Zinc release of Zn_7-metallothionein-3 induces fibrillar type amyloid-β aggregates†

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Received 2nd July 2010, Accepted 4th October 2010
DOI: 10.1039/c0mt00027b

The reactive oxygen species H_2O_2 promotes the Zn_7-metallothionein-3 induced $A\beta_{40}$ aggregation of fibrillar type structures *via* slow cysteine oxidation and Zn^{2+} release, whereas amorphous aggregates are formed by addition of Zn^{2+} to $A\beta_{40}$.

In the central nervous system, specifically the brain, relatively high concentrations of zinc are found with regional selectivity. While approximately 80% of zinc in the brain is protein-bound zinc, the remainder is concentrated in presynaptic vesicles of zinc-enriched glutamergic neurons, containing millimolar Zn^{2+} concentrations. This vesicular zinc is released into the synaptic cleft upon neuronal activity, where it can through an interaction with many different postsynaptic targets modulate their activity.[1]

In Alzheimer's disease (AD) altered Zn^{2+} homeostasis is well documented. This observation along with the presence of Zn^{2+} in senile plaques, where its concentration can be as high as ~1 mM, indicated that this metal could play an important role in this neurodegenerative disorder.[2–4] Aggregation of the peptide amyloid-β (Aβ) has been proposed to be a key event in AD. Aggregates are believed to promote a neuronal dysfunction, and later on dementia *via* the production of reactive oxygen species (ROS).[5] Studies on AD animal models have also shown that genetic ablation of synaptic Zn^{2+} greatly reduces the amount of amyloid plaques.[6] *In vitro* studies showed that Zn^{2+} ions bind stoichiometrically to Aβ with an apparent dissociation constant of 1–10 μM at pH 7.4. (for review see ref. 7). Zn^{2+} can induce Aβ aggregation within time scale of milliseconds[8] and can promote synaptic targeting of oligomeric Aβ.[4] Generally, Zn^{2+} induced Aβ aggregates are rather amorphous with a lower content of fibrils and are less reactive with the amyloid marker thioflavin T (ThT) than aggregates of Aβ alone (reviewed in ref. 7 and 9). Although Zn^{2+} induces Aβ aggregation and destabilizes oligomers, it is not clear if Zn^{2+} has a protective or an exacerbating effect on the neurotoxicity of Aβ.[9,10]

In the brain, a molecule involved in zinc metabolism and linked to AD is a small metalloprotein metallothionein-3 (Zn_7MT-3).[11–13] Zn_7MT-3 is mainly expressed in hippocampal glutamergic neurons that release Zn^{2+} from synaptic vesicles.[14] Evidence has been provided that MT-3 occurs intra- and extracellularly.[15] Structural studies revealed that the protein can bind 7 Zn^{2+} ions *via* its 20 cysteines, forming two metal-thiolate clusters a Zn_3–Cys_9 cluster in the N-terminal β-domain and a Zn_4–Cys_{11} cluster in the C-terminal α-domain.[13,16]

Oxidative stress is implicated in the pathogenesis and/or progression of neurodegenerative diseases. In general, ZnMTs, including the mammalian Zn_7MT-3, Zn_7MT-2 and Zn_7MT-1 isoforms, through quenching of reactive oxygen and nitrogen species (ROS and RNS) protect cells from oxidative stress. This reaction leads to cysteine oxidation/modification and Zn^{2+} release (for recent review see ref. 12). However, structural differences among Zn_7MT-3 and Zn_7MT-1/-2 are responsible for their differing reactivity and biological activity.[17] Zn_7MT-3, but not Zn_7MT-1 and Zn_7MT-2 protects cultured neurons from the toxicity of Aβ.[18] This protective effect has been accounted for by the metal swap between Zn_7MT-3 and Cu^{2+}–Aβ, leading to the suppression of ROS production caused by the redox cycling of Cu^{2+}–Aβ.[19] These literature reports strongly indicate that ZnMT-3 and Aβ are co-localized near the synapses of zinc-enriched glutamergic neurons and are linked to AD and oxidative stress.

Here, we investigated if Zn^{2+} transfer from Zn_7MT-3 to $A\beta_{40}$ is possible in the absence and presence of H_2O_2. The term transfer was used in the sense to describe the Zn^{2+} movement from Zn_7MT-3 to $A\beta_{40}$ without an interaction between the two peptides. We found that Zn_7MT-3 donates Zn^{2+} to $A\beta_{40}$ not only in the presence of H_2O_2, but also in its absence. Moreover, we could show that Zn^{2+} transfer from Zn_7MT-3 modulates $A\beta_{40}$ aggregation differently than the addition of free Zn^{2+}.

First, we checked by absorption spectroscopy whether Zn^{2+} may be released from Zn_7MT-3 upon addition of H_2O_2. We have followed the CysS–Zn^{2+} charge-transfer band at 230 nm over time as a function of different H_2O_2 concentrations. The observation that the charge-transfer band decreased with time in a concentration dependent manner indicated that Zn^{2+} is slowly released from the Zn^{2+}-thiolate clusters in Zn_7MT-3 (Fig. 1A).

To obtain a more direct measure of Zn^{2+} release from Zn_7MT-3 in the presence of H_2O_2, the protein sample was incubated with increasing concentrations of H_2O_2 and the Zn^{2+} release monitored by the complexing dye zincon. Fig. 1B shows the H_2O_2 concentration dependent Zn^{2+} release from 20 μM Zn_7MT-3, obtained by following the formation of Zn^{2+}–zincon complex at 620 nm over 1 day. We also observed that a small amount of Zn^{2+} (about 0.1 mole equivalent) was apparently released even in the absence of H_2O_2. To confirm this result the kinetics of Zn^{2+} release was measured directly

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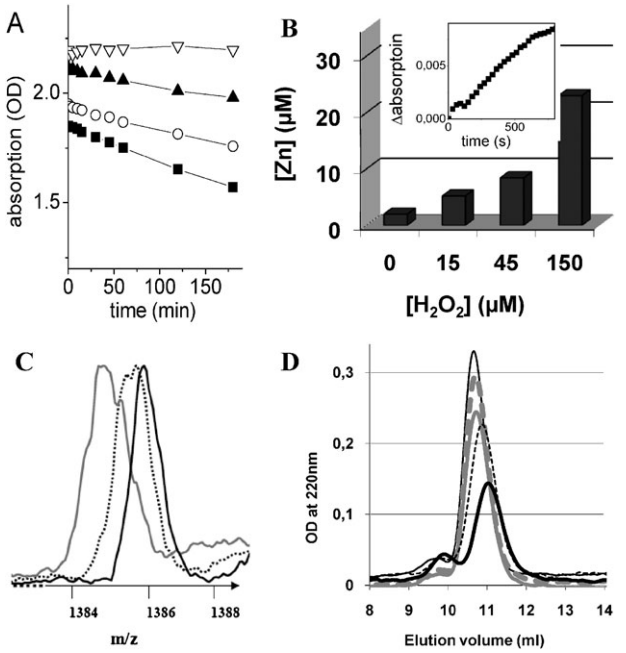

Fig. 1 (A) Time dependence of the Zn_7MT-3 absorption at 230 nm as a function of different H_2O_2 concentrations. The Cys–Zn^{2+} charge-transfer band at 230 nm was followed over 3 h. Conditions: Zn_7MT-3 20 μM, Hepes 20 mM, NaCl 20 mM, at pH 7.4. H_2O_2 concentrations used 0 μM (▽), 50 μM (▲), 100 μM (○), and 200 μM (■). (B) H_2O_2 induced Zn^{2+} release from Zn_7MT-3 monitored by complexing dye zincon. The Zn^{2+} release from Zn_7MT-3 as a function of different concentrations of H_2O_2 after a 1 day incubation. Conditions: 20 μM Zn_7MT-3, H_2O_2: 0, 15, 45 or 150 μM, Hepes 20 mM, NaCl 20 mM, pH 7.4. Prior to measurement the sample was 20 times diluted with the same buffer containing 20 μM zincon. The presented Zn^{2+} release relates to the original Zn_7MT-3 concentration of 20 μM; *Inset*: Time dependence Zn^{2+} release from 10 μM Zn_7MT-3 in the absence of H_2O_2 monitored trough the absorption of 20 μM zincon at 620 nm. (C) Mass spectra of apoMT-3 after incubation with different H_2O_2 concentrations. Conditions: 40 μM Zn_7MT-3 in 20 mM Hepes, 20 mM NaCl, at pH 7,4 was incubated for 1 day with 0 (black), 40 (dotted), and 200 μM (gray)) H_2O_2. Prior to analyses the samples were diluted with two volumes of H_2O–acetonitrile (50% v/v) containing 0.1% formic acid. The measurements were performed at pH 3, which induced the release of Zn^{2+}. Only the 5 times charged peaks are shown. (D) Size exclusion chromatography (SEC) of Zn_7MT-3 after reaction with H_2O_2. The Zn_7MT-3 sample was incubated for 1 day with 0 (thick dotted gray), 20 (thick solid gray), 100 (thin dotted black), or 200 μM (thick solid black) H_2O_2. A freshly prepared solution of Zn_7MT-3 without H_2O_2 was used as a control (thin solid). Conditions: 20 μM Zn_7MT-3, 20 mM Hepes, 20 mM NaCl, at pH 7.4.

using a higher Zn_7MT-3 concentration (10 μM) (Fig. 1B, inset). Indeed, the absorption band of the Zn^{2+}–zincon complex at 620 nm increased linearly with time. A similar Zn^{2+} release from Zn_7MT-3 in the presence of increasing zincon concentrations has also been reported by Chen *et al.*[17]

To examine whether the Zn^{2+} release from Zn_7MT-3 by H_2O_2 is due to a cysteine oxidation and not its removal by zincon, a mass spectrometry (ESI-MS) was applied. We analyzed the apoMT-3 form after the Zn^{2+} depletion of the holoprotein at acidic pH. Although no deconvoluted peaks could be obtained, the results suggest that cysteine oxidation occurred by adding H_2O_2 (Fig. 1C). The apoMT-3 form has a theoretical mass of 6927.1 Da. In the absence of H_2O_2, an average mass of 6928.0 Da was obtained. However, upon addition of H_2O_2 a shift to smaller average masses was seen, in agreement with the formation of disulfide bonds (loss of two protons leads to a decrease of mass by 2 Da). In addition, a broadening of the mass peak was also observed indicating a mass heterogeneity due to the formation of a varying number of disulfide bonds in different protein molecules. Consequently, the Zn_7MT-3 structure is susceptible to oxidation by H_2O_2. Taken together, the results of zincon, ESI-MS, and the CysS–Zn^{2+} charge-transfer band absorption measurements all indicate that H_2O_2, through cysteine oxidation to disulfides, releases Zn^{2+} from Zn_7MT-3 in a concentration dependent manner.

Next we used size-exclusion chromatography (SEC) to examine whether the disulfide bridges formed upon oxidation of Zn_7MT-3 by H_2O_2 were intra- or intermolecular. In general, while in the case of intermolecular disulfide bridges the formation of dimers or higher polymers of Zn_7MT-3 should be observed, in the case of intramolecular disulfide bridges a decrease of the hydrodynamic radius of ZnMT-3, due to a more compact disulfide bridged structure would be expected.[20] Fig. 1D shows the results of the SEC of Zn_7MT-3 after a 1 day incubation with different concentrations of H_2O_2. The freshly prepared Zn_7MT-3 eluted at about a 10.5 ml volume, a typical value for monomeric metal loaded Zn_7MT-3 with an apparent molecular mass of about 22 kDa due to its ellipsoid shape.[21] A small amount of ZnMT-3 dimers, eluting with a volume of 9.7 ml, was also observed in the control experiments as reported.[20] Incubation of Zn_7MT-3 with increasing H_2O_2 concentrations resulted in two effects. First, the monomeric peak shifted to a higher elution volume. The latter effect reflects a decrease in the apparent molecular mass of the protein consistent with the increase in structure compactness brought about by a disulfide formation and Zn^{2+} release.[20] Second, an increase in size of the dimeric peak, in line with the formation of some intermolecular disulfide bridges, was also observed. However, since the monomeric peak even at 200 μM H_2O_2 was still much higher, this indicates that the disulfide bridges are predominantly formed intramolecularly. This conclusion is in agreement with the ESI-MS data (see above), where molecular masses consistent with predominately intramolecular disulfide bonds were obtained. Note that the apparent decrease in peak intensity seen in the elution profile of Zn_7MT-3 at a higher H_2O_2 concentration does not reflect a protein loss, but is due mainly to the decrease in the molar extinction coefficient of the CysS–Zn^{2+} charge-transfer band upon protein oxidation (see above).

The above results indicate that cysteine oxidation in Zn_7MT-3 by H_2O_2 results in a release of Zn^{2+} from the protein. Next we investigated whether the released Zn^{2+} can stimulate aggregation of the $A\beta_{40}$ peptide. The aggregation process was followed by turbidimetry, ThT assay, and transmission electron microscopy. The turbidimetry is a simple, but crude assay to follow the formation of aggregates. The turbidity experiments were performed at 300 nm over 100 min and are summarized in Fig. 2A. $A\beta_{40}$ alone showed a very small turbidity increase under the conditions used

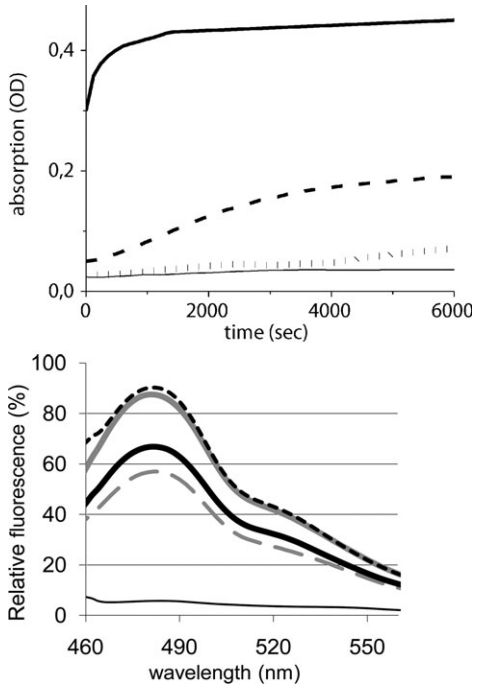

Fig. 2 (Upper panel) Turbidity assay: Turbidity of $A\beta_{40}$ was measured at 300 nm over 100 min (thin solid line), Zn-Aβ (thick solid), $A\beta_{40}$ and Zn_7MT-3 (dotted grey), and $A\beta_{40}$, Zn_7MT-3 and H_2O_2 (dashed black) Conditions: Aβ 30 μM, Zn_7MT-3 20 μM, 100 μM H_2O_2, Hepes 20 mM, NaCl 20 mM, pH 7.4. (Lower panel) Thioflavin T fluorescence: Emission spectra of ThT (10 μM), excitation at 435 nm. ThT was added after sample incubation for 1 day to $A\beta_{40}$ (gray dashed), Aβ and 1 equiv Zn^{2+} (gray solid), $A\beta_{40}$ and Zn_7MT-3 (black thick solid), $A\beta_{40}$, Zn_7MT-3 and H_2O_2 (black dotted), ThT alone (thin solid); Conditions: 30 μM $A\beta_{40}$, 20 μM Zn_7MT-3, 100 μM H_2O_2, 20 mM Hepes, 20 mM NaCl, at pH 7.4.

(Fig. 2A, thin solid line). In contrast, the Zn^{2+}–$A\beta_{40}$ complex, formed upon addition of 1 mole equivalent of Zn^{2+} solution, exhibited a fast and dramatic increase in turbidity (Fig. 2A, thick solid line), in agreement with the aggregation accelerating effect of Zn^{2+} described in the literature.[9,22] $A\beta_{40}$ in the presence of Zn_7MT-3 and without H_2O_2 showed a small but significant turbidity increase compared to that of $A\beta_{40}$ alone (Fig. 2A, dotted line). However, upon addition of H_2O_2 to the latter sample the turbidity increase was much higher (Fig. 2A, dashed line), but did not reach the intensity and rapidity of aggregation of the Zn^{2+}–$A\beta_{40}$ complex (Fig. 2A, thick solid line). The latter result indicates that the Zn^{2+} release from Zn_7MT-3 with H_2O_2 results in an intermediate turbidity between $A\beta_{40}$ alone and Zn^{2+}–$A\beta_{40}$. Furthermore, in accordance with the Zn^{2+} release from Zn_7MT-3 in the absence of H_2O_2 (see above), the aggregation promoting effect has also been seen.

The measurement of turbidity only provides information about the global aggregation state. In contrast, the fluorescence dye ThT is relatively specific for amyloid structures such as that of the fibrils of Aβ. The corresponding ThT fluorescence spectra were measured after 1 day of sample incubation (Fig. 2B). In this case, $A\beta_{40}$ alone (Fig. 2B, gray dashed line) showed ThT fluorescence that increased upon binding of 1 mole equivalent of Zn^{2+} solution (Fig. 2B, gray solid line) in line with the Zn^{2+} amyloid promoting effect. Interestingly, although Zn^{2+} release from Zn_7MT-3 with H_2O_2 resulted in an intermediate turbidity between $A\beta_{40}$ alone and Zn^{2+}–$A\beta_{40}$ (see above), the corresponding ThT fluorescence (Fig. 2B, black dotted line) revealed a value closely similar to that of the Zn^{2+}–$A\beta_{40}$ complex prepared upon addition of Zn^{2+} solution, suggesting that the amounts of fibrilar structure are similar.¶ Moreover,

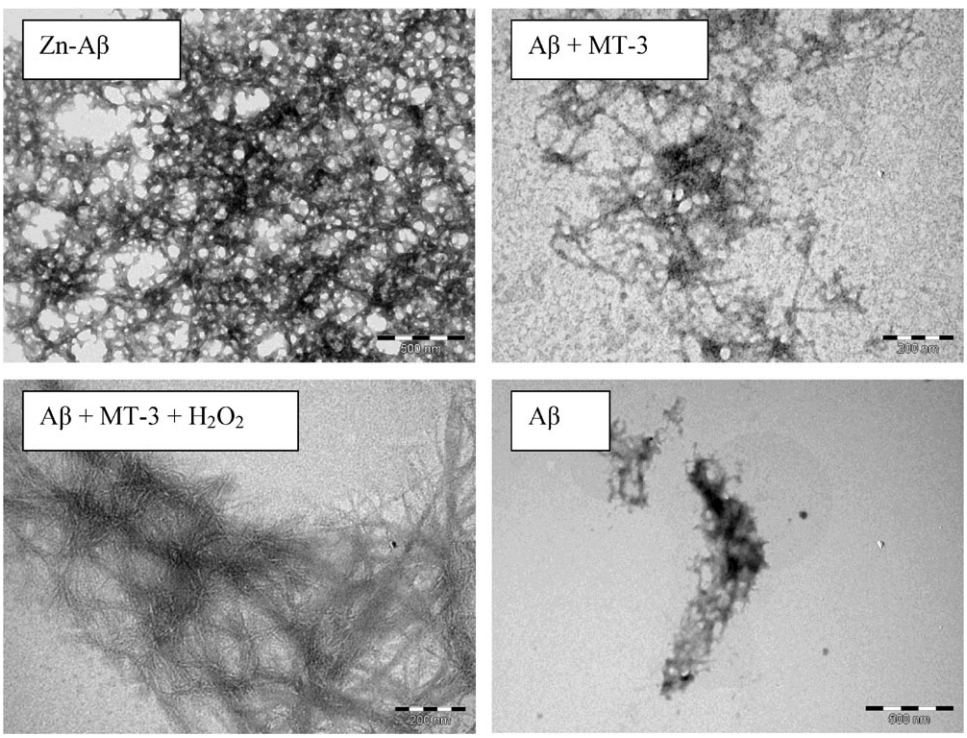

Fig. 3 Transmission electron microscopy (negatively stained) of $A\beta_{40}$, Zn^{2+}–$A\beta_{40}$, $A\beta_{40}$ plus Zn_7MT-3, and $A\beta_{40}$, Zn_7MT-3 plus H_2O_2 after 1 day incubation. Concentrations: 30 μM $A\beta_{40}$, 30 μM $ZnSO_4$, 20 μM Zn_7MT-3, and 100 μM H_2O_2.

since a considerable ThT fluorescence of aggregated $A\beta_{40}$ was also seen in the presence of Zn_7MT-3 without H_2O_2 (Fig. 2B, black solid line), this indicates that released Zn^{2+} under these conditions is also able to promote significantly the formation of $A\beta_{40}$ fibrils (see below).

Transmission electron microscopy was used to investigate the $A\beta_{40}$ aggregates formed under the different conditions (Fig. 3). The estimated amount of aggregates follows the order Zn^{2+}–$A\beta_{40}$ > $A\beta_{40}$ plus Zn_7MT-3 plus H_2O_2 > $A\beta_{40}$ plus Zn_7MT-3 > $A\beta_{40}$ alone. These results parallel the turbidity measurements as expected, since turbidity measures the $A\beta_{40}$ aggregation. However, the content of fibrillar structures reflects more the ThT fluorescence. Thus, the solution of $A\beta_{40}$ and Zn_7MT-3 in the presence and absence of H_2O_2 showed the highest content of fibrillar structures, explaining why the ThT fluorescence was as high as for the Zn^{2+}–$A\beta_{40}$ complex, although the latter form is more aggregated. This further suggests that while in the former case $A\beta_{40}$ aggregates slowly form substantial amounts of fibrillar structures, in the latter case fast Zn^{2+} binding to $A\beta_{40}$ results in more amorphous type aggregates.

In summary, the results presented above suggest that cysteine oxidation in Zn_7MT-3 in the presence and absence of H_2O_2 leads to Zn^{2+} release, which in turn can induce aggregation of $A\beta_{40}$. Moreover, the slow release of Zn^{2+} promotes the formation of amyloid-type fibrils in contrast to amorphous $A\beta_{40}$ aggregates formed by the addition of Zn^{2+}. As a consequence it is the rate of cysteine oxidation in MT-3 that regulates the assembly of $A\beta_{40}$ aggregates and their morphology. The results are in line with the observation that zinc concentrations modulate the rate of assembly and the type of morphology of $A\beta_{40}$ aggregates. (See *e.g.* ref. 8 and 23). This effect may have biological relevance since both Aβ and Zn_7MT-3 have been linked to AD, oxidative stress and are likely to be co-localized in the brain. Zn_7MT-3 might be involved in the protection of cells not only by its capacity for ROS scavenging,[24] but also *via* Zn^{2+} release and subsequent binding to Aβ. However, at present it is not clear whether Zn^{2+} binding has a beneficial or rather detrimental effect on Aβ toxicity. Thus, the biological significance of the *in vitro* findings remains to be established.

Acknowledgements

This work was supported by the programme blanc NT09-488591 (Neurometals) of Agence national de la recherche (ANR) (P.F.) and Swiss National Science Foundation Grant 3100A0-111884 (M.V.). We would like to thank Cathy Claperols and Vincent Colliere (LCC Toulouse) for measuring mass spectrometry and transition electron microscopy, respectively. Dr Fatima Bousejra-El Garah (LCC) is acknowledged for measuring effect of H_2O_2 on ThT fluorescence.

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¶ We verified that H_2O_2 does not affect the ThT fluorescence under the conditions used here. Thus the increase of ThT fluorescence in samples containing $A\beta_{40}$, Zn_7MT-3 and H_2O_2 can not be ascribed to a direct interaction of H_2O_2 with ThT. This supports the view that the increase in ThT fluorescence is due to increased Zn-release from Zn_7MT-3 upon reaction with H_2O_2.

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