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Cysteine and Glutathione trigger the Cu-Zn swap between Cu(II)-Amyloid- β_{4-16} peptide and Zn₇-Metallothionein-3

Alice Santoro,^a Nina Wezynfeld,^b Milan Vařák,^c Wojciech Bal,^b and Peter Faller^{a,d}

Cysteine and glutathione are able to reduce Cu(II) coordinated to the peptide Amyloid β_{4-16} , and shuttle the resulting Cu(I) to partially replace Zn(II) in the protein Zn₇-Metallothionein-3. The released Zn(II) in turn binds to amyloid- β_{4-16} . Thus cysteine and glutathione are modulators of Cu-distribution between metallothionein-3 and amyloid- β_{4-16} .

Copper is an essential element for humans as it is involved in several important biological processes, mainly as a catalytic center in enzymes. However, free or loosely bound Cu is a potent catalyst for reactive oxygen species, hence it can also be toxic. To avoid Cu-toxicity the Cu metabolism is tightly controlled and Cu remains bound almost exclusively to proteins.¹⁻³ Cu-mis-metabolisms have been related to several diseases, including Alzheimer's disease (AD).^{4,5} In AD patients Cu is found to be bound to the peptide amyloid- β (A β) that accumulates in extracellular amyloid-plaque.⁶ Under healthy conditions A β seems not to be bound to Cu, and hence the question is why Cu is bound to A β under AD but not healthy conditions.^{7,8} Thus interest arose in natively present metalloproteins that could protect A β from aberrant Cu binding. Metallothioneins (MTs) are one of the most predominant zinc and copper-binding proteins in the body, with Metallothionein-3 (MT-3) as the brain specific form. MTs bind mostly seven Zn(II) ions (Zn₇MT) but can also bind Cu(I).^{9,10} Indeed, Zn₇MT-3 is able to protect against Cu-A β induced cell toxicity, related to ROS production, through the formation of an oxygen stable and redox silent complex, Cu(I)₄Zn(II)₄MT-3. During this process, parallel to the binding to the β -domain of MT-3, Cu(II) is reduced to Cu(I) by the thiolate system of Zn₇MT-3. Moreover, Zn(II) is released from MT-3 and binds to A β .¹¹ The abundant form of A β are 40 or 42 amino acids long (A β_{1-40} and A β_{1-42} , respectively) and their Cu(II) and Cu(I)-binding sites are relatively well characterized.^{12,13} In a recent study Wezynfeld et al. proposed a physiological role of an abundant truncated A β species, A β_{4-42} as Cu(II) scavenger into the synaptic cleft, through its binding to Cu(II) ions.¹⁴ This truncated A β_{4-42} has a different and stronger Cu(II) binding motif compared to A $\beta_{1-40/42}$ ($K_a = 10^{13.5} \text{ M}^{-1}$ and 10^{10} M^{-1} at pH 7.4, respectively).¹⁵ This motif is known as ATCUN motif¹⁶ and only consists of the first three amino-terminal amino acids, Xxx-Yyy-His in general and Phe-Arg-His for A $\beta_{4-40/42}$. Cu(II) bound to this motif in the peptides Cu^{II}(A $\beta_{4-16/42}$) was not able to catalyze the production of ROS, opposite to the non-truncated A $\beta_{1-16/40/42}$. Interestingly, Cu(II) bound to this motif in the peptide Cu^{II}(A β_{4-16}) (model for A $\beta_{4-40/42}$) was completely resistant to react with Zn₇MT-3 (and hence no Cu-Zn swap occurred). Ascorbate was able to facilitate partial Cu transfer, but only at non-physiological high concentrations (Ref 14). Consequently, Wezynfeld et al. hypothesized that A β_{4-42} and MT-3 may play parallel roles in handling copper, depending on the environment conditions, i.e. oxidizing or reducing.^{14,15} In this context, we were interested in other compounds that can be found in the same location as A β and MT-3, and might modulate their interaction on the level of Cu. Glutathione (GSH) is a highly abundant intracellular tripeptide, that can reduce Cu(II) and bind strongly Cu(I) mainly via its cysteine side chain.^{17,18} Cu(I)-GSH complexes were proposed as Cu(I) shuttles between proteins, like Ctr1 and Cu-chaperons, and the *in vitro* studies showed that the Cu(I)-GSH complex can transfer Cu(I) to MT-1.^{19,20}

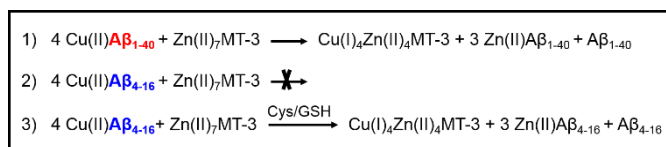


Fig 1. Reactivity of Cu^{II}A $\beta_{x-16/40/42}$ complexes with Zn₇MT-3. Reaction 1 from reference¹¹, reaction 2 from reference¹⁴, reaction 3 from this work.

However, GSH is also present extracellularly in its reduced form and has been proposed to act as a neurotransmitter.²¹ Free cysteine (Cys) is the main reduced thiol extracellularly, but is also present intracellularly.²²

Therefore, we investigated the impact of Cys and GSH concerning their ability to extract Cu(II) from Cu^{II}(A β_{4-16}) in the presence of Zn₇MT-3. Furthermore, we probed the use of these molecules as shuttles for the metal ion transport between the two peptides,

with a concomitant release of Zn(II) from the β -domain of Zn₇MT-3. We show that Cys and to a lesser extent GSH are able to trigger the release and shuttle Cu from Cu^{II}(A β ₄₋₁₆) to Zn₇MT-3, the latter forming a Cu(I)₄Zn(II)₄MT-3 complex. We started by repeating the reaction of the Cu^{II}(A β ₄₋₁₆) complex with Zn₇MT-3, which was monitored by UV-Vis spectroscopy. The Cu^{II}(A β ₄₋₁₆) complex was generated in phosphate buffer with a Cu(II) to peptide ratio 0.9:1, to avoid the presence of free copper. Cu^{II}(A β ₄₋₁₆) showed the characteristic *d-d* band of an ATCUN-Cu(II) complex, with a maximum at 525 nm.¹⁶ Zn₇MT-3 was then added to the Cu^{II}(A β ₄₋₁₆) complex and the course of the reaction was monitored over a period of 30 min. The *d-d* band was stable as previously reported, clearly in line with the absence of an unassisted Cu transfer from Cu^{II}(-A β ₄₋₁₆) to Zn₇MT-3.¹⁴ Next we added Cys to the Cu^{II}(A β ₄₋₁₆) / Zn₇MT-3 mixture (Figure 2A). The *d-d* band decreased in a time dependent manner. The band completely disappeared in about 30 min. This indicates that Cys is able to react with Cu^{II}(A β ₄₋₁₆), by reducing Cu(II) to Cu(I) and concomitantly release Cu from A β ₄₋₁₆. An analogous experiment with GSH under the same conditions resulted in a

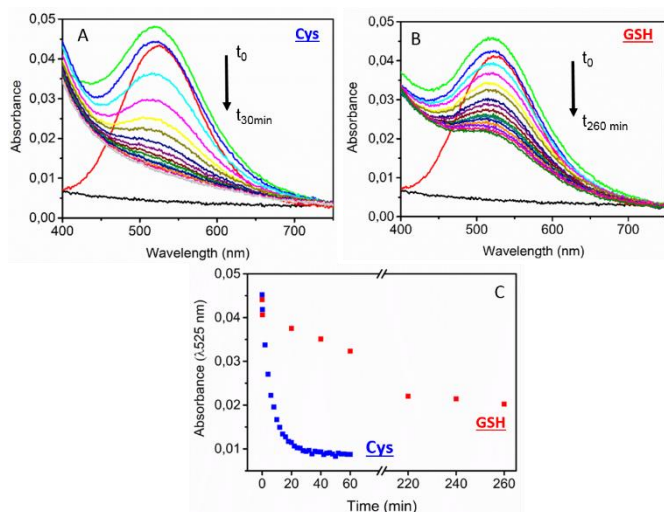


Fig 2. Absorption spectroscopy of Cu(II) release from Cu^{II}(A β ₄₋₁₆) complex, in the presence Zn₇MT-3, with L-Cys (A) and GSH (B), in phosphate buffer, 50 mM, pH 7.4. A, B) Uv-Vis spectra of A β ₄₋₁₆ (black line), A β ₄₋₁₆ / Cu(II), 1:0.9 (red line), A β ₄₋₁₆ / Cu(II) / Zn₇MT-3, 1:0.9:0.2 (light green line). In case of L-Cys (A) the reaction is monitored over time from t₀ (blue spectra) to t_{60 min} (grey spectra, t_{30 min}); each intermediate spectrum was collected at 2 min intervals. For GSH (B) from t₀ (blue spectra) to t_{260 min} (green spectra); each intermediate spectrum was collected at 10 min intervals. Reaction conditions (final concentrations): 500 μ M A β ₄₋₁₆, 450 μ M Cu(II), 100 μ M Zn₇MT-3, 3 mM LCys (A) or GSH (B). C) Corresponding kinetics for the two reactions with L-Cys and GSH: absorbance at 525 nm (maximum of the *d-d* band) versus time.

qualitatively similar behavior. Also GSH was able to react with Cu^{II}(A β ₄₋₁₆) and inducing Cu-release. However, the reaction was slower and did not reach completion after 260 min (Figure 2B). The higher reactivity of Cys compared to GSH can be mainly related to the lower pK_a value of the thiol function in Cys (pK_a ~ 8.2) compared to GSH (pK_a ~ 9.2), key determinant factor for their activity. The deprotonated thiol is the form undergoing the oxidation to cystine and is also the form that can bind metal ions. These experiments strongly suggest that in the reaction mixture Cu^{II}(A β ₄₋₁₆) / Zn₇MT-3 Cys or GSH can extract Cu(II) from the A β ₄₋₁₆ and reduce it to Cu(I). Furthermore, in order to elucidate if the extracted Cu(I) is transported to the Zn₇MT-3, thus generating Cu(I)₄Zn(II)₄MT-3, we employed circular dichroism (CD) and UV-Vis spectroscopy. Evidence for the Cu(I)₄Zn(II)₄MT-3 species formation was provided by the appearance of its characteristic CD bands in the low energy region of the spectra (Uv-region, 250-360 nm), where the Cu^{II}(A β ₄₋₁₆) complex and Cys-Cu(I)/cystine present different CD signals. Here again, the Cu^{II}(A β ₄₋₁₆) complex was generated first in a 0.9:1 ratio. Its characteristic spectrum is shown in Figure 3 (blue line). Then, Zn₇MT-3 was added to the mixture and its lack of copper transfer reactivity was monitored for 30 min. Insert in Figure 3A shows the two spectra recorded before and after the addition of Zn₇MT-3 (blue and red lines, respectively) which perfectly overlap, except for the 250-270 nm region, where the increase in ellipticity is attributed to the Zn₇MT-3 absorption in that region. Then Cys was added to the reaction mixture Cu^{II}(A β ₄₋₁₆) / Zn₇MT-3. After the reaction was mainly completed (i.e. 30 minutes, see above), the CysS-Cu(I) charge transfer CD bands, characteristic of Cu(I)₄Zn(II)₄MT-3, were detected at about (+) 255 nm and (-) 285 nm (cyan line), identical in position and intensity to that observed for the reaction of free Cu(II) with Zn₇MT-3 (light green spectra).²³ By comparison, in the reaction with GSH, circular dichroism confirmed that it could not quantitatively reduce Cu(II) to Cu(I) from Cu^{II}(A β ₄₋₁₆) over a period of 260 min and therefore its transfer to the Zn₇MT-3 metalloprotein was incomplete. In fact, as shown by the purple spectrum in Figure 3A, copper is still distributed between the two peptides, as Cu(II) with Cu^{II}(A β ₄₋₁₆) and as Cu(I) with Cu(I)₄Zn(II)₄MT-3.

In order to confirm the Cys and GSH induced Cu transfer from $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ to $\text{Zn}_7\text{MT-3}$, we compared the reaction with and without $\text{Zn}_7\text{MT-3}$ (Figure 3B). Adding Cys to the preformed $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ complex resulted in the initial disappearance of the $d-d$ band of $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$. However, after 20 min the re-formation of $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ was observed. After 60 min this reaction was completed and the obtained spectrum was almost the same in intensity and λ_{max} as the initial one. This indicates that $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ was completely re-formed and therefore $\text{A}\beta_{4-16}$ was not degraded during the reaction. Moreover, the reversal of the reaction in the absence of $\text{Zn}_7\text{MT-3}$ is in agreement with the instability of the $\text{Cu}(\text{I})$ -Cys complex. The re-oxidation to $\text{Cu}(\text{II})$ and to cysteine allow the re-creation of the $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ complex. In contrast, in the presence of $\text{Zn}_7\text{MT-3}$, $\text{Cu}(\text{I})_4\text{Zn}(\text{II})_4\text{MT-3}$ was formed and remained stable towards air oxidation. Hence the reaction is not reversible.

It is also important to note that the kinetics of the disappearance of the $d-d$ band were identical within the experimental error regardless of the presence or absence of $\text{Zn}_7\text{MT-3}$ (Figure 3). This fact indicates that the rate limiting step in the Cu-transfer from $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ to $\text{Zn}_7\text{MT-3}$ is the reduction of $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ by Cys (rather than the formation of the $\text{Cu}(\text{I})$ -Cys complex or the Cu transfer to $\text{Zn}_7\text{MT-3}$). In line with that also the kinetics of the disappearance of the $d-d$ band of $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ with GSH was hardly dependent on the presence of $\text{Zn}_7\text{MT-3}$ (results not shown).

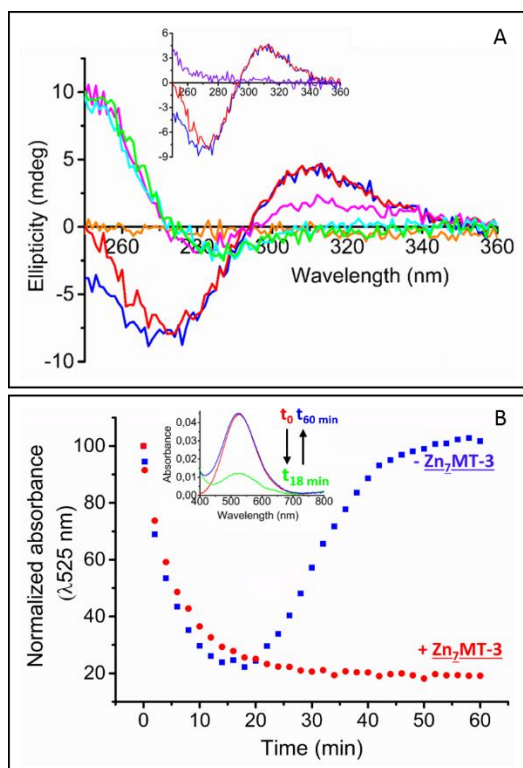


Fig. 3: Formation of the $\text{Cu}(\text{I})_4\text{Zn}(\text{II})_4\text{MT-3}$ species. Figure A) Circular Dichroism spectroscopy. CD spectra of $\text{A}\beta_{4-16}$ (orange line); $\text{A}\beta_{4-16}/\text{Cu}(\text{II})$, 1:0.9 (blue line); $\text{A}\beta_{4-16}/\text{Cu}(\text{II})/\text{Zn}_7\text{MT-3}$, 1:0.9:0.2 (red line, spectra registered after 30 min); $\text{A}\beta_{4-16}/\text{Cu}(\text{II})/\text{Zn}_7\text{MT-3}/\text{L-Cys}$ (cyan line, spectra recorded after 30 min); $\text{A}\beta_{4-16}/\text{Cu}(\text{II})/\text{Zn}_7\text{MT-3}/\text{GSH}$ (magenta line, spectra recorded after 260 min); $\text{Cu}(\text{II})/\text{Zn}_7\text{MT-3}$, 0.9:0.2 (light green line). Inset (panel A) Comparison of the $\text{Cu}(\text{II})/\text{A}\beta_{4-16}/\text{Zn}_7\text{MT-3}$ mixture (red line) with the single components $\text{Zn}_7\text{MT-3}$ (violet line), and $\text{Cu}(\text{II})/\text{A}\beta_{4-16}$ (blue line). Figure B) Kinetics for $\text{Cu}(\text{II})$ release from $\text{Cu}^{\text{II}}(\text{A}\beta_{4-16})$ with L-Cys in the presence (red profile) and absence (blue profile) of $\text{Zn}_7\text{MT-3}$. The normalized absorbance at 525 nm is plotted versus time. Inset Figure B) Uv-Vis spectra of $\text{A}\beta_{4-16}/\text{Cu}(\text{II})$, 1:0.9 (red line), $\text{A}\beta_{4-16}/\text{Cu}(\text{II})/\text{L-Cys}$ at $t_{18 \text{ min}}$ (light green line), $\text{A}\beta_{4-16}/\text{Cu}(\text{II})/\text{L-Cys}$ at $t_{60 \text{ min}}$ (blue line). Reaction conditions for CD experiment (final concentrations): 100 μM $\text{A}\beta_{4-16}$, 90 μM $\text{Cu}(\text{II})$, 20 μM $\text{Zn}_7\text{MT-3}$, 3 mM L-Cys or GSH. Reaction conditions for UV-Vis experiments: $[\text{A}\beta_{4-16}] = 500 \mu\text{M}$, $[\text{Cu}(\text{II})] = 450 \mu\text{M}$ (ratio 1:0.9), $[\text{L-Cys}] = 3 \text{ mM}$; $[\text{A}\beta_{4-16}] = 500 \mu\text{M}$, $[\text{Cu}(\text{II})] = 450 \mu\text{M}$, $[\text{Zn}_7\text{MT-3}] = 100 \mu\text{M}$ (ratio 1:0.9:0.2), $[\text{L-Cys}] = 3 \text{ mM}$.

To investigate whether $\text{Zn}(\text{II})$ released from MT-3 was bound to $\text{A}\beta_{4-16}$ peptide at the end of the reaction we decided to follow the overall reaction with Cys by $^1\text{H-NMR}$. Indeed we expected that $\text{Zn}(\text{II})$ binding to $\text{A}\beta_{4-16}$ would impact the NMR signatures of the coordinating groups in the peptide, like it was the case for $\text{Zn}(\text{II})$ -binding to $\text{A}\beta_{1-16}/28/40$. (e.g. ²⁴⁻²⁶ and review in ²⁷)

In the aromatic region shown in Figure 4, protons of A β ₄₋₁₆ from His(6,13,14), Phe4 and Tyr10 are present. Addition of paramagnetic Cu(II) to A β ₄₋₁₆ resulted in the broadening of His protons and disappearance of those from Phe and Tyr, due to the paramagnetic effect of Cu(II). As expected, upon Zn₇MT-3 addition to Cu^{II}(A β ₄₋₁₆), no change in the spectrum was observed, in line with the absence of aromatic amino acids in MT-3 and with the lack of reactivity of

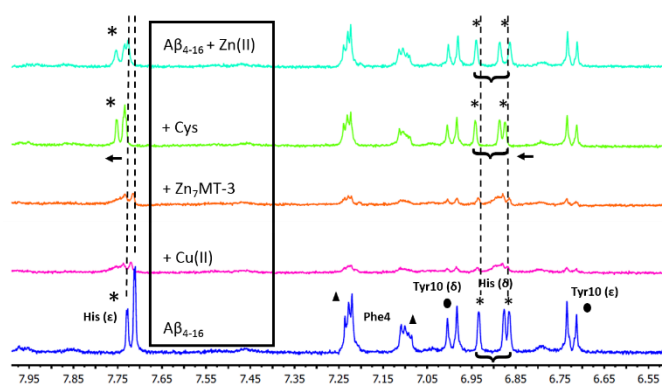


Fig. 4: Zn binding to the A β ₄₋₁₆ peptide. ¹H-NMR spectra were obtained from 10%D₂O/90%H₂O solutions in 50mM phosphate buffer at ν =400 MHz. Aromatic region of the spectra of A β ₄₋₁₆ (blue line) and after the addition of i) Cu(II) (purple line), ii) Zn₇MT-3 (orange line) iii) L-Cys (light green line); control of A β ₄₋₁₆ after the direct addition of 0.6 eq of Zn(II) (cyan line). Reaction conditions (final concentrations): [A β ₄₋₁₆]=300 μ M, [Cu(II)]=270 μ M, [Zn₇MT-3]=60 μ M (ratio 1:0.9:0.2), [L-Cys]=3 mM. The details for the amino acid residues are reported in Scheme S1 of Supporting Information.

Zn₇MT-3 in the Cu-Zn swap with A β ₄₋₁₆. However, when Cys was added to the mixture Cu^{II}(A β ₄₋₁₆) / Zn₇MT-3 we observed the recovering of the A β ₄₋₁₆ signatures, in line with the reduction of Cu(II) to diamagnetic Cu(I) and its release from A β ₄₋₁₆. Compared to the A β ₄₋₁₆ at the beginning, the protons of three His rings (H δ , H ϵ) were slightly down shifted. This was assigned to the binding of Zn(II), released from MT-3, to A β ₄₋₁₆. Indeed, direct addition of Zn(II) to A β ₄₋₁₆ gave rise to similar shifts of the His resonances, in line with the direct coordination of His to Zn. These data are consistent with at least partial Zn binding to A β ₄₋₁₆ at the end of the reaction and therefore suggest a Cu-Zn swap between the two peptides triggered by Cys.

From these experiments, with the complementary use of Uv-Vis, CD and ¹H-NMR we demonstrate that Cys and GSH can trigger the Cu transfer from Cu^{II}(A β ₄₋₁₆) complex to Cu(I)₄Zn(II)₄MT-3, by reduction of Cu(II) from the Cu^{II}(A β ₄₋₁₆) complex to Cu(I) and shuttling of the latter to MT-3. The full transfer of Cu(II) from A β ₄₋₁₆ was observed for Cys over a period of 30 min, whereas GSH under the same conditions reacted slower and only a partial transfer was observed. Furthermore, ¹H-NMR are consistent with the hypothesis that Zn released from MT-3 is at least partially bound to A β ₄₋₁₆.

The possibility to shuttle copper between the A β ₄₋₁₆ peptide and MT-3 in the presence of Cys and GSH indicate that they can modulate the copper/zinc distribution between the two species. Considering that intracellular and extracellular Cys and GSH fluctuations have been reported and that extracellular Cys/GSH concentrations are impacted by intracellular metabolism, such reaction might have relevance in the extracellular space, where A β peptide and MT-3 are mostly found together. A report of potential relevance is that extracellular deposition of A β peptide increases the extracellular concentration of reduced Cys.²⁸ Furthermore, the reaction with GSH, although sluggish under our *in vitro* experimental conditions, suggests a possibility of coupling the Cu(II)/A β biology with physiological processes mediated by GSH neurotransmission.

There are no conflicts to declare.

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