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Disease Mechanisms

Copper(II) Coordination to Amyloid β: Murine versus Human Peptide**

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In Alzheimer's disease (AD), the amyloid β (Aβ) peptide seems to play a causative role. Aβ is the major constituent of amyloid plaques, a hallmark of AD. According to the amyloid cascade hypothesis, in AD, the aggregation of Aβ leads to the formation of toxic species, which induce neuronal cell death. It has been proposed that reactive oxygen species (ROS) are produced, and that these species mediate cell toxicity.[1,2] Although still under debate,[3] a large body of evidence suggests that metallic ions (copper, zinc, and iron) play a role in the etiology of AD.[3–6] For example, amyloid plaques extracted from human brains contain high amounts of Cu^{II} and Zn^{II} ions[7] bound to the Aβ peptide.[8,9] Chelators were able to partially solubilize the plaques,[8] and studies on neuronal cell culture and transgenic mice supported the involvement of ions in Aβ metabolism.[10,11] Copper(II) can be released in the synaptic cleft and can reach concentrations up to 15 μM.[5] This value is in line with the possibility of Cu^{II} binding to Aβ in vivo, since a dissociation constant in the picomolar range has been determined for the Cu^{II}–Aβ species.[12] Furthermore, in vitro aggregation of the Aβ peptide can be modulated by Cu and Zn ions,[12–14] and because of its redox nature, Cu may play a role in ROS production.[15] These observations and hypotheses explain the intensive research on the modulation of metal-ion homeostasis as a therapeutic approach.[3,16]

A better understanding of the AD mechanisms requires investigations on mouse and rat animal models.[17,18] However, these animals, whose peptide differs from the human Aβ peptide by three point mutations, do not show amyloid deposition.[17,18] Consequently, studies are performed on transgenic mice or rats that produce the human Aβ (hAβ) peptide in addition to their own peptide (mAβ). Cu^{II} coordination to murine and human peptides has been proposed to differ.[19–21] Thus, in the present study, to explain the distinct Cu^{II} coordination to hAβ and mAβ, we used complementary spectroscopic techniques to determine the crucial mutation(s). We also propose Cu^{II}–mAβ structural models. Finally, we discuss possible consequences of such differences in Cu^{II} coordination with respect to the use of mice or rats as AD animal models.

We examined the coordination of Cu^{II} to six peptides: hAβ (DAEFRHDSGYEVHHQK; see Scheme S1 in the Supporting Information), Y10F-hAβ, H13R-hAβ, R5G-hAβ, mAβ (DAEFGHDSGFEVRHQK; see Scheme S2 in the Supporting Information), and F10Y-mAβ (or R5G-H13R-hAβ). These shorter 16-residue peptides were used as valuable models of Cu^{II} binding to the full-length peptides.[22–24] Indeed, no differences in spectroscopic signature,[22] binding affinity,[24] or ROS production[23] have been observed between the truncated and full-length hAβ peptides. Two peptide families can be distinguished from the spectroscopic signatures of their Cu^{II} complexes (see Figures S1–S5 and Table S1 in the Supporting Information): hAβ, Y10F-hAβ, and H13R-hAβ (humanlike family), and mAβ, F10Y-mAβ, and R5G-hAβ (murine-like family). Thus, the key mutation between the hAβ and mAβ peptides with regard to Cu^{II} binding is the R5G mutation. For both families, two Cu^{II} complexes that differ in the protonation state of the peptide are present near the physiological pH value, namely, components **I** and **II**. Figure 1 shows the differences between the CD and EPR spectroscopic signatures of Cu^{II}–hAβ and Cu^{II}–mAβ solutions at pH 6.7 and 5.4, at which **I** is predominant, and at pH 8.7 and 7.6, at which **II** is predominant. The pKa(**I**/**II**) values are close to pH 7.7 for Cu^{II} complexes of the human-like peptides and close to pH 6.2 for the murine-like family (see Figures S3 and S5 and Table S1 in the Supporting Information).

We previously described copper(II)-induced modification of the peptide NMR spectroscopic signature to determine the Cu^{II}-binding sites of hAβ.[25] The results obtained were in line with most previous studies[12,19,22,26,27] and showed that the equatorial binding site of component **I** is formed by the NH_2 group of Asp1, two of the three imidazole rings of His6, His13, and His14, and a CO function. At higher pH values, deprotonation of the Asp1−Ala2 peptide bond leads to the replacement of one imidazole ring with the Asp1−Ala2 deprotonated amide (amidyl) ligand.

In this study, we used NMR spectroscopy to gain more insight into Cu^{II} coordination to the mAβ peptide. We recorded ^{1}H, ^{13}C, and 2D NMR spectra of mAβ peptide at pH 5.4 and 7.6 with or without a substoichiometric amount of Cu^{II} ions (see Figures S6–S14 in the Supporting Information). At pH 5.4 and in the presence of Cu^{II}, the side chains of Asp,

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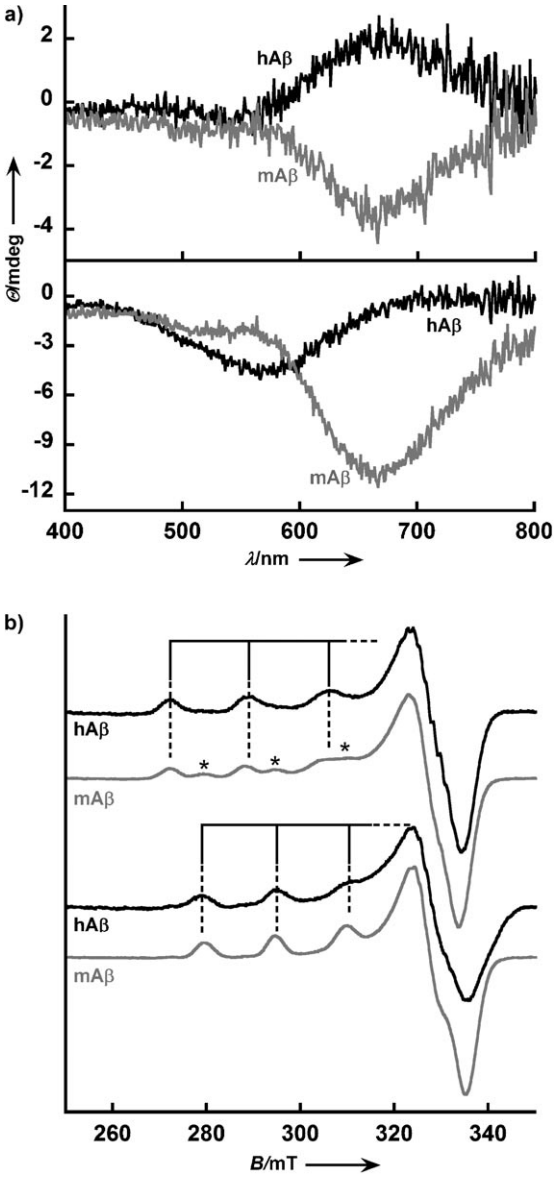

Figure 1. a) CD and b) EPR spectra of Cu^{II}–hAβ (black lines) and Cu^{II}–mAβ complexes (gray lines) at pH 6.5 and 5.4, respectively (top) and at pH 8.7 and 7.6, respectively (bottom). a) $T = 20\,°C$, $[Cu^{II}–Aβ] = 0.45$ mM; b) $\nu = 9.5$ GHz, modulation amplitude: 0.45 mT, $T = 110$ K, $[Cu^{II}–Aβ] = 0.9$ mM. * indicates residual component **II**.

Glu, and His residues were strongly affected; the signals for the CO and C_α carbon atoms of Asp1, Asp7, His6, and His14 were also broadened, but to a lesser extent. When the pH value was increased to 7.6, the main changes occurred in the CO region, in which only the CO group of Asp1 was strongly affected, and in the C_α region, in which the α positions of Asp1, His6, and to a lesser extent His14, were affected. The CD data provide evidence that an amide bond is deprotonated near pH 6.1, and the NMR spectroscopic data indicate that deprotonation occurs in close vicinity to His6. We propose deprotonation of the Gly5–His6 rather than the His6–Asp7 peptide bond, mainly because 1) a six-membered metallacycle is more favorable than a seven-membered metallacycle and 2) Cu^{II} coordination to the shorter peptide models Aβ6 (DAEFGH) and Aβ9 (DAEFGH**D**SG) is similar.[20] This result is consistent with the fact that the R5G mutation is the key mutation between hAβ and mAβ.

The crucial information deduced from the NMR spectroscopic data is that the Gly5–His6 amide bond is deprotonated during the transition between **I** and **II**: a main mechanistic difference to the deprotonation of the Asp1–Ala2 bond observed in the Cu^{II}–hAβ complex.[25] This difference is even more evident from the ^{13}C NMR spectra in Figure 2, in which

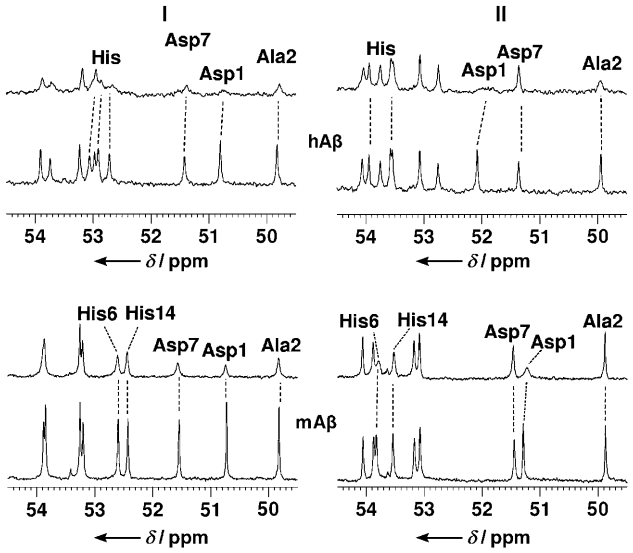

Figure 2. C_α regions of the $^{13}C\{^1H\}$ NMR spectra of hAβ peptide (10 mM) in D_2O (bottom) and in the presence of Cu^{II} (top) at pH 6.6 (left) and 8.7 (right), and mAβ peptide (10 mM) in D_2O (bottom) and in the presence of Cu^{II} (top) at pH 5.4 (left) and 7.6 (right); $T = 25\,°C$, $\nu = 125.8$ MHz; 0.1 equivalent of Cu^{II} was used, except with mAβ at pH 5.4 where 0.02 equivalent of Cu^{II} was used. The shifting of some peaks is due to a slight change in the pH value as a result of the addition of Cu^{II}.

the effect of Cu^{II} on the C_α regions of hAβ and mAβ peptides are shown for **I** and **II**. For **I**, there is no significant difference between hAβ and mAβ in the broadening of the signals for the His, Asp, and Ala2 residues upon Cu^{II} binding. In contrast, in the case of **II**, several ^{13}C nuclei behave differently: the signal for the C_α atom of Ala2 of hAβ but not mAβ is broadened, whereas broadening of the signal for the C_α atom of His6 was observed only for mAβ. Divergences between hAβ and mAβ with respect to copper(II)-induced NMR line broadening are illustrated in Figure 3 (see also Schemes S4 and S5 in the Supporting Information). Besides the differences described above, we observed broadening of all signals for carboxylate ^{13}C nuclei in the case of mAβ (for **I** and **II**), whereas in hAβ, only that of Asp1 was affected by switching to **II**. This result is in line with the formation, in the case of Cu^{II}–hAβ, of a tridentate pincer (NH_2 (Asp1), N^-(Asp1–Ala2), COO^- (Asp1)), which is not present after deprotonation of the Gly5–His6 peptide bond in the Cu^{II}–mAβ species. Finally, for **II**, the carbonyl function affected in the case of hAβ is predominantly that of the Ala2–Glu3 peptide bond, that is, the carbonyl group adjacent to the Asp1–Ala2 amidyl

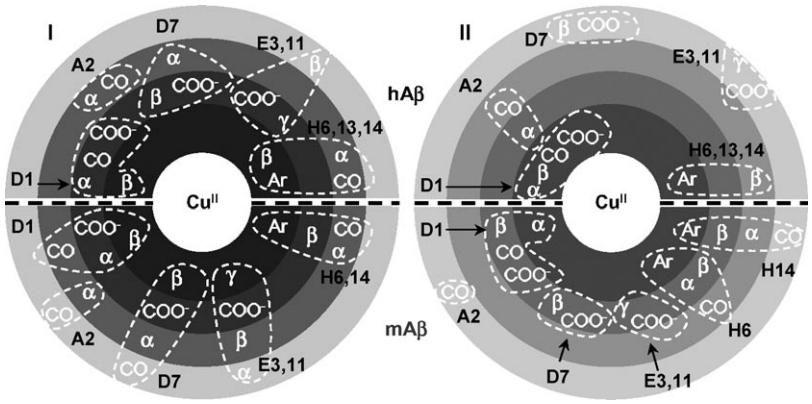

Figure 3. Schematic representation of the C and H atoms most affected by the binding of Cu^{II} to hAβ (top) and mAβ peptides (bottom) for **I** (left) and **II** (right). The intensity of the rings increases according to the extent of broadening of the NMR signals in the presence of Cu^{II}.

function, whereas the Asp1–Ala2 carbonyl function (thus, not that adjacent to the Gly5–His6 amidyl function) is most affected in mAβ.

A plausible explanation for the deprotonation of the Asp1–Ala2 peptide bond in the Cu^{II}–hAβ complex (at pH values close to 7.7) and deprotonation of the Gly5–His6 peptide bond in the Cu^{II}–mAβ complex (at pH values close to 6.2) is the increased pK_a value of the Arg5–His6 peptide bond relative to that of Gly5–His6. This increased pK_a value precludes deprotonation of the peptide bond in the case of the hAβ peptide and induces deprotonation near another anchoring site, that is, the N terminus. The increase in the pKa value induced by the bulky Arg residues may be due to an ineffective orientation of the NH amide with respect to the Cu^{II} ion as a result of steric constraints.

The two binding sites of Cu^{II} bound to mAβ in best agreement with the data obtained in this study are depicted in Figure 4 (see also Scheme S6 in the Supporting Information). They were deduced by comparison with reported results for Cu^{II}–hAβ. For **I**, we propose the involvement of one COO^- group in an equatorial position (instead of a second His residue, as in the case of hAβ) in a 2N2O binding site, rather than a 3N1O binding site (hAβ), in line with the differences observed in the EPR parameters (see Table S1 in the Supporting Information).[29] Thus, the second His residue lies in an apical position. For **II**, the main difference with respect to the Cu^{II}–hAβ binding site is the additional presence of the His14 side chain in an apical position. This structure is in line with the very similar EPR parameters observed for the Cu^{II} complexes of both peptides and with the broadening of the His14 signal detected by NMR spectroscopy in the presence of Cu^{II} ions. The presence of the CO group of Asp1–Ala2 in the equatorial position is proposed on the basis of geometric constraints (five-membered metallacycle with the NH_2 group), which favor the equatorial over the apical position. These models are mostly in line with results reported by Kowalik-Jankowska et al. in their pioneering studies,[19,20] although we propose the involvement of an equatorial carbonyl group (instead of a carboxylate) in **II**. Furthermore, the NMR spectroscopic data obtained in this study enable the type of binding functions to be attributed to a specific residue. Such data is key to a good description of Cu^{II} coordination to the peptide and a better understanding of the biological implications of this coordination. Gaggelli et al. did not propose coordination of the amidyl function on the basis of their NMR spectroscopic data obtained at pH 7.5 in a micellar solution.[21] The most plausible reason for this discrepancy with our results is that, as we previously detailed,[25] broadening of the side-chain signals as a result of Cu^{II} binding is more important than the broadening of backbone signals.

The comparison of Cu^{II} coordination to mAβ and hAβ near physiological pH values uncovered three major features:

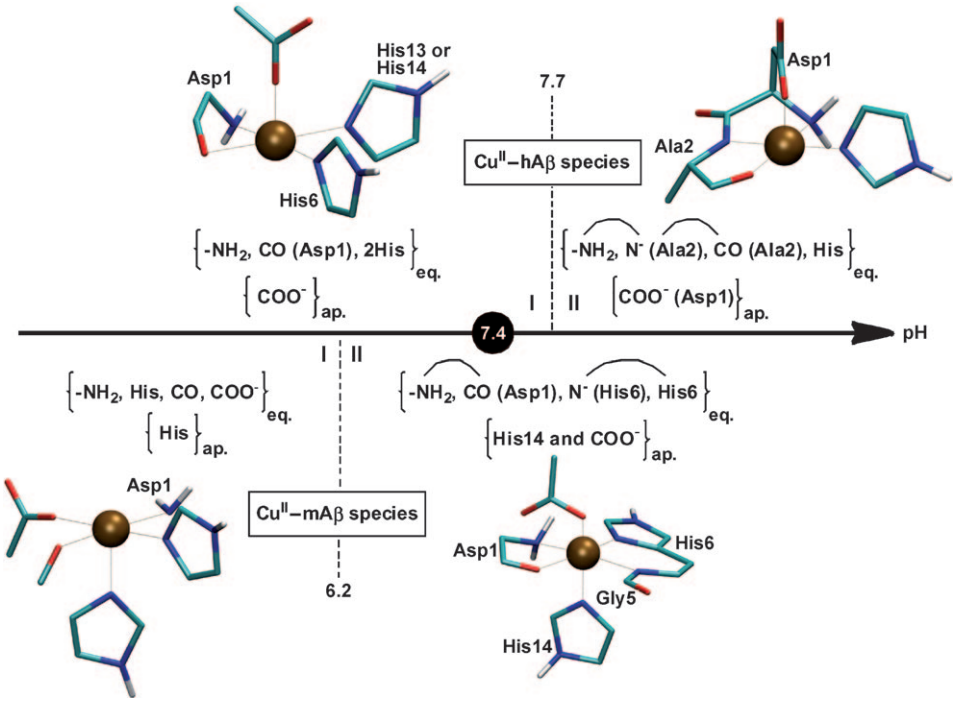

Figure 4. Proposed pH-dependent Cu^{II} coordination to hAβ and mAβ peptides. Curved lines stand for metallacycles formed upon Cu^{II} binding. As previously observed for hAβ, the exchange of chemically equivalent ligands at a given coordination position also occurs in the case of mAβ. Hence, when the residue is not specified, several residues (in equilibrium) fulfill the binding function. The structures of the Cu^{II}–Aβ complexes were drawn with the VMD software.[28] For the sake of clarity, only hydrogen atoms bound to nitrogen atoms are represented; eq. = equatorial, ap. = apical.

1) dynamic equilibria between equivalent binding functions for one coordination position were detected for both Cu^{II}–hAβ and Cu^{II}–mAβ complexes, even if, in the case of **II**, the Cu^{II} ion is more constrained in hAβ (in the N-terminal part) than in the mAβ peptide, in which all carboxylate functions are still involved in Cu^{II} binding; 2) the proportions of **I** and **II** differ strongly, whereby **I** is predominant in the case of hAβ, and almost only **II** is present for mAβ; 3) species **II** of hAβ and mAβ mainly differ in terms of the formation of the metallacycle either between the NH_2 group and the amidyl N^- atom (Asp1−Ala2; hAβ) or between the imidazole ring of His6 and the amidyl N^- atom (Gly5−His6; mAβ).

The first direct consequence of these differences concerns the formation of the deleterious N-terminally truncated pyroglutamate forms (p3E-hAβ).[30] Cu^{II} ions bound to both Asp1 and Ala2 in the hAβ peptide might enhance the formation of p3E-hAβ by assisting with the hydrolysis of the Ala2−Glu3 bond.[25,27] Such an effect cannot apply in the case of the mAβ peptide.

A second consequence is that the affinity of Cu^{II} for mAβ and hAβ and the redox response of Cu^{II}–mAβ and Cu^{II}–hAβ complexes should differ significantly. To evaluate the amplitude of such variations, we performed a competition experiment by CD. We found that Cu^{II} is bound about three times more strongly by mAβ than by hAβ under relevant biological conditions (Figure 5). Moreover, in a preliminary assessment of the redox properties of the two complexes, we measured the time necessary to reduce Cu^{II} to Cu^{I}: the first step of any mechanism for the production of ROS.[15] We found that Cu^{II} bound to mAβ was reduced two to three times more slowly than Cu^{II} bound to hAβ (Figure 5). These two observations suggest that the situation is very different when both peptides are present (in transgenic mice and rats) to that when only hAβ is present (in human AD patients): 1) The Cu^{2+} ion is preferentially coordinated to mAβ; as a consequence, it is expected that less copper is present in amyloid plaques composed of hAβ.[31] This hypothesis fully agrees with reports that amyloid plaques in transgenic mice contain less copper than those of human patients.[32] 2) Since even trace amounts of Cu^{2+} binding to hAβ influence the aggregation behavior of the peptide,[33] the partial withdrawal of Cu^{2+} by the mAβ peptide may modulate the aggregation of hAβ significantly. This hypothesis is in accord with results indicating that the morphology of amyloid plaques is modified in transgenic mice,[31] and that different phenotypes of plaques are obtained in transgenic mice when injected with brain extracts from either AD patients or AD transgenic mice.[34] 3) ROS production will be reduced in transgenic mice relative to that in humans.

Hence, we can conjecture that the interaction of Cu^{II} ions with hAβ is altered in murine models as a result of the concomitant presence of the mAβ peptide. Although many factors other than copper binding to Aβ are important in AD, this interference might be worth considering in studies with AD murine models, in particular when addressing metal-ion homeostasis.[35]

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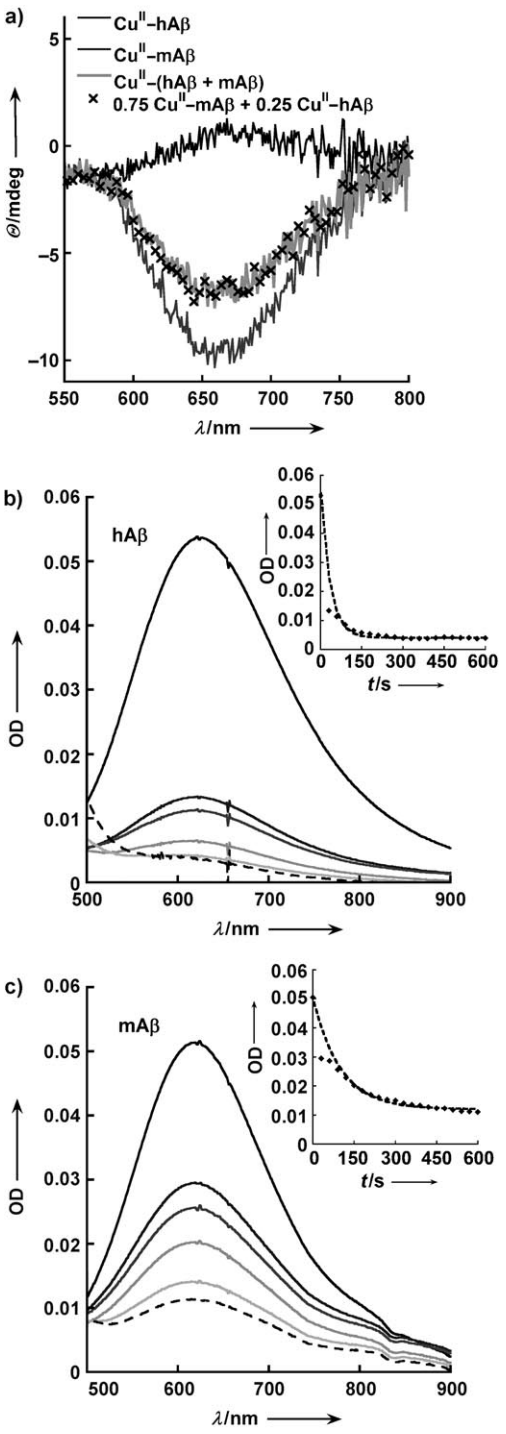

Figure 5. a) CD signatures of Cu^{II}–hAβ (black), Cu^{II}–mAβ (dark gray), and Cu^{II} in the presence of an equimolar mixture of both peptides (light gray); $[Cu^{II}] = 0.45$ mM, [peptide] = 0.5 mM, $l = 1$ cm, $T = 20$ °C, 20 mM phosphate, pH 7.4; **x** is the calculation of the light-gray spectrum as a linear combination of the two CD signatures of the Cu^{II}–hAβ and Cu^{II}–mAβ complexes in a 3:1 ratio. b,c) UV/Vis spectra recorded at $t = 0$, 0.5, 1, 2, 5, and 10 min after the addition of ascorbate (1.5 equiv) to the complexes Cu^{II}–hAβ (b; 1 mM) and Cu^{II}–mAβ (c; 1 mM) in 50 mM phosphate at pH 7.4. Insets show the decrease in optical density as a function of time, and the corresponding fit using exponential decay with $\tau = 34$ (hAβ) and 100 s (mAβ). OD = optical density.

Keywords: amyloid β peptides · coordination modes · copper · NMR spectroscopy · peptides

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[35] Note added in proof: During the processing of this manuscript, an article was published supporting the present findings that the R5G mutation is responsible for the difference in Cu^{II} binding between human and murine Aβ peptides: : L. Hong, T. M. Carducci, W. D. Bush, C. G. Dudzik, G. L. Millhauser, J. D. Simon, *J. Phys. Chem.* **2010**, *114*, 11261 – 11271.

