Amyloid-β Peptides

Deutsche Ausgabe: DOI: 10.1002/ange.201511968
Internationale Ausgabe: DOI: 10.1002/anie.201511968

Resistance of Cu(Aβ4–16) to Copper Capture by Metallothionein-3 Supports a Function for the Aβ4–42 Peptide as a Synaptic Cu^{II} Scavenger

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Abstract: *Aβ4-42 is a major species of Aβ peptide in the brains of both healthy individuals and those affected by Alzheimer's disease. It has recently been demonstrated to bind Cu^{II} with an affinity approximately 3000 times higher than the commonly studied Aβ1-42 and Aβ1-40 peptides, which are implicated in the pathogenesis of Alzheimer's disease. Metallothionein-3, a protein considered to orchestrate copper and zinc metabolism in the brain and provide antioxidant protection, was shown to extract Cu^{II} from Aβ1-40 when acting in its native Zn_7MT-3 form. This reaction is assumed to underlie the neuroprotective effect of Zn_7MT-3 against Aβ toxicity. In this work, we used the truncated model peptides Aβ1-16 and Aβ4-16 to demonstrate that the high-affinity Cu^{II} complex of Aβ4-16 is resistant to Zn_7MT-3 reactivity. This indicates that the analogous complex of the full-length peptide Cu(Aβ4-42) will not yield copper to MT-3 in the brain, thus supporting the concept of a physiological role for Aβ4-42 as a Cu^{II} scavenger in the synaptic cleft.*

The Aβ peptides are considered to be key pathological species in Alzheimer's disease (AD), a form of fatal dementia that affects millions of patients worldwide.[1] These peptides are derived from a precursor protein, APP, by proteolysis.[2] The two most prominent of these peptides, Aβ1-42 and Aβ1-40, are found in brain tissues and cerebrospinal fluid (CSF), and their aggregation to oligomers and higher structures is currently believed to be a key step in AD pathology according to the amyloid cascade hypothesis.[3] N- and C-terminally truncated forms of these peptides are also found and have been assigned toxic properties. One of these truncated peptides, Aβ4-42, is very abundant in the tissues of both healthy and AD brains, and is found at levels similar to or exceeding those of Aβ1-42.[4–7]

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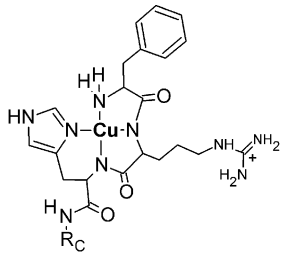
Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201511968.

Cu^{II} ions are present transiently in the synaptic cleft. They participate in neurotransmission, reaching peak concentrations as high as 100–250 μM.[8] In vitro experiments demonstrated that Aβ1-*x* peptides ($x = 42$ or 40 for major brain species, and 28 or 16 for model peptides used in metal-binding studies) bind one Cu^{II} ion with relatively high affinity ($K_a \approx 10^{10}\,M^{-1}$ at pH 7.4) and another Cu^{II} ion with an affinity approximately 100 times weaker.[9,10] The high-affinity site is heterogeneous in terms of Cu^{II}-binding ligands, which include the N-terminal (Asp1) amine and two out of three His residues, present at positions 6, 13 and 14 of the peptide chain.[11,12] Co-exposure of cultured neurons to Cu^{II} ions and Aβ1-42 or Aβ1-40 peptides significantly augments the cytotoxicity of the peptides, thus supporting the copper hypothesis in AD.[13] The ability of Cu^{II} complexes of Aβ1-*x* peptides to generate reactive oxygen species (ROS) prompted the proposal that they are responsible for the widespread oxidative stress observed in AD brains post mortem.[14,15]

Metallothioneins (MTs) are small Cys-rich metalloproteins implicated in the storage and distribution of Zn^{II} ions in cells and in the extracellular space.[16,17] The maximum capacity of MT to bind Zn^{II} under physiological conditions is seven (Zn_7MT).[18] Metallothionein-3 (MT-3), the brain-specific MT, participates in processes of regeneration and degeneration of neurons, and Zn_7MT-3 (but neither Zn_7MT-1 nor Zn_7MT-2) has been shown to rescue cultured neurons from Aβ1-40 toxicity.[19] Unlike other MTs, apo-MT-3 binds Cu^{I} ions avidly, up to a Cu_{11}MT-3 stoichiometry, and is involved in brain copper metabolism.[20] Zn_7MT-3 was shown to swap Cu^{II} with proteins related to neurodegenerative conditions: α-synuclein,[21] prion protein,[22] and Aβ1-*x* peptides,[23–25] to form a mixed species $Cu^{I}_4(\beta)Zn^{II}_4(\alpha)$-MT-3. This finding links the neuroprotective role of Zn_7MT-3 with brain copper metabolism and a putative copper involvement in Aβ pathology.

Recently, we demonstrated that Aβ4-16, which is used to model the Aβ4-42 peptide, binds one Cu^{II} ion with very high affinity ($K_a = 10^{13.5}\,M^{-1}$ at pH 7.4) through its N-terminal Phe-Arg-His sequence (Scheme 1).[26] The resulting complex is redox silent under biologically relevant conditions and does not yield ROS, as has also been confirmed for the Aβ4-42 peptide. Aβ4-16 binds the second Cu^{II} ion through His13 and His14 (the numbering taken from Aβ1–*x* peptides). The resulting complex, loosely termed the "secondary site", is relatively weak ($K_a = 10^{6.7}\,M^{-1}$ at pH 7.4). These properties



Scheme 1. The structure of the primary Cu^{II} binding site, located in the N-terminal tripeptide Phe-Arg-His of Aβ4-*x* peptides. R_c denotes the remaining C-terminal peptide sequence.

suggest a physiological role for the N terminus of the Aβ4-42 peptide as a synaptic scavenger of Cu^{II} ions.

In this study, we sought to reinforce the concept of Aβ4-42 as a physiological Cu^{II}-binding peptide by establishing that the N-terminal Cu^{II}(Aβ4-16) complex is fully resistant to copper/zinc swap with Zn_7MT-3. In our experiments, we used recombinant human MT-3 overproduced in *E. coli*, purified as apoprotein, and reconstituted with $ZnSO_4$ as Zn_7MT-3. We began by monitoring by UV/Vis spectroscopy whether Zn_7MT from our preparation was able to react with Cu^{II} ions. Figure 1 shows the Cu^{II} titration of a 5 μM Zn_7-MT3

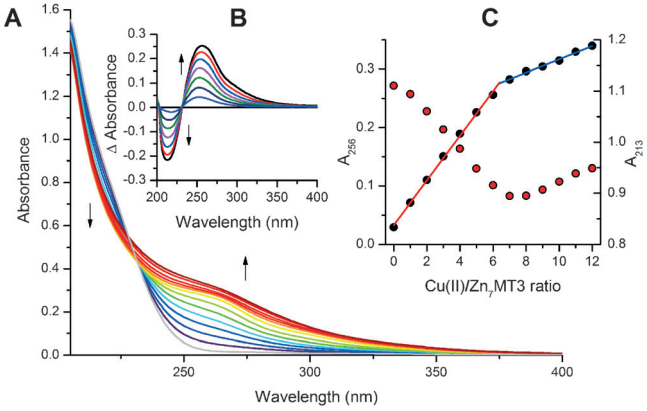


Figure 1. A) Titration of Zn_7MT-3 (5 μM in 20 mM Tris-HCl and 100 mM NaCl, pH 7.4) with $CuCl_2$ from 0 to 12 mol equiv. B) Difference spectra for the first seven mol equiv. C) Black spheres: titration curve generated by absorbance at 256 nm with linear fits for two branches (0 to 6 and 7 to 12 mol equiv), red spheres: absorption at 213 nm.

sample in a 20 mM Tris-HCl/100 mM NaCl buffer, pH 7.4. The titration revealed a roughly biphasic character for the copper/zinc swap at MT-3. The isosbestic point at 231 nm was maintained up to approximately 6.3 molar equivalents (mol equiv) of added Cu^{II}. Subtraction of the initial Zn_7-MT3 spectrum from the titration spectra (Figure 1B) revealed an increase of absorption at 256 nm, which is assigned to the S–Cu^{I} charge transfer (CT) band, and a corresponding decrease of the band at 213 nm, which can be assigned to the S–Zn^{II} CT band.[27] Analysis of the titration curve (Figure 1C) indicated that 6.3 Cu^{II} equivalents were incorporated into nearly equivalent binding sites in MT-3, as evidenced by a linear increase in the absorption of the S–Cu^{I} CT band (but a slight shift of this band maximum seen in difference spectra indicates some interactions between these sites). Our results are fully consistent with previous studies on the interaction between Cu^{II} and Zn_7MT-3, thus confirming the correctness of our approach.[23] Further Cu^{II} equivalents were incorporated into MT-3 in a clearly different fashion. The band at 213 nm ceased to decrease, thus resulting in loss of the isosbestic point. Also, a more complicated pattern of bands in the S–Cu^{I} CT region emerged. These results suggest that the first 6–7 Cu^{II} ions displace Zn^{II} from MT-3. Cu^{II} ions have to be reduced to Cu^{I} in order to be incorporated into MT3, and the thiolates are the only clear reductant in our experimental system. The reduction of 6.3 mol equiv Cu^{II} requires the same number of thiolates to be oxidized to disulfides. This leaves approximately 13 thiolates per MT-3 molecule, a number about right for the formation of approximately 6.3 S-Cu-S binding sites, which are known from yeast MT studies to be sufficient for efficient Cu^{I} coordination.[28,29]

The reaction of Cu^{II} ions with Zn_7MT-3 was also checked by ESI-MS. The results (Figures S1–S4 in the Supporting Information) corroborate the conclusions from the UV/Vis spectra. At a 1:1 Cu^{II}/MT-3 ratio, two similarly intense peaks were detected: one was for the intact Zn_7MT-3, and another indicated two Cu^{I} ions swapped for one Zn^{II}. At a 4:1 ratio, the major peak indicated four Cu^{I} ions swapped for four Zn^{II} ions, with approximately 2–4 disulfide bridges formed. At an 8:1 ratio, a series of additional peaks were detected, indicating a higher number (up to nine) of Cu^{I} ions. No MT-3 oligomers were detected in the spectra. A more thorough analysis of the ESI-MS results was hampered by overlaps with peaks for adducts with NH_4^+ ions derived from the ammonium carbonate buffer used in these experiments.

Having established the conditions of the copper/zinc swap at Zn_7MT-3, we performed experiments with Cu^{II} complexes of Aβ peptides under identical pH/buffer conditions. The Zn_7MT-3 portions were added to respective complexes at peptide/Cu^{II} molar ratios of 0.9 and 1.8 to avoid oversaturation of the peptide binding sites. The final concentrations of Zn_7-MT3 and the peptides were 5.0 μM and 23.6 μM, respectively. The course of the reactions was monitored by recording whole spectra at 5 min intervals over the 30 minute period. The key results from UV/Vis experiments with one mol equiv of Cu^{II} are presented in Figure 2 for Aβ1-16, and Figure 3 for Aβ4-16. All spectra are provided in Figures S5 and S6 in the Supporting Information. Separate experiments with both Aβ peptides, performed under identical conditions, were monitored through circular dichroism (CD) spectroscopy. The CD spectra are presented in Figures S7 and S8. The copper/zinc swap reactions were fast, with more than 95% of the transfer occurring in all cases within the dead time of sample mixing and recording the first spectrum (ca. 2.5 min).

As expected on the basis of previous studies,[23–25] Cu^{II} ions bound to Aβ1-16 reacted readily with Zn_7MT-3. Evidence of a complete copper/zinc swap was provided by the occurrence of a CT band at 256 nm and loss of the Cu^{II} d–d band (Figure 2), Zn_7MT-3 was also able to extract both Cu^{II} ions

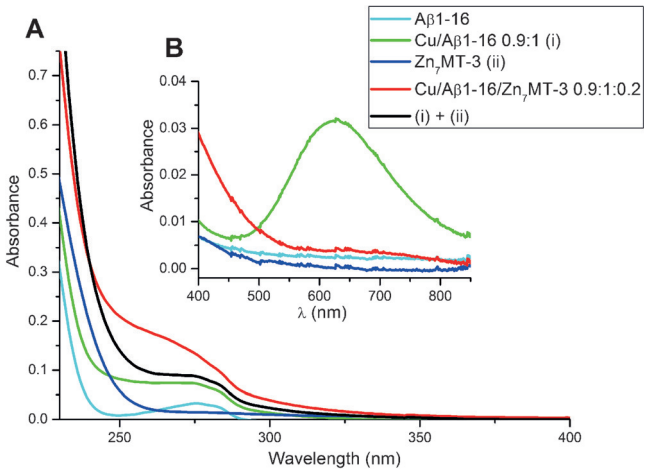


Figure 2. Reaction of the Cu(Aβ1-16) complex with Zn_7MT-3 in 20 mM Tris-HCl and 100 mM NaCl, pH 7.4 observed A) in the UV region for 23.6 μM Aβ1-16 with 21.2 μM Cu^{II} (the Cu(Aβ1-16) complex) and 5 μM Zn_7MT-3, and B) in the d–d bands for 446 μM Aβ1-16 with 401 μM Cu^{II} (the Cu(Aβ1-16) complex) and 80.2 μM Zn_7MT-3. For the UV region, the spectrum of the reaction product at Cu/Aβ1-16/Zn_7MT-3 = 0.9:1:0.2 was compared to the mathematical sum of the Cu(Aβ1-16) complex and Zn_7-MT3 spectra (black line).

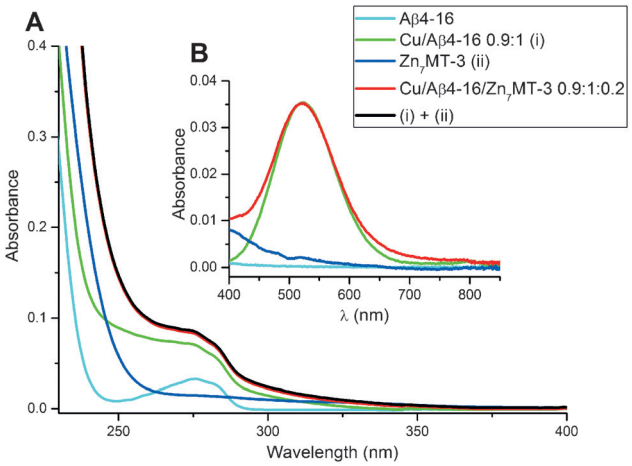


Figure 3. Reaction of the Cu(Aβ4-16) complex with Zn_7-MT3 in 20 mM Tris-HCl and 100 mM NaCl, pH 7.4 observed A) in the UV region for 23.6 μM Aβ4-16 with 21.2 μM Cu^{II} (the Cu(Aβ4-16) complex) and 5 μM Zn_7MT-3, and B) in the visible region for 475 μM Aβ4-16 with 367 μM Cu^{II} (the Cu(Aβ4-16) complex) and 73.4 μM Zn_7MT-3. For the UV region, the spectrum of the reaction product at Cu/Aβ4-16/Zn_7MT-3 = 0.9:1:0.2 was compared to the mathematical sum of the Cu(Aβ4-16) complex and Zn_7MT-3 spectra (black line).

from Cu_2(Aβ1-16) (Figures S5 and S7). The behavior of the first Cu^{II} site in the Aβ4-16 peptide was remarkably different. As shown in Figures 3 and Figures S6 and S8, it was totally unreactive during a 30 min incubation with Zn_7MT-3, while the second mol equiv of Cu^{II} was swapped readily (Figures S6 and S8). This was evidenced by accurate reconstruction of the experimental spectra of these reaction mixtures. We assumed that the (partially) Cu^{I} loaded MT-3 and the Aβ apopeptide were the only species remaining in solution after the copper/zinc swap reaction. Consequently, we summed the spectra of Zn_7MT-3 reacted with corresponding amounts of Cu^{II} ions (presented in Figure 1) and those of respective forms of Aβ peptides remaining in solution. For Aβ1-16 samples, the reconstruction was successful when the spectrum of the Aβ1-16 apopeptide was used, while the spectrum of the Cu(Aβ4-16) complex had to be used instead for the reaction of Cu_2(Aβ4-16).

We also performed additional control experiments. A reverse swap experiment, where apo-Aβ4-16 was added to the preformed (Cu/Zn)MT-3 complex, did not yield Cu^{II} transfer from metallothionein to the peptide (Figure S9), but the addition of Cu^{II} ions to a mixture of Zn_7MT-3 and apo-Aβ4-16 resulted in the partition of copper between these biomolecules, with approximately 30% as Cu(Aβ4-16) (Figure S10). The addition of physiological amounts of ascorbate or hydrogen peroxide did not facilitate the swap, but very high non-physiological concentrations of these redox agents resulted in the transfer of copper from Aβ4-16 or to Aβ4-16 (ascorbate or H_2O_2, respectively, Figure S11). Collectively, these experiments indicate a kinetic as well as thermodynamic basis for the resistance of Cu(Aβ4-16) to copper/zinc swap with Zn_7MT-3.

These results correlate with the redox properties of Cu(Aβ4-16) presented in our recent work.[26] Voltammetric experiments on the N-terminal Cu^{II} complex of Aβ4-16 revealed that this complex could be oxidized irreversibly at a high potential, but could not be reduced to Cu^{I}. Analogous behavior was observed for the Cu^{II} complex of a peptide modeling the Cu^{II} site in human serum albumin (HSA), which has a similar stability,[30] but the much weaker Cu^{II} site in the full-length albumin could be reduced to Cu^{I}.[31] The secondary Cu^{II} complex of Aβ4-16, which supported the Cu^{I}/Cu^{II} redox pair in voltammetric experiments, readily released copper to metallothionein.

The resistance of Cu(Aβ4-16) to Zn_7MT3 reactivity indicates that the analogous complex of the full-length peptide, Cu(Aβ4-42) will not yield copper to MT-3 in the brain extracellular space. The copper/zinc swap was postulated in the literature as a key mechanism of control of the toxicity of copper-Aβ complexes by MT-3.[23–25] This fact, combined with the very high stability of the Cu(Aβ4-42) complex, the ability of its binding site to extract Cu^{II} ions from the binding site of Aβ1-*x* peptides, and its lack of ROS production, strongly supports a physiological role of Aβ4-42 as a Cu^{II} scavenger in the synaptic cleft, as postulated in our recent paper.[26] We can speculate that Aβ4-42 and MT-3 may play parallel roles in synaptic copper clearance: the former handling copper under more oxidizing conditions and the latter in the more reducing environments. It should be noted that Aβ4-42 does not impair the antioxidant function of Zn_7MTs.[32]

Other truncated amino-terminal copper and nickel binding (ATCUN)-type Aβ peptides may have properties similar to those of Aβ4-42. A recent paper reported a 34 fM Cu^{II} affinity for Aβ11-42.[33] Nevertheless, the majority of species truncated at residue Glu11 exist in the pyroglutamate form,[7,34] which blocks ATCUN coordination. Moreover, it remains to be determined whether the ATCUN site of Aβ11-*x* peptides can undergo redox cycling and generate ROS.

Acknowledgements

The study was sponsored by the National Science Centre of Poland, grant no. 2014/15/B/ST5/05229 (W.B.) and the Ministry of Science and Higher Education, grant no. IP2012 018272 (A.K.). The equipment used was sponsored in part by the Centre for Preclinical Research and Technology (CePT), a project cosponsored by the European Regional Development Fund and Innovative Economy, The National Cohesion Strategy of Poland. S.C.D. received a fellowship (FT110100199) administered by the Australian Research Council.

Keywords: amyloid-beta peptides · copper · metalloproteins · metallothionein · zinc

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 8235 – 8238
Angew. Chem. **2016**, *128*, 8375 – 8378

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Received: December 28, 2015
Revised: March 23, 2015
Published online: May 30, 2016

