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# Redox Chemistry of Copper–Amyloid-β: The Generation of Hydroxyl Radical in the Presence of Ascorbate is Linked to Redox-Potentials and Aggregation State

Luc Guilloreau,[a] Sarah Combalbert,[a] Alix Sournia-Saquet,[a] Honoré Mazarguil,[b] and Peter Faller*[a]

*Aggregation of the β-amyloid peptide (Aβ) to amyloid plaques is a key event in Alzheimer's disease. According to the amyloid-cascade hypothesis, Aβ aggregates are toxic to neurons through the production of reactive oxygen species (ROS). Copper ions play an important role, because they are able to bind to Aβ and influence its aggregation properties. Moreover, Cu–Aβ is supposed to be directly involved in ROS production. To get a better understanding of these reactions, we measured the production of HO• and the redox potential of Cu–Aβ. The results were compared to other biological copper–peptide complexes in order to get an insight into the biological relevance. Cu–Aβ produced more HO• than the complex of copper with Asp-Ala-His-Lys (Cu–DAHK), but less than with Gly-His-Lys (Cu–GHK). Cyclic voltammetry revealed that the order for reduction potential is Cu–GHK > Cu–Aβ > Cu–DAHK, but for the oxidation potential the order is reversed. Thus, easier copper redox cycling correlated to higher HO• production. The copper complex of the form Aβ1–42 showed a HO• production five-times higher than that of the form Aβ1–40. Time-dependence and aggregation studies suggest that an aggregation intermediate is responsible for this increased HO• production.*

## Introduction

Amyloid plaques are a hallmark in the brain of Alzheimer's disease (AD) victims.[1] These plaques are constituted mainly of an aggregated peptide called amyloid-β (Aβ).[2] According to the amyloid cascade hypothesis, an increased Aβ accumulation and aggregation leads first to the formation of Aβ oligomers and then to amyloid plaques.[3] These oligomers are supposed to provoke neuronal dysfunction and later on dementia.[3]

Aβ is a peptide of 39 to 43 amino acids. The two major forms found in amyloid plaques are Aβ40 (40 amino acids long) and Aβ42. Although the Aβ40 and Aβ42 are ubiquitous in the biological fluids of humans, it is thought that the longer Aβ42 is more pathogenic. Aβ42 has a higher propensity to aggregate than Aβ40 and is accumulated in amyloid plaques; it has also been shown to be more neurotoxic than Aβ40.

Copper has also been found in amyloid plaques at high concentrations (~0.4 mM).[4] Increasing evidence supports an important role of this metal ion in the metabolism and aggregation of Aβ, which is linked to Alzheimer's disease.[5–8] Aβ binds two equivalents of $Cu^{II}$ with an apparent $K_d$ of ~0.1 μM and 10 μM.[9–12] Probably only the the first equivalent is biologically relevant, because copper has been found substoichiometricly in amyloid plaques. This first equivalent of $Cu^{II}$ is in a square-planar environment with the putative ligands His6, His12, His13 and Asp1.[9,10,12]

Copper has been shown to increase Aβ toxicity towards neuronal cell cultures,[13] but the precise mechanism responsible for the Aβ toxicity that leads to the death of neurons in the diseased brains is still unclear. Nevertheless, more and more evidence indicates that the cause of neuronal cell loss in AD might be linked to excessive free radical generation. Reactive oxygen species (ROS) such as HO• and $O_2^{\bullet-}$, which are generated under oxidative stress conditions might play a key role in the neurotoxicity of Aβ. Due to the close relationship between redox-active metal ions and ROS, the complex of Cu–Aβ could be involved in the toxicity of Aβ towards neurons.[7] Initially it was reported that Aβ could spontaneously produce radicals in the absence of metals,[14] but subsequent work showed that at least trace amounts of metals were necessary for the production of ROS.[15–17]

In vitro studies have shown that in the presence of metals, Aβ is capable of producing about an equimolar concentration of $H_2O_2$ by reducing molecular oxygen.[18] Methionine35 was proposed to act as an electron donor for the reduction of Aβ-bound $Cu^{II}$ to $Cu^{I}$, which in turn reduces the molecular oxygen.[18–21] Interestingly, Tabner et al. showed that this pro-

[a] Dr. L. Guilloreau, S. Combalbert, A. Sournia-Saquet, Prof. Dr. P. Faller
Laboratoire de Chimie de Coordination, CNRS UPR 8241
associated with the University of Toulouse III
205 route de Narbonne, 31077 Toulouse Cedex 4 (France)
Fax: (+33) 561553003
E-mail: faller@lcc-toulouse.fr

[b] Dr. H. Mazarguil
Institut de Pharmacologie et de Biologie Structurale
205 route de Narbonne, 31077 Toulouse Cedex 4 (France)

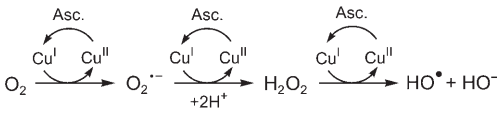
duction of $H_2O_2$ is correlated to the early aggregation process; this indicates that the very early states of aggregation are responsible for the production of $H_2O_2$.[22] This supports the importance of $H_2O_2$ in Aβ neurotoxicity, because according to the amyloid cascade hypothesis, the early stages of aggregation are the toxic forms.[3] On the other hand, when one considers the low concentration of Aβ aggregates (nM) in the early stage, it is not clear how relevant this equimolar $H_2O_2$ production is to the pathology of the disease.

$H_2O_2$ quantities largely beyond the Cu–Aβ and Fe–Aβ concentrations were produced in the presence of a reducing agent.[23] Cu–Aβ and Fe–Aβ complexes were able to catalyze the production of $H_2O_2$ not only with ascorbate, L-dopa and dopamine, but also with cholesterol, progesterone and NADPH. Aβ42 was twice as reactive as Aβ40, which also paralleled its toxicity towards neurons (the truncated Aβ28 was about two times less active than Aβ40) and its capacity for aggregation.[23] Moreover, in the absence of Aβ, copper did not generate detectable amounts of $H_2O_2$.

$Cu^{I}$–Aβ and $Fe^{II}$–Aβ can react further with $H_2O_2$ to produce hydroxyl radicals by Fenton-type reaction.[18] Tabner et al. showed that Aβ42 and the shorter peptide Aβ40 can produce $H_2O_2$ and hydroxyl radicals in the presence of $Fe^{II}$, which acts as a catalyst and reductant.[24] Dikalov et al. showed that $Cu^{II}$–Aβ42 can form hydroxyl radicals upon the addition of $H_2O_2$.[25]

In a related study, Murray et al. showed that Cu–Aβ promotes lipid oxidation in the presence of ascorbate.[26] This reaction probably occurs via the production of $HO^{\bullet}$ (although $HO^{\bullet}$ was not directly measured). Intriguingly, they found that $Cu^{II}$–Aβ42 had an antioxidant effect on the oxidation of lipids by $H_2O_2$ in the absence of ascorbate.

All these studies indicate that Cu–Aβ and Fe–Aβ are able to catalyze the production of ROS in the presence of a reductant via the following reactions (Scheme 1).[27]




**Scheme 1.** Oxygen reduction by copper redox cycling in the presence of ascorbate.

$$M^{ox}\text{–}A\beta + e^{-}\ (\text{reducing agent}) \rightarrow M^{red}\text{–}A\beta \quad (1)$$

$$M^{red}\text{–}A\beta + O_2 \rightarrow M^{ox}\text{–}A\beta + O_2^{\bullet -} \quad (2)$$

$$M^{red}\text{–}A\beta + O_2^{\bullet -} + 2H^{+} \rightarrow M^{ox}\text{–}A\beta + H_2O_2 \quad (3)$$

$$M^{red}\text{–}A\beta + H_2O_2 \rightarrow M^{ox}\text{–}A\beta + HO^{-} + HO^{\bullet} \quad (4)$$

For Cu–Aβ, in contrast to Fe–Aβ,[24] the production of $HO^{\bullet}$ in the presence of a reducing agent (i.e., Reactions 1–4) has not been investigated. Only parts have been analysed so far, that is, the production of $H_2O_2$ in the presence of an reducing agent (Reactions 1–3) or the $HO^{\bullet}$ generation in the presence of $H_2O_2$ (Reaction 4) have been measured only separately. The investigation of the entire reaction sequence (Reactions 1–4) is relevant because 1) it is close to the biological conditions in the brain, which contains between 200 μM and 10 mM ascorbate,[28] 2) $HO^{\bullet}$ is one of the most dangerous radicals in biology[27] and 3) the entire sequence of reactions, 1–4 is not necessarily a simple addition of the two partial reactions, 1–3 and 4, because ascorbate can be a prooxidant as well as an antioxidant reagent. Ascorbate can react with $HO^{\bullet}$ and interfere with Reaction 4. Indeed, it has been reported that Cu–Aβ42 had an antioxidant effect on the oxidation of lipids by $H_2O_2$ (in the absence of ascorbate, e.g., Reactions 3–4), but it promoted oxidation in lipids in the presence of ascorbate and $O_2$ (Reactions 1–4).[26]

Thus, in the present work we investigated the generation of $HO^{\bullet}$ by Cu–Aβ in the presence of physiological concentrations of ascorbate with the aim to compare quantitatively the $HO^{\bullet}$ production with other biologically relevant copper–peptide complexes. This is of importance because if the $HO^{\bullet}$ production by Cu–Aβ is playing a role in its neurotoxicity, it would have to be more efficient than other biologically relevant copper complexes. Moreover, because copper undergoes a redox cycling in Reactions 1–4, and cyclic voltammetry indicated that Cu–Aβ42 has an unusually high redox potential,[13] the redox potential of several copper–peptide complexes were measured to see if they could be correlated to the reactivity.

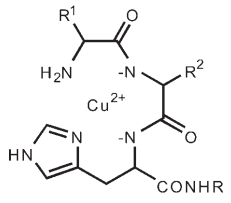
## Results

### Measurement of hydroxyl radical production

With the knowledge that Cu–Aβ complexes could be involved in the oxidative stress that leads to neuronal degeneration, we assessed the capability of $Cu^{II}$–Aβ to generate $HO^{\bullet}$ in the presence of ascorbate and under aerobic conditions. These conditions were chosen because the brain is well supplied with oxygen, and ascorbate is available at high concentrations in brain cells (neuron: 10 mM, glia: 1 mM). Also, extracellular ascorbate is quite abundant; concentrations of 500 μM in cerebrospinal fluid and 200–400 μM in extracellular fluid have been reported.[28]

The present experiments were performed in the presence of 300 μM of freshly prepared ascorbate under aerobic conditions. $HO^{\bullet}$ production was measured by its reaction with coumarin-3-carboxylic acid (CCA), which generated 7-hydroxy-CCA. This product exhibits a fluorescence emission at 452 nm. In order to assess the production of $HO^{\bullet}$ by $Cu^{II}$–Aβ, we compared it with two well-characterized copper–peptide complexes: Cu–GHK and Cu–DAHK (Scheme 2).[29,30]

Figure 1 shows the time course of the $HO^{\bullet}$ generation as measured by the fluorescence of 7-hydroxy-CCA. The highest $HO^{\bullet}$ production was measured for "free" copper in the buffer (▼ in panel A). The fluorescence increased linearly for about 5 min and reached a plateau




**Scheme 2.** Structure of the ATCUN motive found in $Cu^{II}$–DAHK.

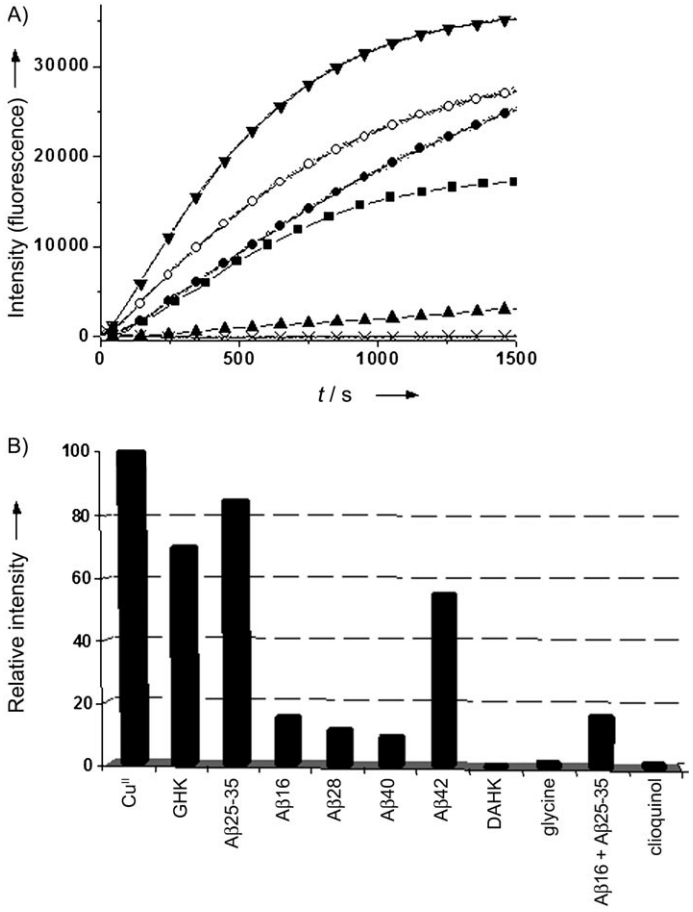



**Figure 1.** HO• generation of different Cu-complexes. HO• formation was followed by fluorescence detection of 7-hydroxy-coumarin-carboxylic acid formed by the reaction of OH• with 3-coumarin-carboxylic-acid (excitation wavelength: 395 nm; emission wavelength: 450 nm). Upper Panel: Time course of the HO• generation by the copper peptides: $Cu^{II}$ alone (▼), $Cu^{II}$–GHK(●), Cu–Aβ25–35 (○),$Cu^{II}$–Aβ42 (■),$Cu^{II}$–Aβ16 (▲), and $Cu^{II}$–DAHK (×). Lower panel: Relative fluorescence intensity of 7-HO-CCA for different copper complexes after 1500 s incubation. Free $Cu^{II}$ was taken as 100%. Conditions: copper compexes (10 μM), ascorbate (300 μM), desferal (1 μM), CCA (10 mM) in PBS buffer pH 7.4.

at around 30 min. This termination of the HO• generation was probably due to the consumption of all of the ascorbate, because when fresh ascorbate was added, the HO• generation started again (not shown).

In order to assess the HO• generation of $Cu^{II}$–Aβ in the presence of ascorbate, two small copper-binding peptides were used for comparison. The peptide Gly-His-Lys (GHK) occurs as a copper complex in plasma, and is involved in wound healing.[31] In the presence of ascorbate, the Cu–GHK complex showed a marked production of HO•, almost as much as the free copper. In contrast, the peptide Asp-Ala-His-Lys (DAHK, which is the N terminus of HSA) binds copper in the well-characterized ATCUN motive (Scheme 2),[30,32] and does not show any significant HO• production. This clearly shows that the copper-containing peptide complexes differ in their HO• production ability, and this difference depends on their coordination chemistry.

The copper complexes of native Aβ peptides Aβ40 and Aβ 42 were also tested. Both peptides generated significant amounts of HO•, but less than free copper or Cu–GHK. Intriguingly, Aβ42 displayed much higher HO• production than Aβ40; this is in accord with the increased toxicity that is described in the literature.[23] Two truncated peptides that contain the metal-binding site (residues 1–16) were also tested. Both Aβ16, which is relatively hydrophilic and well soluble under the present conditions, and the longer Aβ28, which is less hydrophilic but still relatively soluble, showed similar HO• production as Aβ40. The residues 17–28 and 17–40, which are not involved in the binding copper, do not influence the HO• production. Peptide Aβ25–35 has been suggested to be the most toxic part of the amyloid peptide, but it does not contain the specific copper-binding site; its HO• generation was about 70% of the free copper. In the absence of a specific copper-binding site Aβ25–35 behaved more like free copper. The small difference could be due to some non-specific interaction with the peptide. To see if the Met35 has an effect on the HO• generation, a mixture of Aβ25–35 and Cu–Aβ16 in a 1:1 ratio was measured. The HO• generation of Cu–Aβ16 was not significant in the present or absence of Aβ25–35; this indicates that under these conditions Met35 seems not have a high impact and the result is in line with the about equal HO• production that has been observed for Aβ40 and Aβ16.

In order to confirm these HO• generation results, another method has been applied. EPR has been often used to measure radicals by the spin trap method. Here we used α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN), which is widely used to measure HO• (for details see the Experimental Section). These experiments are shown in Figure 2. Panel A exhibits the spectra that were obtained after 30 min of incubation of "free" copper, Cu–Gly2, Cu–GHK, Cu–DAHK, Cu–Aβ40 and Cu–Aβ42 in the presence of 300 μM ascorbate (same conditions as in the fluorescence measurements). The spectrum of free copper was the most intense and showed the typical six-line spectrum with two coupling constants of 16 Gauss and 3 Gauss.[33] The intensity of the EPR signal of ethoxy-POBN in the presence of the different copper-containing peptide complexes after 30 min are shown in panel B. They are very similar to the measurements that were obtained by trapping HO• with CCA, followed by fluorescence (see Figure 1B). This corroborates that $Cu^{II}$–Aβ16, 28 and 40 produce significant amounts of HO•, but less than free copper and $Cu^{II}$–GHK. Moreover, Aβ42 produced about 5 times more HO• than Aβ40.

In order to verify that $H_2O_2$ is an intermediate in the reaction that produces HO• by $Cu^{II}$ in the presence of ascorbate, catalase was added at different time points to the sample with copper and ascorbate. Immedialtly after the addition of catalase, the production of HO• (as measured by CCA) stopped (Figure 3). Note that the catalase concentration was about 50 nM, which is 200 times less than the $Cu^{II}$–Aβ concentration; this indicates that the effect was due to its catalysis and not due to a direct interaction with copper.

This establishes that $H_2O_2$ is the precursor of the HO•. In the literature, often the $H_2O_2$ and not HO• production has been measured in the presence of ascorbate.[18,22] It has been found

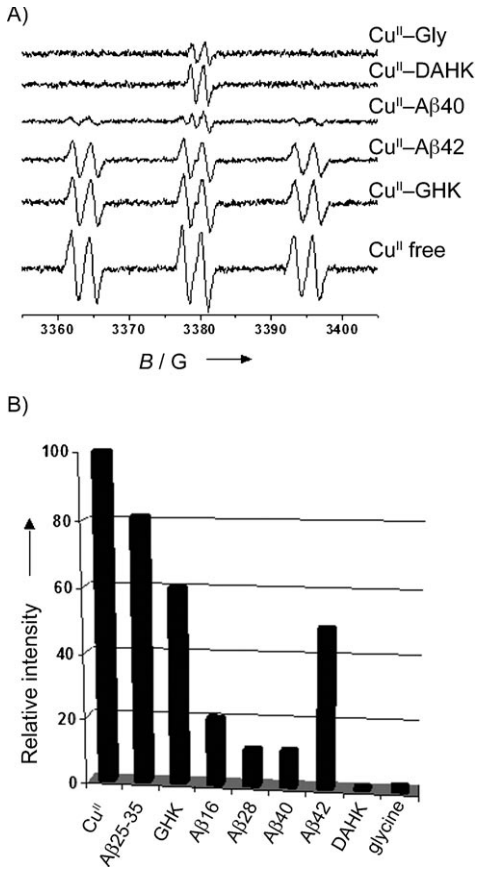



**Figure 2.** HO$^{\bullet}$ generation of different copper complexes. HO$^{\bullet}$ formation was followed by EPR detection via the spin trap POBN. Upper panel: EPR spectra after 15 min. HO$^{\bullet}$ generation by the copper peptides: Cu$^{II}$ alone (▼), Cu$^{II}$–GHK (●), Cu$^{II}$–Aβ25–35 (○), Cu$^{II}$–Aβ16 (▲), and Cu$^{II}$–DAHK (×). Lower panel: Relative EPR intensity HO$^{\bullet}$ generation of the different copper complexes as measured by the spin POBN. Free Cu$^{II}$ was taken as 100%. Conditions: copper compexes (10 μM), ascorbate (300 μM), desferal (1 μM), POBN (10 mM) ethanol (5%) in PBS buffer pH 7.4.

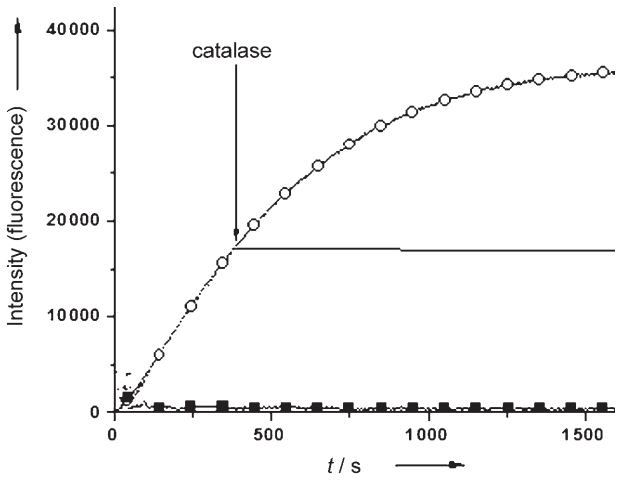



**Figure 3.** Effect of catalase addition on the HO$^{\bullet}$ production of Cu$^{II}$ measured by fluorescence detection of 7-hydroxy-coumarin-carboxylic acid, which was formed by the reaction of OH$^{\bullet}$ with 3-coumarin-carboxylic-acid. Without catalase (○); catalase added at the beginning (■); catalase added at the time indicated by the flash (——). Conditions as in Figure 1. Catalase concentration 50 nM.

that Cu$^{II}$–Aβ produce more $H_2O_2$ than copper, which has been related to the higher neurotoxicity of Cu$^{II}$–Aβ compared to "free" Cu$^{II}$. However, the comparison with copper was actually measured in a complex with glycine (Cu$^{II}$–$Gly_2$) and not with from the free copper; furthermore, $H_2O_2$ production does not necessarily mean that more HO$^{\bullet}$ production has also occurred. Thus, we studied the HO$^{\bullet}$ production of the Cu$^{II}$–$Gly_2$ complex as well (Figures 1 and 2). No significant HO$^{\bullet}$ production by Cu$^{II}$–$Gly_2$ was observed; this is similar to the Cu–DAHK result. Thus Cu$^{II}$–$Gly_2$ generated less HO$^{\bullet}$ than all Cu$^{II}$–Aβ complexes. Therefore copper that is bound to Aβ produces more $H_2O_2$ and HO$^{\bullet}$ than Cu$^{II}$–$Gly_2$, but less than "free" copper or Cu–GHK.

Clioquinol (CQ), a copper chelator has been shown to reduce plaque burden in transgenic mice (AD model), and is able to retrieve copper from Aβ. However, in order to be non-offensive, the copper that is bound to CQ should not be able to produce radicals, because otherwise it would be as dangerous as Cu–Aβ. Thus, we measured the affect of the addition of CQ to free copper at the beginning or during the experiment. When CQ was added, no increase in fluorescence was observed; this indicates that CQ was able to bind copper and that Cu–CQ is not able to catalyze the production of HO$^{\bullet}$ under the present conditions.

### Time dependence of HO$^{\bullet}$ production and aggregation of Aβ42:

The results that show that Aβ42 generates around five times more HO$^{\bullet}$ than Aβ40, seem to be particular interesting. The additional two amino acids at the C terminus are not likely to interfere directly with the metal binding, which could explain the difference in HO$^{\bullet}$ generation. So it seems more likely that the difference in the aggregation state could be responsible for the reactivity. In order to test this, fresh Cu–Aβ40 and Cu–Aβ42 at concentrations used in the HO$^{\bullet}$ assay (10 μM) were analyzed by gel filtration (not shown).[32] The results suggest that under these conditions Cu$^{II}$–Aβ40 is predominantly monomeric, but Aβ42 is a mixture of three forms: 1) monomers, 2) intermediate aggregates with an apparent molecular mass of $>$70 kDa and 3) larger aggregates that are too big to enter in the column resin. This excludes the possibility that the monomer is responsible for the higher HO$^{\bullet}$ generation.

To test if the increased HO$^{\bullet}$ generation correlates with the intermediate aggregates or the larger aggregates, the time dependence of the HO$^{\bullet}$ generation was measured with regard to the aggregated state (Figure 4). Already after 15 min incubation of Cu$^{II}$–Aβ42 at 37 °C, the HO$^{\bullet}$ generation was reduced. After 3 h, the HO$^{\bullet}$ generation decreased by a factor of 10. This is about the level of fresh Cu$^{II}$–Aβ40. Further incubation for 1 day did not significantly change the level of HO$^{\bullet}$ generation. Analysis of the Cu$^{II}$–Aβ42 that had been incubated for 3 h indicated that all of the Cu$^{II}$–Aβ42 was in the form of larger aggregates. No significant amount of monomer or intermediate aggregates could be detected (not shown). Thus, the only species paralleling the elevated HO$^{\bullet}$ generation, that is, is present in a fresh sample of Aβ42 (but not Aβ40), and is absent after 3 h incubation are the intermediate aggregates. This indicates that

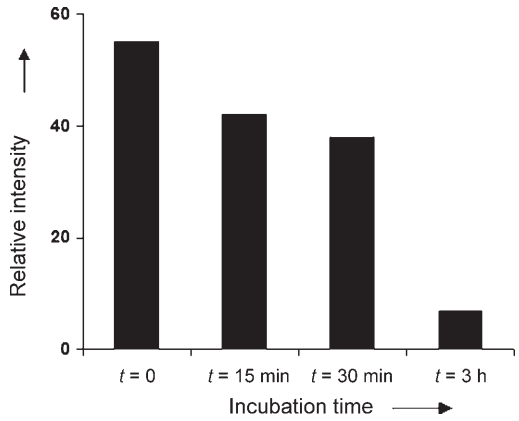



**Figure 4.** HO• generation of Cu–Aβ42 after different incubation times. HO• formation was followed by fluorescence detection of 7-hydroxy-coumarin-carboxylic as in Figure 1; Conditions: Cu–Aβ42 (10 μM), ascorbate (300 μM), desferal (1 μM), CCA (10 mM) in PBS buffer pH 7.4.

these intermediate aggregates of Aβ42 are responsible for the difference in the HO• generation compared to the monomeric Aβ40 (or 28/16).

Interestingly, when Aβ42 was incubated for 1 day without copper, and then one equivalent of copper was added, the HO• generation was similar to fresh Cu–Aβ42, and not as one would expect to find from Cu–Aβ42 after 1 day of incubation. This is somehow surprising and merits further investigation. Possible explanations for that could be that 1) Aβ42, which is known to aggregate more slowly in the absence of copper, forms an oligomeric state after one day of incubation, and the addition of copper leads to the same intermediate structure as the incubation of Cu–Aβ42 for a much shorter period; 2) the copper sites of aggregated Aβ42 might not be accessible, and some free or loosely bound copper is responsible for the increased HO• generation; Or 3) the Aβ42-only aggregates have a different structure and bind the copper differently, that is, in a coordination form that is able to generate high amounts of HO•.

### Cyclic voltammetry

Because the production of HO• from ascorbate and molecular oxygen that is catalyzed by the copper complexes requires oxidation and reduction of the copper centers, the redox potentials of the different copper–peptide complexes were measured by cyclic voltammetry. The high concentration needed (~1 mM) did not allow the analysis of the Aβ40 and 42. Nevertheless, at least for the soluble form of Aβ40, Aβ16 and Aβ28 have been shown to be good models.

Figure 5 shows the cyclic voltammetry measurements of $Cu^{II}$–GHK, $Cu^{II}$–DAHK and $Cu^{II}$–Aβ16. $Cu^{II}$–Aβ28 was very similar to Cu–Aβ16 (not shown). The deduced values for the oxidation and reduction potentials for $Cu^{I}/Cu^{II}$ are summarized in Table 1.

For Cu–GHK, $Cu^{I}/Cu^{II}$ and $Cu^{I}/Cu^{0}$ can be observed. The reduction occurred at 0.14 V and the oxidation at 0.20 V (vs. NHE). The peak-to-peak difference of 60 mV is in agreement with a quasi-reversible process. The large peak at around

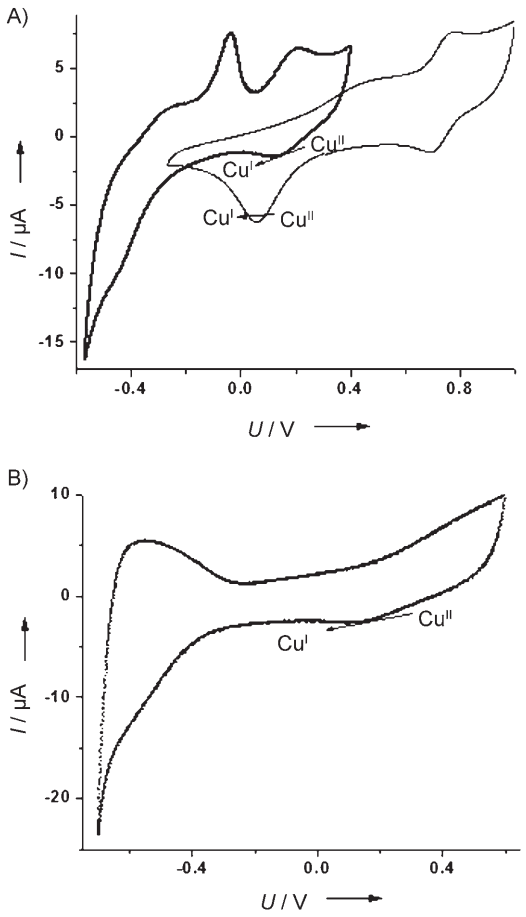



**Figure 5.** Cyclic voltammetry of Cu–GHK (thin line, upper panel) and Cu–DAHK (thick line, upper panel) and Cu–Aβ16 (lower panel). Reference: saturated calomel electrode. Conditions: 1 mM copper peptides, 50 mM Tris/HCl pH 7.4, 100 mM NaCl.

**Table 1.** Reduction and oxidation potential of the peptide–copper complexes deduced by electrochemistry.

| Species | Potential vs. NHE | |
|---|---|---|
| | Reduction | Oxidation |
| Cu–DAHK | 0.30 V | 0.71 V |
| Cu–GHK | 0.39 V | 0.45 V |
| Cu–Aβ16 | 0.34 V | 0.65 V |
| Cu–Aβ28 | 0.33 V | 0.63 V |

−30 mV is probably due to the deposition of $Cu^{0}$ on the electrode, as indicated by its absence at the first cycle, and its increase after each subsequent cycle.

For $Cu^{II}$–DAHK, the reduction potential is 0.05 V and the difference from peak to peak is 0.42 V. This large difference indicates a major rearrangement upon reduction of the complex. This is expected, because the relatively rigid square-planar coordination site of the ACTUN motive is not favorable to accommodate $Cu^{I}$. Thus, $Cu^{I}$ will not bind to the same site as $Cu^{II}$.

$Cu^{II}$–Aβ16 and $Cu^{II}$–Aβ28 were very similar; they showed a reduction potential of 0.09 and a peak-to-peak difference of

0.30 V. These features are in between the features of $Cu^{II}$–GHK and $Cu^{II}$–DAHK, but are closer to the latter.

## Discussion

Here, the capability of generating $HO^{\bullet}$ radicals by different $Cu^{II}$–Aβ and model peptides in the presence of ascorbate has been investigated. Generally the $Cu^{II}$–Aβ showed moderate $HO^{\bullet}$ generation compared to the $Cu^{II}$–GHK, but substantially more than $Cu^{II}$–DAHK. This dependence of $HO^{\bullet}$ production of the different $Cu^{II}$–peptide complexes can be correlated to the electrochemistry data obtained (Table 1). Cu–GHK has the highest reduction potential (0.39 V) and the lowest oxidation potential (+0.44 V). The two potentials are only about 60 mV apart. Hence, in terms of thermodynamics, Cu–GHK undergoes redox-cycling the most easily. In contrast, Cu–DAHK has a lower reduction potential (+0.30 V) and a higher oxidation potential. (+0.70 V), that is, there is difference of about 0.4 V. The redox cycling is thus more difficult for Cu–DAHK. Because redox cycling of copper is the underlying mechanism to produce the radicals, it seems reasonable that Cu–GHK produced higher quantities of $HO^{\bullet}$ than Cu–DAHK. However these are thermodynamic considerations and do not include kinetic aspects. Moreover, in contrast to the $HO^{\bullet}$ generation measurements, the redox potential had to be measured in the absence of oxygen and ascorbate. Thus, it could be possible that oxygen and/or ascorbate could coordinate to copper and influence the redox potential.

Because the midpoint potential of ascorbate (+0.28 V) is more negative than the reduction potential of all of the $Cu^{II}$ peptides measured, ascorbate will readily reduce $Cu^{II}$ peptides, and the redox potential will be even lower when most of the ascorbate is in the reduced form. The next step towards the $HO^{\bullet}$ production is the reduction of molecular oxygen to the superoxide anion, which has a relatively low midpoint potential of −0.16 V. Thermodynamically, this is the most difficult step. However, under typical concentrations in a biological environment, the redox potential will be increased due to the much higher concentration of $O_2$ compared to $O_2^{\bullet-}$.

The reason for this reversal of the order in reduction and oxidation potential between $Cu^{II}$–GHK, $Cu^{II}$–Aβ, $Cu^{II}$–DAHK is due to the differences between the two potentials. In the case of $Cu^{II}$–GHK, the difference is 0.06 V which is in accord with a quasireversible redox event. This is in agreement with a small conformational change of the Cu–GHK complex upon redox reaction, which is conceivable for distorted square-pyramidal coordination. For $Cu^{II}$–Aβ and $Cu^{II}$–DAHK the difference is much larger, at 0.30 V and 0.42 V respectively. The likely reason for that is that $Cu^{II}$–DAHK is bound in a square-planar geometry by four nitrogens, in a relatively rigid complex.[30,32] Although there is no structural study on $Cu^{I}$–DAHK, this same binding site is unlikely to accommodate $Cu^{I}$, because $Cu^{I}$ has a strong preference for tetrahedral geometries. Thus, it is likely that upon reduction of $Cu^{II}$–DAHK to $Cu^{I}$–DAHK, the copper migrates to a tetrahedral binding site. Once in that site, the reoxidation is more difficult because of the geometry. As a consequence, copper must migrate back to its initial site before oxidation can occur. This fits with the electrochemical data and with the large observed difference between oxidation and reduction potential.

$Cu^{II}$–Aβ also has a large difference between the reduction and oxidation potential, although not as high as $Cu^{II}$–DAHK. This indicates that $Cu^{II}$ and $Cu^{I}$ do not have the same binding site in Aβ. $Cu^{II}$–Aβ is in a square-planar complex. The ligands are likely the three imidazol of the histidines and the carboxylate and/or amine of Asp1.[9,10,12] This coordination site is less rigid and more heterogeneous than $Cu^{II}$–DAHK, which could explain the smaller difference between the oxidation and reduction potential of Cu–Aβ. No information is available on the structure of $Cu^{I}$–Aβ. The present data indicate that this site is not exactly the same as for $Cu^{II}$.

Cyclic voltammetry measurements have been reported for Cu–Aβ42.[13] A redox potential between +0.50–55 V, which represents a reduction at ~0.42 V and oxidation at ~0.63 V (against Ag/AgCl) was determined. This is not in agreement with the redox potential for Cu–Aβ16/28 of around +0.25 V (against Ag/AgCl). This could be because 1) the structure Cu–Aβ42 is aggregated and the coordination chemistry of copper is changed, which is not the case for Aβ16/28, 2) different conditions were used,[13] or 3) as the authors pointed out, they had indication that the potential was measured for surface-adsorbed complexes. However, this very high redox potential, and in particular the high oxidation potential, is not in agreement with the increased $HO^{\bullet}$ generation by Aβ42 that we observed. One would expect to find behavior more like that observed for Cu–DAHK. Further studies are warranted to sort out this issue.

In general, all Cu–Aβ complexes generated significant $HO^{\bullet}$ levels in the presence of physiologically relevant ascorbate concentrations. If the $HO^{\bullet}$ generation by Cu–Aβ is of relevance to its neurotoxicity, one would expect that Cu–Aβ produces more radicals than other copper complexes. Cu–Aβ produces more $HO^{\bullet}$ than Cu–DAHK and Cu–Gly, but was below "free" copper and Cu–GHK. "Free" intracellular copper concentrations are probably too low to be relevant,[36] (little is known about extracellular copper concentration in the brain). Cu–GHK concentrations might be relevant, but $OH^{\bullet}$ production not, since GHK occurs in the plasma, which is low in reductant. Thus, principally, Cu–GHK should be more dangerous than Cu–Aβ to cells. In this context, it is important also to take into account the localization of Aβ. Cu–Aβ with its hydrophobic C-terminal part has been shown to interact with the membrane.[26,36,38] Hence, although the $HO^{\bullet}$ generation of Aβ is below Cu–GHK or "free" copper, its localization in the membrane can explain the particular toxicity of Aβ, because $HO^{\bullet}$ generation inside/or close to the membrane can cause lipid oxidation and would be particularly dangerous. As the studies by Murray et al. have shown, lipid oxidation could be higher for Cu–Aβ than for free copper.[26] Moreover, catalase was not able to protect lipids from oxidation by Cu–Aβ; this illustrates the danger of radical production inside membranes.

There are reports in the literature that claim that Cu–Aβ generates more $H_2O_2$ than "free" copper.[23] This seems to contradict our results, although we measured $HO^{\bullet}$. However, in

this reported case, "free" copper referred to a Cu–$Gly_2$ complex because copper was added in the presence of four equivalents of glycine. Our results showed that Cu–$Gly_2$ did not produce high levels of HO•, and it produced clearly less HO• than Cu–Aβ. In conclusion, the different results do not contradict each other, and suggest that $H_2O_2$/HO• production is highest by "free" copper, than Cu–Aβ, and than Cu–$Gly_2$.

Whereas Aβ16, Aβ28 and Aβ40 did not show any significant difference in the generation of HO•, Aβ42 produced up to five-times more HO• than Aβ40. This HO• generation of Cu–Aβ42 over Cu–Aβ40 is more drastic than the one reported for $H_2O_2$ generation, where only an about twofold increase was reported.[23] The increased HO• generation of Cu–Aβ42 could be correlated with the existence of an aggregation intermediate that is small enough to enter the gel-filtration column, but is larger than 70 kDa. A similar peak was obtained from Aβ40 in the absence of copper, and has been assigned to protofibrils.[39]

This is of interest because neuronal dysfunction has been proposed to be mediated predominantly by aggregation intermediates rather than by fully formed fibrils of Aβ; further, Aβ42 is known to be more neurotoxic than Aβ40 (or truncated peptides; for a review see ref. [3]). Several intermediates have been described that range in size from dimers up to large aggregates (diameter of ~10 nm).[40] The properties of the present oligomeric intermediate have escaped further characterization so far because it was formed only transiently and at relatively low concentration.

## Conclusions

In this work, a correlation was found between HO• generation, redox potentials and coordination chemistry of three biologically relevant copper-binding peptides: Aβ, GHK and DAHK. Cu–DAHK has the lowest reduction potential but the highest oxidation potential, with a difference of ~0.4 V. This means that it does not easily redox cycle and hence, does not produce much HO•. This can be related to the rigid square-planar geometry of $Cu^{II}$–DAHK, which is not a favorable site for $Cu^{I}$ coordination. In contrast, GHK binds $Cu^{II}$ less rigidly, and in a rather distorted square-planar geometry. It undergoes relatively facile redox cycling, with a difference of 0.06 V between reduction and oxidation potential; this could explain the high production of HO•. Cu–Aβ exhibited an intermediate behavior in term of redox potentials and HO• generation.

Moreover, the copper complex of the highly toxic Aβ1–42 showed a production of HO• five-times higher than that of the less toxic Aβ1–40 form. Time-dependence and aggregation studies suggest that an aggregation intermediate is responsible for this increased HO• production. This is in accordance with the current view that aggregation intermediates (oligomers), rather than mature aggregates are the most toxic species.

## Experimental Section

**Peptides and sample preparation**: Aβ42 (sequence: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala) and Aβ16 (amino acids 1–16), Aβ28 (amino acids 1–28) and Aβ40 (amino acids 1–40) were either synthesized by standard Fmoc chemistry and purified by using HPLC with a C8 column, or they were purchased from EZBiolab (Westfield, IN, USA), Geneshere Biotechnologies (Paris, France) or Activotec (Cambridge, UK). ESI-MS of Aβ16, Aβ28, Aβ40, and Aβ42 showed masses of the monocharged species, which were within ±0.3 units of their calculated masses. The stock solutions of peptides were prepared by dissolving the peptides in water at high pH to facilitate the dissolution, and to be sure that the peptide was in its monomeric form. Then pH was adjusted at 7.4, and the solution was stored at −20 °C. The peptide concentrations of Aβ16, Aβ28, Aβ40 and Aβ42 were determined by absorption spectroscopy by using the extinction coefficient of the Tyr residue at 276 nm, $\varepsilon = 1410\ cm\ M^{-1}$. (Note that the peptides are predominantly in a random coil conformation (see below) and hence the Tyr probably was surrounded by water, as for the free Tyr).[41]

The peptides GHK (sequence: Gly-His-Lys) and DAHK (sequence: Asp-Ala-His-Lys) were purchased from Bachem. The stock solutions (0.1 M) were prepared by dissolving the appropriate mass of peptides in water. They were stored at −20 °C.

A stock solution (1 mM) of desferrioxamine (Sigma) was prepared by dissolving an appropriate mass of desferal powder in phosphate (20 mM), NaCl (100 mM) buffer (PBS) at pH 7.4 at room temperature. This stock solution was stored at −20 °C.

A stock solution (10 mM) of ascorbic acid was prepared in PBS at pH 7.4 at room temperature just before beginning the experiment and was used immediately. Because ascorbate degrades very quickly, a new solution was prepared for each experiment. Catalase (0.1 mM) from bovine liver (Sigma) was prepared in PBS at pH 7.4 at room temperature. Further dilution was done in the same buffer. The final concentration in the sample was about 50 nM.

**Measurements of HO•**: Measurements of HO• production and detection by the copper peptides were performed in PBS at pH 7.4. The concentration of peptide and copper was 10 μM. The reaction was started by the addition of ascorbate (300 μM). In all these experiments desferrioxamine (at a final concentration of 1 μM) was added. This was necessary because ascorbate in the buffer always generated HO• without the addition of metals. In the absence of metal, ascorbate does not autooxidize.[42] We attribute this to trace amounts of iron (and copper) in the buffer. Residual HO• production by the buffer persisted even though the buffer was passed over Chelex (Biorad, Marnes La Coquette, France) to remove trace metals. Thus Desferrioxamine, which is known to suppress HO• generation by iron,[43] was added to chelate trace metals. Desferrioxamine (1 μM) was sufficient to abolish HO• generation by the trace metals in the buffer. Desferrioxamine might also chelate a part of the copper added (10 μM) if the traces of metals in buffer are overestimated, but this can not exceed 10%. Although this adds uncertainty to the estimation of the actual copper bound to the peptides, it does not change the general conclusions.

**Absorption spectroscopy (UV/Vis)**: UV–visible absorption spectra were recorded at room temperature on a Spectrometer Cary 2300 in a 1 cm path length quartz cuvette. UV absorption was used to determine the concentration of peptides Aβ16, Aβ28, Aβ40, Aβ42.

**Fluorescence spectroscopy**: Fluorescence spectra were collected by using a Cirad fluorescence spectrophotometer. The samples were excited at 395 nm and the time course of emission at 452 nm was measured over a period of about 45 min. Samples were

placed in a four-sided quartz fluorescence cuvette, and data was recorded at room temperature.

Coumarin-3-carboxylic acid (3-CCA) (Sigma) was used to detect hydroxyl radicals (HO•) in PBS buffer.[41] HO• reacts with 3-CCA to form 7-OH-CCA, which is fluorescent at 452 nm upon excitation at 395 nm. The intensity of the fluorescence signal is linearly proportional to the number of the formed 7-hydroxy-coumarin-3-carboxylic acid molecules, which in turn is proportional to the HO• radicals generated.

A stock solution of 3-CCA (5 mM) was prepared in PBS at pH 9 at room temperature. When the 3-CCA was fully dissolved, the pH was adjusted back to 7.4. This stock solution was stable at −20 °C for at least 6 months. No traces of precipitation or changes in the absorbance spectra were observed. Working solution (100 μM) was made by diluting the stock solution with an appropriate buffer at pH 7.4.

**EPR spectroscopy**: X-band Electron Paramagnetic Resonance (EPR) data were recorded by using an Elexsys ESP 500 that operated at a microwave frequency of ~9.5 GHz. All spectra were recorded by using a microwave power of 20 mW across a sweep width of 1500 Gauss (centered at 3100 Gauss) with a modulation amplitude of 10 Gauss. Experiments were carried out at ambient temperature and data were recorded every five minutes about 40 min.

POBN (α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone) (Sigma) was used as a spin trap for the detection of hydroxyl radicals (HO•) in aqueous solution. HO• radicals are short lived and thus react inefficiently with POBN. In order to increase the sensitivity, ethanol (5%) was added.[33] Ethanol reacts with HO• to form an ethoxy radical, which in turn reacts with the spin trap. The latter product is then detected by EPR. POBN was a commercial product from Sigma. A stock solution of POBN (100 mM) was prepared in PBS at pH 7.4 at room temperature. This stock solution was stable at −20 °C for several weeks. A working solution was made by diluting the stock solution with PBS at pH 7.4 to a final concentration of 10 mM.

**Electrochemical measurements**: Voltammetric measurements were carried out with a potentiostat Autolab PGSTAT100 controlled by GPES 4.09 software. Experiments were performed at room temperature in an airtight three-electrode cell connected to a vacuum/argon line. The reference electrode consisted of a saturated calomel electrode (SCE) that was separated from the solution by a bridge compartment. The counter electrode was a platinum wire of ca. 1 $cm^2$ apparent surface. The working electrode was a Pt electrode (1 mm diameter). Tris buffer (50 mM) NaCl (100 mM) pH 7.4 was the supporting electrolyte.

Cyclic voltammetry was performed in the potential range of −0.8 to 0.8 V vs. SCE at 0.1 $V s^{-1}$. Before each measurement, the solutions were degassed by bubbling argon through them, and the working electrode was polished with a polishing machine (Presi P230).

Cyclic voltammetry was measured with the peptides (1 mM) GHK, DAHK, Aβ16, and Aβ28 with none, one or two equivalents of copper at a concentration of in Tris/HCl (50 mM), NaCl (100 mM) pH 7.4. Voltammograms of the buffer only, and of copper (1 mM) in buffer were also acquired.

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