was not concomitantly detected in the 6-pulse HYSCORE spectrum as expected. However, this could be due to other suppression effects, because the ^{15}N signal of the labeled-H14 sample was also not detected in the 6-pulse HYSCORE spectrum (Figure S9 in the Supporting Information). Through comparison with what was described at pH 9.0, we propose that the carbonyl group of D1 is an equatorial ligand of the Cu^{II} ion in component I. When HYSCORE experiments were performed on the labeled-D1 peptide, ridges centered on the ^{13}C Larmor frequency were detected (Figure 2). Again, although readily detectable, the intensity of the ^{13}C cross-peaks was low compared to that of the histidine nitrogen signals, but it was significantly increased by using 6-pulse HYSCORE (Figure 3). The contour plot obtained near $g_\parallel$ in this case indicates that three ^{13}C nuclei are detected. The two with stronger couplings (cross peaks at (5.1, 1.8 MHz) and (4.3, 2.5 MHz)) are attributed to the C_α and C_δ atoms following the binding of the NH_2 terminus and the carbonyl group from D1, whereas the third one, with a weaker coupling showing only around the ^{13}C Larmor frequency, is attributed to the C_γ atom; this result supports the binding of the carboxylate side-chain of D1 in an axial position.

By using a wide range of advanced EPR techniques in conjunction with specific isotopic labeling, we were able to directly detect and thus identify the ligands of the Cu^{II} ion coordinated to Aβ16 in the two forms relevant at physiological pH value, component I and component II. In component I, two histidines, the NH_2 terminus, and the carbonyl group from D1 compose the equatorial coordination plane, while the side-chain carboxylate group of D1 is in an axial position. In component II, the equatorial ligands are the NH_2 terminus, the amide and carbonyl groups of A2 (due to deprotonation of the amide nitrogen atom of A2 upon pH increase), and one histidine. The side-chain carboxylate group of D1 is still present in an apical position. Our model for component I mostly agrees with the data reported by Drew et al.,[11,12] except that distinct detection of the ^{13}C nuclei and ^{14}N amide signatures with 6-pulse HYSCORE allowed us to place the carbonyl group of D1 as the equatorial oxygen ligand, while the side-chain carboxylate group of D1 is bound in an axial position. By contrast, our structural model for component II is significantly different from that of Drew et al.[11,12] Indeed, they failed to detect ^{13}C signals from D1, concluded the non-coordination of the NH_2 terminus, and proposed the simultaneous binding of the three histidine residues instead. Such a coordination sphere is unable to explain the variation of $g_\parallel$ and $A_\parallel$ ($A_\parallel$) for the Cu^{II} ion observed in the cw EPR spectra of the two components and is in contradiction with the 3-pulse ESEEM results with regard to the number of histidine residues bound. In addition, it is also mismatched with other results from the literature, with

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which our model agrees well: 1) binding of the NH_2 terminus in component II explains the observations made on acetylated Aβ[6,8,9] and is consistent with the fact that the NH_2 terminus is also an equatorial ligand at lower (pH 6.5) and higher (pH 11)[14] pH values; 2) coordination of the deprotonated amide group from A2 agrees with an earlier potentiometric study,[8] with the study by Karr and Szalai showing that the pK_a value of this deprotonation is sensitive to mutation on D1[5] and with the detection of an amide-to-Cu^{II} transition in the CD spectrum;[6,8] 3) the presence of only one histidine is supported by the spectroscopic signature detected for the truncated Aβ10 peptide missing H13 and H14, which is similar to that of the native peptide.[8,20] The structural models we have determined here are in agreement with most of the results obtained in the literature regarding chemical modification or mutation of D1.

The unambiguous identification of the Cu^{II} ligands in components I and II achieved in this study underlines the key role of residues D1 and A2 in the coordination of the metal ion. A recent NMR study showed that for the rat, which does not develop AD, the Cu^{II} coordination of the carboxylate group from D1 is indirect.[21] Hence, the difference between humans and rats in the development of AD could be the nature of the Cu^{II} coordination by D1. More importantly, the D1 and A2 residues may also have a predominant role in the aggregation process of Aβ, as evidenced in the study of Aβ40(D1E/A2V).[22] Further work will be aimed at probing the dynamic nature and related reactivity of the coordination of these two residues to the Cu^{II} ion.

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