Peptide Binding

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Deprotonation of the Asp1–Ala2 Peptide Bond Induces Modification of the Dynamic Copper(II) Environment in the Amyloid-β Peptide near Physiological pH**

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Aggregation of the amyloid-β (Aβ) peptide and the production of reactive oxygen species by aggregates are two key features in Alzheimer's disease.[1] Copper ions have been linked to both of these events,[2,3] and hence determination of the basic interaction of Cu and Aβ is essential for understanding its roles in the development of the pathology. The native Aβ peptides consist of 39 to 43 amino acid residues and have been shown to be strongly prone to aggregation (from a few μM concentration). However, the Cu^{II} binding site has been localized in the N-terminal part of the peptide encompassing the first 16 amino acid residues (see Scheme S1 in the Supporting Information for the peptide sequence),[4,5] a truncated peptide that is highly soluble. Hence, this shortened peptide is accepted as a valuable model of Cu^{II} coordination to full-length Aβ and its high solubility allows classical spectroscopic methods, such as those of the present study, to be used. While most techniques aim at identifying the Cu^{II} ligands (for a review, see reference [6] and for very recent reports, see references [7,8]), NMR spectroscopy is among the few methods also able to reveal dynamical processes in the coordination of Cu^{II} to Aβ.

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Indeed, the paramagnetism of the Cu^{II} ion induces an enhancement of the relaxation rate of the peptide nuclei, this effect diminishing according to the inverse sixth power of the interatomic distance (for reviews, see references [9,10]). Consequently, selective broadening of the NMR signals of nuclei spatially close to the metal-ion binding site(s) is observed. In the case of Cu^{II}, the line broadening is severe and the effect of the largely substoichiometric ratio of the paramagnetic ion is detectable in the case of fast exchange of the paramagnet between sites. This is also true for ^{13}C NMR signals despite the lower sensitivity to broadening effects for this nucleus as a result of its lower gyromagnetic ratio compared to that of the proton.

As concerns Cu^{II} coordination to Aβ, only a few NMR studies have been reported and they are limited to ^{1}H NMR[11,12] or ^{1}H–^{15}N heteronuclear single quantum correlation (HSQC) experiments.[13,14] Fast amide proton exchanges are responsible for the loss of the signals of several amino acids (including Asp1 and the three His residues) in apo–Aβ peptide in the latter cases, an effect that precludes the analysis of Cu^{II}-induced signal broadening. For those reasons, herein we focus on $^{13}C\{^{1}H\}$ NMR spectroscopy, which is a straightforward way to inspect the effect of Cu^{II} on Aβ peptide signals. Furthermore, it is known that near physiological pH, two Cu^{II} complexes of Aβ coexist, which differ in the protonation state of the peptide and their spectroscopic signatures.[6,15] They are referred to below as "low-pH" and "high-pH" species. We identify the amino acid residues involved in Cu^{II} binding, and give clear-cut evidence for the presence of equilibria between different ligands in both forms. We also give new insights into the dramatic change undergone by the Cu^{II} binding sites in Aβ between pH values of about 6.6 and 8.7, which arises from the deprotonation and binding of the Asp1–Ala2 peptide bond amide.

Figure 1 shows the evolution of the $^{13}C\{^{1}H\}$ NMR spectra of the Aβ peptide (sequence DAEFRHDSGYEVHHQK) upon addition of 0.1 equivalents of Cu^{II} at pH 6.6 and 8.7 (see also Figure S4 in the Supporting Information for spectral domains that concern His residues).[16,17] Addition of Cu^{II} leads to broadening of several signals that is more selective at high pH (right-hand spectra in Figure 1) with only Asp1, Ala2, and the side chain of His mainly affected. More precisely, at pH 6.6 peaks of the carboxylate groups from Asp1, Asp7, Glu3, Glu11, and the unprotected C terminus are significantly broadened with a slight preference for that of Asp7. Only that of Asp1 is broadened at pH 8.7. Peaks of the

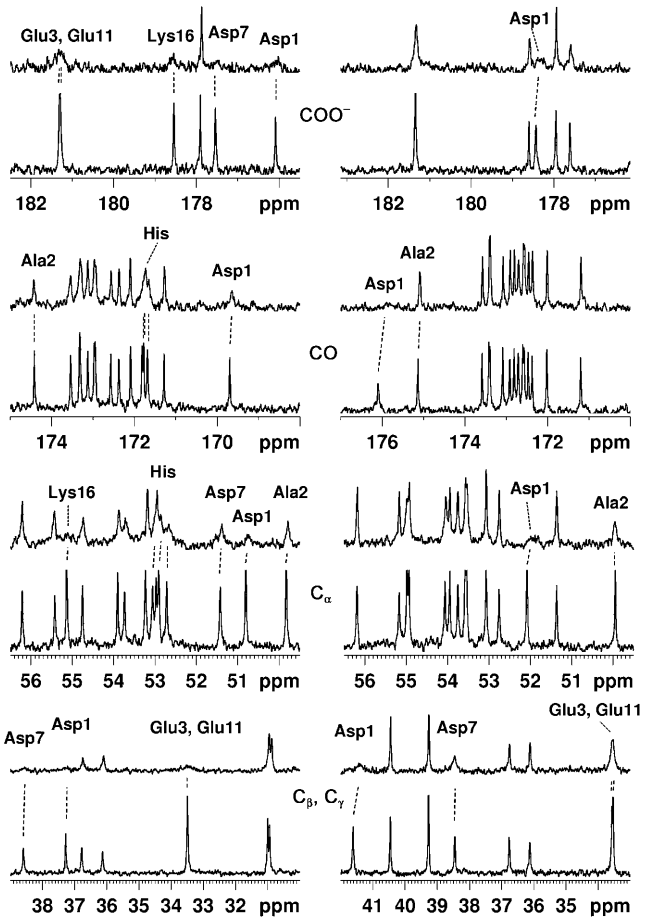


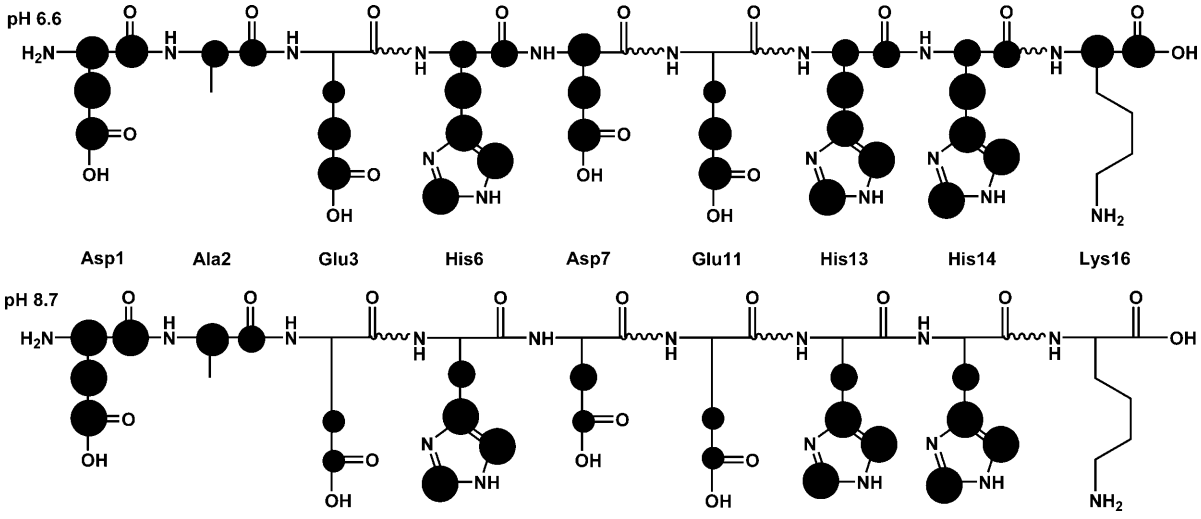
Figure 1. $^{13}C\{^1H\}$ NMR shifts of 10 mM Aβ peptide in D_2O (bottom spectra) and in the presence of 0.1 equiv of Cu^{II} (top spectra) at pH 6.6 (left) and 8.7 (right). $T = 25\,°C$, $\nu = 125.8$ MHz. The shift of some peaks is a result of slight modification in the pH value induced by Cu^{II} addition.[16]

carbonyl functions from Asp1, His6, His13, and His14, and to a lesser extent from Ala2, are affected at pH 6.6.

Increasing the pH to 8.7 leads to a more pronounced broadening of the carbonyl peak of Asp1, the peaks of the His carbonyl are no longer affected, while that of Ala2 is as broadened as at pH 6.6. Peaks of the C_α atom from Asp1, Asp7, Ala2, Lys16, and the three His residues, of the C_β atom from Asp1, Asp7, Glu3, and Glu11 (Supporting Information, Figure S4), and of the C_γ atom from Glu3 and Glu11 are all broadened at pH 6.6, whereas the broadening remains significant only on peaks of Asp1 and Ala2 and to a lesser extent on the C_β peak of Asp7 at pH 8.7. 2D ^1H–^{13}C experiments were also performed and the results are consistent with those from the $^{13}C\{^1H\}$ NMR experiments (Supporting Information, Figures S6–S11). Scheme 1 summarizes the 1D and 2D NMR results and gives a qualitative view of the atoms for which the NMR signals are broadened by the addition of Cu^{II}.

At pH 6.6, the broadenings observed support the involvement of the side chains of Asp1, Asp7, Glu3, Glu11, His6, His13, and His14, the carbonyl groups from Asp1, Ala2, Lys16, and the three His residues, and the N and C termini in Cu^{II} coordination, thus inducing the presence of equilibria between several binding sites. The backbone carbon nuclei of Lys16 are affected, but this may not be relevant for the full-length peptide because the C terminal was not protected. At pH 8.7, Asp1, the imidazole rings of the three His residues, and to a lesser extent Ala2 are the functions mostly affected. Hence, when the pH is increased the Cu^{II} binding site is shifted toward the N-terminal part of the peptide with side chains of His in equilibrium to complete the coordination sphere. The propensity of several equivalent ligands to be in equilibrium for Cu^{II} binding is still observed but there are fewer conformers than at pH 6.6.

To gain complementary insights into the Cu^{II} coordination, spectra were analyzed in the X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) regions. EXAFS data (Supporting Information, Figure S12) are best fitted with five N/O neighbors, one being significantly more distant than the other four, which indicates a square-base pyramid (SBP) geometry for

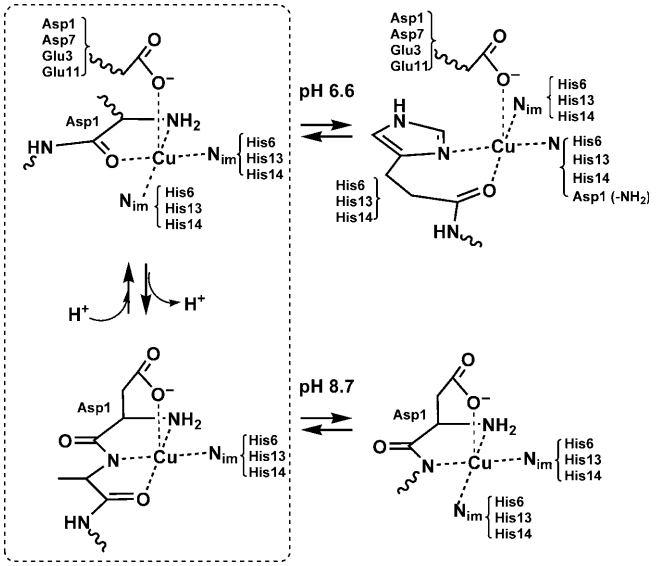


Scheme 1. Schematic representation of the regions in Aβ peptide most affected by the addition of Cu^{II}. The size of the black circles increases according to the importance of the broadening of the NMR signal.

both the low-pH and high-pH forms. Qualitative XANES analysis fully agrees with the SBP geometry determined by EXAFS (see Figure S13 in the Supporting Information and the corresponding description). Although EXAFS can hardly distinguish between N and O atoms because of their similar amplitude and phase functions, best fits are obtained in the present case when taking a $(3N1O)_{eq}(1O)_{ap}$ (eq = equatorial, ap = apical) first coordination shell at pH 6.6 and either a $(3N1O)_{eq}(1O)_{ap}$ or a $(4N)_{eq}(1O)_{ap}$ shell at pH 8.7.

Cu–neighbor distances are given in Table S3 in the Supporting Information. The most significant differences observed between the two pH values is the shortening of the Cu–O apical distance (from 2.46 to 2.34 Å) and of the associated Debye–Waller factor (from 0.010 to 0.002 $Å^2$) when increasing the pH. At pH 6.6, these EXAFS results best agree with those of Shearer and Szalai[18] who ruled out the possibility of a fifth Cu^{II} neighbor within 2.1 Å, as was previously proposed by other groups.[5,19] However, Shearer and Szalai did not detect a fifth ligand even further away,[18] a discrepancy with the present work that may be related to differences in recording conditions and/or analysis methods.

The results obtained in the present study are most consistent with the coordination models proposed in Scheme 2. We note that: 1) on the basis of size-exclusion chromatography, $Cu^{II}(A\beta)$ species are mainly mononuclear and even with a substoichiometric ratio of Cu^{II}, no Cu^{II}-bridged peptide complexes were detected;[16] and 2) since fast exchange of the Cu^{II} ion is observed between peptides, broadening of signals from peptide groups may occur either because they are a ligand of the Cu^{II} ion in the mononuclear complex (main effect), or because they are transiently involved in the transfer of the Cu^{II} ion between one peptide and another. In the latter case, side chains may have a predominant role because of their enhanced accessibility compared to backbone groups.



Scheme 2. Proposed binding sites for Cu^{II} in Aβ at pH 6.6 and 8.7. These models well match the present results and literature data (see text for details) but they are not the only ones consistent with our data. At pH 6.6 and 8.7, several species are in equilibrium but the deprotonation event occurs on the Asp1–Ala2 bond (dotted box).

At low pH (ca. 6.6), the three N atoms are provided either by the NH_2 terminus and two out of the three His side chains or the three His side chains, in line with the two most pertinent models discussed in the literature[6] and in agreement with the pK_a values of the ionizable peptide groups.[16] However, involvement of the NH_2 instead of a third His is favored on the basis of the 285 nm band detected in the circular dichroism (CD) spectrum[15,16] (Supporting Information, Figure S2). O atoms from either carboxylate side chains or carbonyl groups occupy the last equatorial and the apical positions. From the reported coiling of prion peptides around Cu^{II},[20,21] the situation in which the carbonyl groups occupy the equatorial position may be favored. The carbonyl groups adjacent to the NH_2 or the His side chains may then be in equilibrium in the fourth equatorial position, thus creating five- and seven-membered metallacycles, respectively. Consequently, the carboxylate groups are in equilibrium in the apical position, with a preference for that of Asp7.

At high pH (ca. 8.7), the situation is significantly different and the Cu^{II} ion is mainly bound via ligands from Asp1, the side chain of the three His residues, and to a lesser extent Ala2. The presence of a deprotonated amide function is deduced from the observation of the 314 nm band in the CD spectrum[15,16] (Supporting Information, Figure S2). As a consequence, NH_2 and the deprotonated amide as well as the carboxylate group from Asp1 are bound, together with at least one His side chain. As the broadening is stronger for the carbonyl group from Asp1 and for C_α(Ala2), the deprotonation of the amide function is thought to happen on the Asp1–Ala2 peptide bond rather than on peptide bonds adjacent to His. This may be because of the tighter metallacycle thus obtained.

Formation of the $\{NH_2, N^-, COO^-\}$ tripod pincer that would ground the Asp1 carboxylate group and the absence of equilibrium between the five possible carboxylates for the apical position agree with the detection of a strongly decreased Debye–Waller parameter at pH 8.7 compared to pH 6.6 in the EXAFS data. Logical candidates for the remaining equatorial position are the carbonyl group from Ala2, thus reproducing previously described peptide coiling around Cu^{II},[20,21] or a second His side chain. Very few results are reported in the literature concerning this high-pH species.[8,11,15] One recent study on labeled peptides by pulsed EPR techniques reveals that the carbonyl group from Ala2 is bound to Cu^{II}, in line with our observation.[8] However, the three His side chains are then proposed to complete the equatorial Cu coordination, a result in contradiction with other published data,[11,15] which proposed the coordination of a deprotonated amide function. The results obtained in the present study confirm the involvement of a deprotonated amide function in the Cu^{II} coordination and favor its identification as originating from the Asp1–Ala2 bond.

The results described herein are a new step towards the understanding of the complex picture of Cu^{II} coordination to Aβ. Experiments were all performed at room temperature but the results are in line with most of the spectroscopic data on the low-pH and high-pH species reported in the literature for frozen-solution samples, and bring insights into the deproto-

nation of the Asp1–Ala2 peptide bond. More importantly, NMR spectroscopy reveals the presence of equilibria between equivalent ligands on a given coordination position of the Cu^{II} ion that can hardly be observed with other techniques. Such equilibria are mostly present in solution at pH 6.6, while at pH 8.7 only the side chains of His compete for an equatorial position. The difference in the number of equivalent Cu^{II} binding sites detected for the two forms present at physiological pH, as well as the strong reorganization of the Cu^{II} site towards the N-terminal portion of the peptide when increasing the pH, may be anticipated to contribute to the different aggregation propensities of the two forms.

The presence of equilibria between different ligands observed at pH 6.6 for human Aβ also differs from the unique binding set in rat Aβ,[22] a mammal that does not develop Alzheimer's disease. Again, this may be related to different aggregation behavior. The presence of an Asp residue in position 1 in the peptide sequence can be linked to the high pH value of the NH(Ala2) deprotonation, in line with the NMR detection of an interaction with the carboxylate side chain (Supporting Information, Figure S15 and Scheme S3). This is also fully consistent with the pH-dependent EPR study of D1N-Aβ mutant, which revealed that in the Cu^{II}(D1N-Aβ) case, deprotonation occurs at lower pH than in the native species.[23] The pK_a(NH/N^-(Ala2)) value may be crucial for the aggregation process. Indeed, it has been shown that N-terminally truncated (Aβ3–40/42) and the resulting pyroglutamate forms show stronger aggregation behavior and cell toxicity than the full-length Aβ40/42 peptides.[24] On the basis of the binding models proposed here, these two truncated species may be more easily obtained from the high-pH form of the Cu^{II}(Aβ) complex as a result of Cu^{II}-assisted amide-bond hydrolysis,[25] the proportion of which is controlled by the pK_a(NH/N^-(Ala2)) value.

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