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Communication

Copper Transporters? Glutathione Reactivity of Products of Cu–Aβ Digestion by Neprilysin

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ABSTRACT: $A\beta_{4-42}$ is the major subspecies of Aβ peptides characterized by avid Cu(II) binding via the ATCUN/NTS motif. It is thought to be produced *in vivo* proteolytically by neprilysin, but *in vitro* experiments in the presence of Cu(II) ions indicated preferable formation of C-terminally truncated ATCUN/NTS species including $Cu^{II}A\beta_{4-16}$, $Cu^{II}A\beta_{4-9}$, and also $Cu^{II}A\beta_{12-16}$, all with nearly femtomolar affinities at neutral pH. Such small complexes may serve as shuttles for copper clearance from extracellular brain spaces, on condition they could survive intracellular conditions upon crossing biological barriers. In order to ascertain such possibility, we studied the reactions of $Cu^{II}A\beta_{4-16}$, $Cu^{II}A\beta_{4-9}$, $Cu^{II}A\beta_{12-16}$, and $Cu^{II}A\beta_{1-16}$ with reduced glutathione (GSH) under aerobic and anaerobic conditions using absorption spectroscopy and mass spectrometry. We found $Cu^{II}A\beta_{4-16}$ and $Cu^{II}A\beta_{4-9}$ to be strongly resistant to reduction and concomitant formation of Cu(I)−GSH complexes, with reaction times ∼10 h, while $Cu^{II}A\beta_{12-16}$ was reduced within minutes and $Cu^{II}A\beta_{1-16}$ within seconds of incubation. Upon GSH exhaustion by molecular oxygen, the $Cu^{II}A\beta$ complexes were reformed with no concomitant oxidative damage to peptides. These finding reinforce the concept of $A\beta_{4-x}$ peptides as physiological trafficking partners of brain copper.

Aβ peptides are products of extracellular hydrolysis of amyloid precursor protein (APP) present in neuronal synaptic membranes.[1−3] Overproduction or excessive aggregation of Aβ has been long implicated as an upstream cause of neuronal death in Alzheimer's disease (AD).[4,5] This concept gained recent reinforcement when direct deleterious action of Aβ dimers on neuronal glutamate receptors was demonstrated.[6] Aβ peptides are a heterogeneous peptide family, generated by a number of primary (acting on APP) and secondary (acting on Aβ) proteases. The most studied members of the Aβ family are $A\beta_{1-40}$ and $A\beta_{1-42}$, but current analytical studies demonstrated a high abundance of the N-truncated $A\beta_{4-42}$ peptide in both healthy and AD human brains.[7,8]

$A\beta_{1-x}$ peptides (x denotes naturally occurring 42 and 40 species, as well as model peptides 28 and 16) bind a Cu^{II} ion avidly with K_d about 100 pM. The resulting complexes can be easily activated by ascorbate to catalyze the production of reactive oxygen species (ROS).[9−12] Supported by reports on deranged copper metabolism in AD brains and colocalization of copper and aggregated Aβ peptides in amyloid plaques, these properties gave rise to a concept of $Cu^{II}−A\beta_{1-x}$ complexes as neurotoxic species in AD.[5,13,14]

Remarkably, $A\beta_{4-42}$ and its C-terminally truncated analogs are Cu^{II} chelators much more avid (3000 times at pH 7.4 for $A\beta_{4-16}$ vs $A\beta_{1-16}$) and specific than $A\beta_{1-x}$ peptides.[15] This results from a specific character of their N-terminal sequence, Phe-Arg-His, belonging to the ATCUN/NTS family.[16] Furthermore, unlike $Cu^{II}−A\beta_{1-x}$ complexes, $Cu^{II}−A\beta_{4-x}$ did not generate ROS and could not be reduced electrochemically to Cu^{I} species.[15] These findings suggest that $A\beta_{4-42}$ might actually serve as synaptic copper scavenger, helping restore the resting state of glutamatergic synapse, after the physiological Cu^{2+} release during neurotransmission.[17,18]

Digestion of Aβ peptides is considered as one of the major routes of their detoxification. They are thought to be cleaved down to oligopeptides that can cross the blood−brain barrier.[19,20] An Aβ-specific peptidase has not been found. Instead, a number of brain proteases with other known functions have been implicated in this process, including neprilysin (NEP), angiotensinogen converting enzyme (ACE), and insulin degrading enzyme (IDE).[21] NEP action on $A\beta_{1-x}$ has also been indicated as the main source of $A\beta_{4-x}$ in the brain.[22,23] A recent study of $A\beta_{1-16}$ and $A\beta_{1-40}$ cleavage by NEP in the presence and absence of Cu^{II} ions did not quite reproduce such activity, however. Instead a significant extent of peptide fragmentation was observed. The fast digestion of the Gly9−Tyr10 bond yielded the Cu^{II}-complexed short peptide $A\beta_{4-9}$. Additionally, a complex of $A\beta_{12-16}$ was generated abundantly when $A\beta_{1-16}$ was used as a substrate and was also present as a minor species for $A\beta_{1-40}$.[24] $A\beta_{4-9}$ and $A\beta_{12-16}$ are even stronger Cu^{II} chelators than $A\beta_{4-16}$, with K_d of 6.6 fM and 9.5 fM vs 30 fM at pH 7.4.[25] This finding gave rise to an idea that such complexes might serve as shuttles for removing excess copper from the brain.

Crossing the blood−brain barrier (BBB) is a complicated and not fully elucidated process, involving passage through the
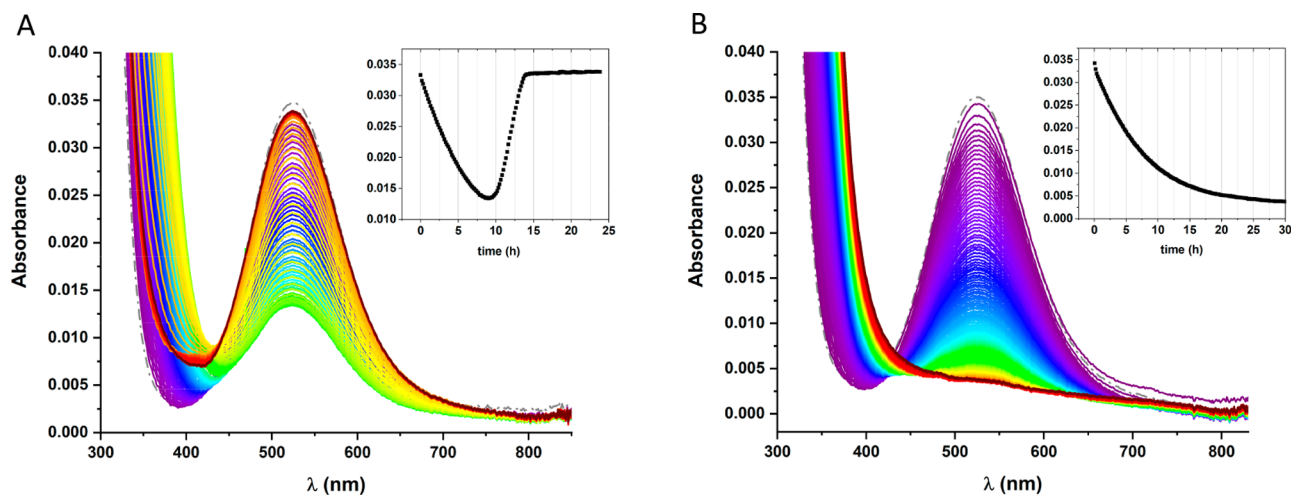
Figure 1. UV−vis spectra of the reaction of 0.315 mM Cu(II) ions with 1.75 mM GSH in the presence of 0.35 mM $A\beta_{4-16}$ in 20 mM ammonium acetate buffer, pH 7.4, carried out for 25−30 h at 25 °C under aerobic (A) and anaerobic (B) conditions. UV−vis spectrum of Cu(II)$A\beta_{4-16}$ showed by dashed line. The spectra were recorded in 10 min intervals. Insets show selected kinetic traces at 525 nm.

Table 1. Initial Reaction Velocities, V_0, and Conversion Degrees of Cu^{II} Reduction to Cu^{I}/GSH in the Presence of $A\beta_{4-16}$ Peptide[a]

	aerobic			anaerobic
	18 °C	25 °C	37 °C	25 °C
V_0	5.0 ± 0.2	10 ± 1	21 ± 3	9 ± 4
conversion degree	0.58 ± 0.03	0.65 ± 0.04	0.64 ± 0.05	0.92 ± 0.03

[a]Determined from the initial decay of the d−d band at 525 nm. Velocities are given in nM/s; all data are shown ± SD.

layer of epithelial cells forming the blood vessel walls.[26] Therefore, the transferred molecule could be exposed for a certain amount of time to intracellular conditions, including millimolar (0.5−10 mM) concentrations of reduced gluta-thione (GSH).[27] GSH is the main reducing agent for Cu^{II} species entering the cell interior and is also implicated in the intracellular Cu^{I} transport.[28−30] It is also present extracellularly in the brain, serving as neuromodulator.[31] GSH facilitated the otherwise very sluggish reductive copper transfer from $A\beta_{4-16}$ to metallothionein-3 (MT-3), indicating that it could reduce the $A\beta_{4-x}$-bound Cu(II) to Cu(I) despite the electrochemical resistance of the parent complex to such reaction.[32]

We therefore decided to follow the reaction of $A\beta_{4-x}$ peptides with GSH in more detail, using $A\beta_{4-16}$ as a suitable soluble, nonaggregating substitute of $A\beta_{4-42}$. We also tested $A\beta_{4-9}$ and $A\beta_{12-16}$. Our experiments were performed under aerobic (21% O_2) and anaerobic (<1% O_2) conditions in order to gain insight into the relation of the studied reaction to oxidative stress conditions. The differential kinetic resistance of the studied complexes to reduction supports their possible roles in Cu^{II} transport in the brain.

In initial experiments, 0.315 mM Cu^{2+} ions were reacted for 24 h with 1.75 mM GSH in 20 mM ammonium acetate at 25 °C under aerobic conditions, with and without 0.35 mM $A\beta_{4-16}$ (0.9/5/1 and 0.9/5 molar ratios, Figure 1A and Supporting Information Figure S1, respectively). In control experiments 1.75 mM glutathione disulfide (GSSG) was used instead of GSH (Figure S2), and Cu^{2+} ions were omitted from the reaction of GSH with $A\beta_{4-16}$ (Figure S3). These experiments allowed us to identify and assign the spectral changes occurring in the course of reactions of $Cu^{II}(A\beta_{4-16})$ with GSH. New bands in the near-UV range between 315 and 265 nm (Figure S4) appeared gradually in the presence of

$A\beta_{4-16}$, at the expense of the Cu(II) band of the 4N complex at 525 nm. In the absence of the peptide, the same bands emerged rapidly. They could be assigned to the Cu(I) complex of GSH, reported previously by others.[33,34]

This Cu^{II} reduction phase lasted for about 9 h and reproducibly reached about 65% Cu^{II} conversion at 25 °C, as calculated from the intensity of the $Cu^{II}(A\beta_{4-16})$ d−d band at 525 nm (Table 1). It was followed by the shorter reoxidation phase, which led to a practically full restoration of $Cu^{II}(A\beta_{4-16})$. In the absence of $A\beta_{4-16}$, the Cu^{II}GSSG complex absorbing at 625 nm was the final reaction product (Figure S1). It was not formed in the presence of $Cu^{II}(A\beta_{4-16})$, because of the log K difference at pH 7.4 in favor of the latter, 10.37 vs 13.53.[15,35] The reaction rates increased with temperature (Figure S5). The ESI-MS analysis of reaction products indicated the absence of covalent oxidative modification of $A\beta_{4-16}$ (Figures S6 and S7). The only change in its mass spectrum was due to partial detachment of bound Cu^{II} ion resulting from its capture by GSH. The mass deficit of 2 Da, seen only in the copper-containing species, indicated the native ATCUN/NTS complex with two deprotonated, Cu^{II}-bound amide nitrogens.[15] A transient spectral feature at 390−405 nm accompanied the Cu^{I} reoxidation phase. The same feature was present during the reoxidation phase of Cu^{II}/GSH reaction in the absence of $A\beta_{4-16}$ (Figure S8); hence it involves neither $A\beta_{4-16}$ nor its Cu^{II} complex. A similar band was seen previously in a study of Cu^{I} complexes in MT-3 and interpreted to originate from $Cu^{I}−Cu^{I}$ interactions in the Cu_4-thiolate cluster.[36] Indeed, Cu^{I} preferentially forms a Cu_4GSH_6 cluster at the molar excess of GSH.[33] However, the selective appearance of this low-energy band during oxidative decomposition of Cu_4GSH_6 by molecular oxygen

suggests a contribution from partially oxidized species such as disulfide or Cu^{II}. This issue will be investigated separately.

The next series of reduction experiments was performed under the effectively anaerobic conditions, and indeed only the reduction phase of the reaction was observed during the 24 h incubation, leading to full Cu^{II} reduction (Figure 1B). Upon extending the incubation to 50 h, however, the reoxidation phase was observed after about 36 h of the incubation (Figure S9). This effect was due to ambient oxygen penetration of the samples residing in the spectrophotometer. The comparison of kinetic traces indicated the similarity of the early phase of the reduction process between the aerobic and anaerobic conditions (Figure S10). These traces exhibited the mathematical form of first order kinetics for all conditions, only differing by the degree of Cu^{II} reduction: ca. 65% under aerobic and nearly 100% under anaerobic conditions. However, as the actual reaction order was not determined, we compared the kinetics of individual reactions using initial velocities. The rate of Cu(II) reduction did not depend on the presence of ambient oxygen (Tables 1 and 2).

Table 2. Initial Reaction Velocities, V_0, and Conversion Degrees of Cu^{II} Reduction to Cu^I/GSH Performed at 25 °C in the Presence of $A\beta_{4-9}$ and $A\beta_{12-16}$ Peptides[a]

	aerobic	anaerobic
$A\beta_{4-9}$		
V_0	7 ± 2	10 ± 2
conversion degree	0.54 ± 0.05	0.91 ± 0.04
$A\beta_{12-16}$		
V_0	2200 ± 500	1600 ± 400
conversion degree	0.97 ± 0.02	0.98 ± 0.02

[a]Determined from the initial decay of the d−d band (527 and 524 nm, respectively). Velocities are given in nM/s; all data are shown ± SD.

Figure 2 presents examples of experiments performed aerobically with Cu(II) complexes of $A\beta_{4-9}$ and $A\beta_{12-16}$ peptides. The $CuA\beta_{4-9}$ reduction was similarly slow, but that of $CuA\beta_{12-16}$ was about 200 times faster than that of $CuA\beta_{4-16}$ (Table 2). The reoxidation phase occurred, however, similarly

in all three cases (Figures 1 and 2). The reduction of $CuA\beta_{1-16}$ under the same conditions was too fast for quantitation (Figure S11).

The kinetic, but not thermodynamic, resistance of $Cu^{II}A\beta_{4-16}$ to reduction to Cu^I species by thiols has been indicated in previous experiments.[32,37] Its kinetic character is reinforced by fast reduction of $Cu^{II}A\beta_{12-16}$, which has higher thermodynamic stability than $Cu^{II}A\beta_{4-16}$.[15,25] A clue for the mechanistic basis of this behavior is provided by the accelerating role of glutamic acid in both reductive copper transfer to MT-3 and nonreductive transfer to EDTA, along the affinity gradient.[38] This finding was interpreted in terms of assistance of copper transfer from the ATCUN/NTS motif via a putative partially coordinated intermediate species prone to form a ternary complex with a small transfer catalyst ligand. A similar mechanism was recently proposed in a study of Cu(II) reduction by GSH alone.[39] In the case of $Cu^{II}A\beta_{12-16}$, the fast Cu^{II} reduction is most likely facilitated by the His residue in position two of the peptide chain, able to provide an alternatively coordinated, minor 3N species.[40,41] Such complexes are known to exchange Cu^{II} ions rapidly.[42] They can also stabilize transient Cu^I species.[38] $Cu^{II}A\beta_{1-16}$ is known to facilitate Cu^{II} reduction to Cu^I by a number of mechanisms and is prone to ternary complex formation.[43,44]

Summarizing, the results presented above indicate that $Cu^{II}A\beta_{4-16}$ and especially $Cu^{II}A\beta_{4-9}$ are sufficiently kinetically resistant to reduction by physiological concentrations of GSH to survive in the cell cytosol for hours without eliciting oxidative damage, while $Cu^{II}A\beta_{12-16}$ and $Cu^{II}A\beta_{1-16}$ do not have this ability. Therefore, $Cu^{II}A\beta_{4-x}$ complexes are good candidates to shuffle Cu^{II} across the blood−brain barrier and in and out of the brain cells.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.inorgchem.0c00427.

Experimental details, additional spectroscopic experiments, mass spectrometry data (PDF)
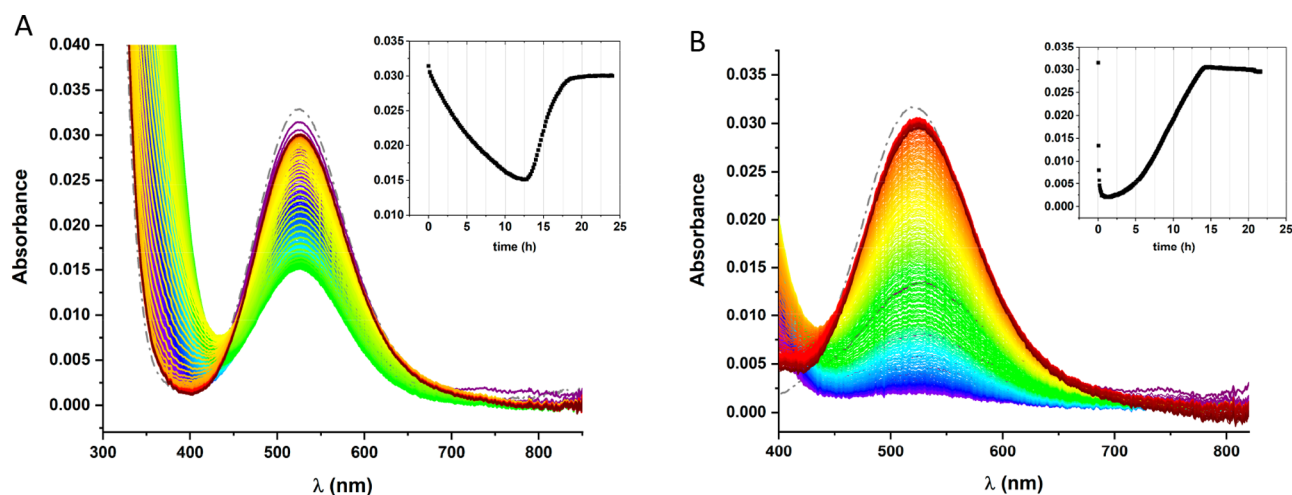

Figure 2. UV−vis spectra of the reaction of 0.315 mM Cu(II) ions with 1.75 mM GSH in the presence of 0.35 mM $A\beta_{4-9}$ (A) and $A\beta_{12-16}$ (B) in 20 mM ammonium acetate buffer, pH 7.4, carried out for 22−24 h at 25 °C under aerobic conditions. The spectra were recorded in 10 min intervals. Insets show selected kinetic traces at 527 nm (A) and 524 nm (B).

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Notes

The authors declare no competing financial interest.

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