Amyloid Peptide

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Free Superoxide is an Intermediate in the Production of H_2O_2 by Copper(I)-Aβ Peptide and O_2

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Abstract: *Oxidative stress is considered as an important factor and an early event in the etiology of Alzheimer's disease (AD). Cu bound to the peptide amyloid-β (Aβ) is found in AD brains, and Cu-Aβ could contribute to this oxidative stress, as it is able to produce in vitro H_2O_2 and $HO^{\bullet}$ in the presence of oxygen and biological reducing agents such as ascorbate. The mechanism of Cu-Aβ-catalyzed H_2O_2 production is however not known, although it was proposed that H_2O_2 is directly formed from O_2 via a 2-electron process. Here, we implement an electrochemical setup and use the specificity of superoxide dismutase-1 (SOD1) to show, for the first time, that H_2O_2 production by Cu-Aβ in the presence of ascorbate occurs mainly via a free $O_2^{\bullet-}$ intermediate. This finding radically changes the view on the catalytic mechanism of H_2O_2 production by Cu-Aβ, and opens the possibility that Cu-Aβ-catalyzed $O_2^{\bullet-}$ contributes to oxidative stress in AD, and hence may be of interest.*

There is a large body of evidence that brains of Alzheimer disease (AD) patients are exposed to oxidative stress during the course of the disease. This is supported through high levels of oxidized proteins, lipid peroxidation, and nuclear and mitochondrial DNA oxidation.[1,2] Increased reactive oxygen species (ROS) concentrations were reported, which could be due to their overproduction or a failure in the *anti* ROS defense systems.[3,4] Although it is not clear to what extent ROS play a causal role, oxidative stress is considered as an important factor and an early event in AD.[5,6]

In amyloid plaques, a hallmark of AD, high concentrations of copper ions are found bound to the peptide amyloid-β (Aβ), the major ingredient of the plaques.[7] The major Aβ peptides are 40 (Aβ40) or 42 (Aβ42) amino acids long. Copper ions can be a very efficient catalyst for the production of ROS[8] and in vitro and in vivo experiments suggested that a misregulation of copper is connected to AD.[7,9,10] In vitro, Cu bound to Aβ (Cu-Aβ) or to the truncated N-terminal domain (Aβ16) was able to catalyze the production of H_2O_2 in the presence of oxygen and biological reducing agents like ascorbate. Initial reports that Aβ without metal ions could produce ROS could not be confirmed, and hence for a substantial ROS production, Cu and a reduction agent is needed[11–15] Under the same conditions, generation of $HO^{\bullet}$ catalyzed by Cu-Aβ was also reported,[16–18] and found to be deleterious for the peptide itself.[19,20] Cu-Aβ is less active in ROS production than Cu in a buffer, but more active than several biological relevant Cu-peptides/proteins.[17] Hence the biological relevance will depend on the so far ill-defined presence of "free" Cu and Cu-peptides/proteins near the localization of Aβ, like in certain synaptic clefts. Recently, Cu-binding was also studied to N-terminal truncated and/or modified Aβ peptides, forms that might be highly relevant in AD.[21,22]

In contrast to the well supported production of H_2O_2 and $HO^{\bullet}$, detection of $O_2^{\bullet-}$ by nitro blue tetrazolium (NBT) or hydroethidine failed. It was generally proposed that production of H_2O_2 from O_2 occurs directly via a 2-electron process without any release of $O_2^{\bullet-}$ (Scheme 1, mechanism 2).[9,14,18,23,24]

This seems coherent, because considering the redox potentials at neutral pH the electron reduction from O_2 to H_2O_2 is a down-hill reaction (exergonic), in contrast to the endergonic one-electron reduction of O_2 to $O_2^{\bullet-}$. Computational chemistry supported that Cu^{I}-Aβ can activate O_2 to form $O_2^{\bullet-}$,[25,26] and that no $O_2^{\bullet-}$ is released during the H_2O_2

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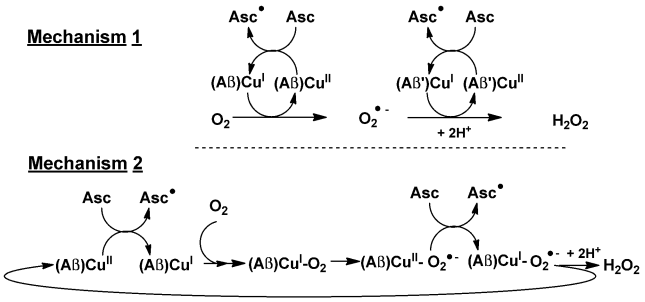


Scheme 1. Two potential mechanisms of H_2O_2 production from O_2 by Cu-Aβ. In mechanism 1 the intermediate $O_2^{\bullet-}$ is released from Cu-Aβ after formation, and then reduced to H_2O_2 by another Cu^{I}-Aβ. In mechanism 2 O_2 is reduced to H_2O_2 when bound to Cu-Aβ without release of the potential intermediate $O_2^{\bullet-}$.

production ($O_2^{\bullet-}$ was proposed as an intermediate bound to Cu-Aβ).[26] Here, we use an electrochemical setup and the specificity of superoxide dismutase-1 (SOD1) to show that the H_2O_2 production of Cu-Aβ proceeds mainly via free $O_2^{\bullet-}$ as intermediate. The impact of these findings on ROS production therapeutic approaches in AD is discussed.

Cu-Aβ is able to catalyze the reduction of O_2 to H_2O_2 by reducing agents such as ascorbate. In order to test whether $O_2^{\bullet-}$ is generated as a released intermediate (Scheme 1), we used the classical test with cytochrome c (cyt c) to detect $O_2^{\bullet-}$.[27] Reaction of $O_2^{\bullet-}$ studied with cyt c leads to the appearance of an absorption band at 550 nm due to reduction of the heme, that is, from ferricytochrome to ferrocytochrome. However, ascorbate could reduce cyt c directly and hence impede the detection of $O_2^{\bullet-}$. Indeed, cyt c was very rapidly reduced in the presence of ascorbate under our conditions (Figure S1 in the Supporting Information). This makes the detection of $O_2^{\bullet-}$ produced by our catalytic system impossible (Cu-Aβ + ascorbate + O_2). To avoid ascorbate that interferes with $O_2^{\bullet-}$ detection, Cu^{II}-Aβ was reduced electrochemically at a constant potential.

Figure 1 top shows the results of the absorption changes of cyt c with Cu-Aβ. Indeed, Cu-Aβ40 and Cu-Aβ16 exhibit an increase in absorption at 550 nm with time upon applying a redox potential of 0 V vs Ag/AgCl, in line with the production of $O_2^{\bullet-}$ from O_2. As there could be the risk that oxygen is directly reduced at the electrode, the measurement was repeated in the absence of Cu-Aβ. Under these conditions, the increase at 550 nm was much lower, showing only little direct electrochemical reduction of oxygen into $O_2^{\bullet-}$. Also in the presence of Cu only, very little $O_2^{\bullet-}$ production was detected. Thus the much higher increase at 550 nm is in line with $O_2^{\bullet-}$ production mediated by Cu-Aβ reduction. To further confirm $O_2^{\bullet-}$ production, the enzyme SOD1 was added to the sample with Cu-Aβ. As SOD1 consumes $O_2^{\bullet-}$ one expects a decrease in the detection of $O_2^{\bullet-}$ by cyt c. Indeed, addition of SOD1 yields a marked decrease of the band at 550 nm (Figure 1 top right). Note that attempts to detect similar $O_2^{\bullet-}$ production by EPR with different spin traps (DMPO, BMPO, and DEPMPO) from different suppliers were hampered by the large and variable background signal of the spin-trap recorded under reducing potential with the electrochemical setup.

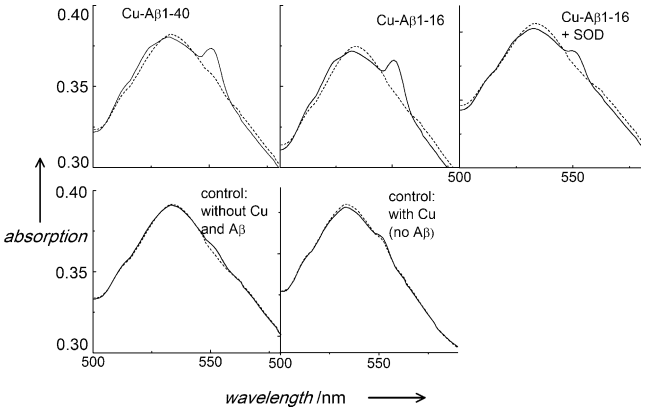


Figure 1. Absorption spectrum of cytochrome c. The narrow band around 550 nm is assigned to the reduction of cyt c by $O_2^{\bullet-}$. Dashed line: time 0, solid line: after 2 min of electrolysis under different conditions: In the presence of Cu-Aβ40 (top left), Cu-Aβ16 (top middle) and Cu-Aβ16 + SOD1 (top right), in the absence of Cu-Aβ (bottom left), and with Cu only (bottom right). Conditions: 100 μM Aβ, 80 μM Cu^{2+}, 100 μM cyt c, 100 mM phosphate pH 7.4; electrolysis: 0 V vs Ag/AgCl, SOD1: 600 u mL^{-1}.

In order to test if $O_2^{\bullet-}$ is produced and released during the well-established Cu-Aβ-catalyzed reduction of O_2 to H_2O_2 by ascorbate, we measured the production of H_2O_2 by using the amplex red assay (Figure 2). This assay is based on the production of the chromophore resorufin (absorbance at 571 nm) by conversion from amplex red with H_2O_2 catalyzed by horseradish peroxidase. Upon addition of ascorbate to Cu-Aβ, resorufin is formed (Figure 2 top, ●) indicative of the formation of H_2O_2.

In order to determine if the production of H_2O_2 proceeds via the released intermediate superoxide (Scheme 1, mechanism 1) or not (mechanism 2), the SOD1 was added. Generally, if the production of H_2O_2 originates directly from O_2 (Scheme 1, mechanism 2), one would not expect any effect of SOD1 as no $O_2^{\bullet-}$ is released. If $O_2^{\bullet-}$ is a released intermediate, the SOD1 would be able to change the kinetics of the reaction.

This is because the rate limiting step is the reduction of O_2 to $O_2^{\bullet-}$ (which is thermodynamically an up-hill process). In the absence of SOD1, for each produced H_2O_2 one slow (O_2 to $O_2^{\bullet-}$) and one faster reduction ($O_2^{\bullet-}$ to H_2O_2) have to occur (Scheme 2). Under conditions where SOD1 reacts with all $O_2^{\bullet-}$ produced (saturating conditions), half of $O_2^{\bullet-}$ reforms O_2 which has to pass again the rate limiting step. In summary, under conditions where all the $O_2^{\bullet-}$ produced reacts with SOD1, the rate limiting step has to be passed twice per average production of H_2O_2 by ascorbate oxidation. Thus twice as slow reaction rate is expected under SOD1 saturation conditions (Scheme 2).

Indeed, addition of SOD1 resulted in a concentration-dependent decrease in the kinetics of H_2O_2 production (measured by resorufin, Figure 2 top). SOD1 addition diminished the rate of H_2O_2 production in a concentration-dependent manner (Figure 2 top), but leveled off at the half kinetically rate (Figure 2 inset). This supports that superoxide is formed and released. Then SOD1 dismutes half of this

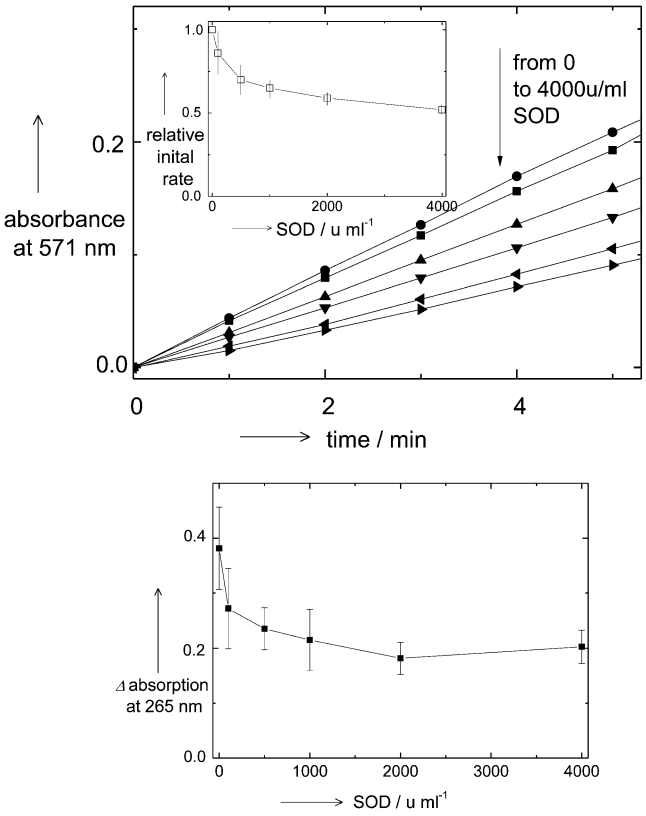
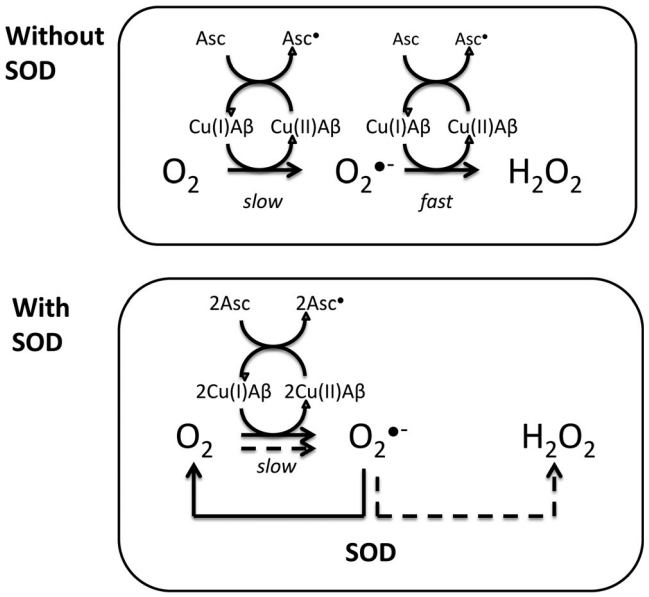


Figure 2. H_2O_2 formation by Cu-Aβ40. Top: Kinetics of production of resorufin measured by the absorption at 571 nm of Cu-Aβ40 with ascorbate (representative experiment). Resorufin is formed from amplex red by oxidation with H_2O_2 catalyzed by horseradish peroxidase. Effect of SOD1: (●) 0, (■) 100, (▲) 500, (▼) 1000, (◀) 1500, and (▶) 4000 u mL^{-1}. Conditions: 100 μM ascorbate, 5 μM Cu^{II}, 25 μM Aβ40, 50 mM KH_2PO_4/NaOH pH 7.4. Inset: SOD1 concentration dependence of the initial kinetics of resorufin production (relative kinetics compared to no SOD1 addition). The average of three independent measurements with standard deviations is given. Bottom: SOD1 concentration dependence of the initial kinetics of ascorbate consumption by Cu-Aβ40 with ascorbate. Ascorbate consumption was measured by the absorption at 265 nm. Conditions: 100 μM ascorbate, 5 μM Cu^{II}, 25 μM Aβ40, 50 mM KH_2PO_4/NaOH pH 7.4.



Scheme 2. Production of H_2O_2 from O_2 via $O_2^{\bullet-}$ in the absence (top) and presence of SOD1 (bottom). Without SOD1 the kinetics of ascorbate oxidation and H_2O_2 production is dominated by the slower first reduction of O_2 to $O_2^{\bullet-}$. The second reaction from $O_2^{\bullet-}$ to H_2O_2 is much faster. In the presence of saturating SOD1 (i.e. SOD1 outcompetes Cu^{I}-Aβ for reacting with $O_2^{\bullet-}$) ascorbate oxidation occurs only via the slow reaction of O_2 to $O_2^{\bullet-}$. Hence, to form H_2O_2 the slow reaction has to be passed twice (in contrast to only once in the absence of SOD1) and the kinetics is twice slower.

superoxide toward O_2 and half toward H_2O_2 (Scheme 2), in line with the maximal inhibition of H_2O_2 by half. Hence this suggests that superoxide is an intermediate and that the production of H_2O_2 catalyzed by Cu^{I}Aβ occurs predominantly by two one-electron reduction steps and released $O_2^{\bullet-}$ (Scheme 1, mechanism 1).

In parallel to the resorufin production, the consumption of ascorbate can be measured as well at 265 nm (Figure 2 bottom). Consistent with the superoxide formation, the rate of ascorbate oxidation dropped also to half, like the resorufin production after addition of SOD1. The slower kinetics of resorufin production upon addition of SOD1 has to be reflected by a slower ascorbate oxidation, because the electrons from ascorbate (via Cu-Aβ) are needed to form H_2O_2, which in turn induce the conversion of amplex red to resorufin (Scheme 2). Note that the same drop to half the rate in ascorbate consumption by adding SOD1 was also obtained in the absence of amplex red (Figure S2), corroborating the results above.

Hydroethidine (also called dihydroethidium) or nitro blue tetrazolium (NBT) colorimetric assays were used in earlier studies to try to detect the presence of $O_2^{\bullet-}$. They did not detect any $O_2^{\bullet-}$ production from O_2 in the presence of Cu-Aβ and ascorbate.[18] Hydroethidine or NBT react with $O_2^{\bullet-}$ by a redox reaction. Thus we hypothesized that Cu-Aβ (either in the Cu^{I} or Cu^{II} state) and/or ascorbate could interfere with the $O_2^{\bullet-}$ detection, as we had observed in the case of cyt c (see above). Indeed, by using the xanthine/xanthine oxidase system that produces $O_2^{\bullet-}$, we could show that hydroethidine detects $O_2^{\bullet-}$ production colorimetrically, but when ascorbate is added, this detection was abolished (see Figure S3). This is likely due to the much faster reaction of $O_2^{\bullet-}$ with the ascorbyl radical (2.6×10^8 M^{-1}s^{-1} at pH 7.8) compared to the reaction with hydroethidine (3×10^5 M^{-1}s^{-1}).[28,29] This means that ascorbate interferes with the detection of $O_2^{\bullet-}$ by hydroethidine. Similarly NBT and cyt c react relatively slow with $O_2^{\bullet-}$ (6×10^4 and 6×10^5 M^{-1}s^{-1}, respectively), and $O_2^{\bullet-}$ could not be detected.[29] In contrast, SOD1 reacts very fast ($\approx1\times10^9$ M^{-1}s^{-1}),[29] which explains the impact of SOD1 addition on the H_2O_2 production as shown above. These measurements and rates explain why the colorimetric assays with hyroethidium and NBT could not detect $O_2^{\bullet-}$ produced by the system Cu-Aβ, ascorbate and O_2.

Here we show for the first time strong evidence that Cu-Aβ can catalyze the production of $O_2^{\bullet-}$ in the presence of dioxygen and ascorbate. Moreover, the reduction of 50% of

H_2O_2 formation and ascorbate consumption by addition of SOD1 suggests that this is the major intermediate species on the way to H_2O_2 production (Scheme 1, mechanism 1). This has mechanistical and biological consequences.

First, it shows that formation of H_2O_2 occurs via two successive one-electron reductions (mechanism 1 in Scheme 1). The first step is the reaction of O_2 with Cu^{I}-Aβ resulting in $O_2^{\bullet-}$ and Cu^{II}-Aβ. $O_2^{\bullet-}$ is released from Cu^{II}-Aβ and can then be reduced again by a Cu^{I}-Aβ to form H_2O_2. To do that, one Cu center that cycles between the Cu^{I} and Cu^{II} state and hence transfer one electron at the time is sufficient. There is no need for a second electron donor, as it would be needed for the direct two-electron reduction from O_2 to H_2O_2 (mechanism 2 in Scheme 1)—as it had been suggested from earlier studies.[30,31] Mechanism 1 is in line with more recent work suggesting that there is no amino acid in Aβ that can serve as an efficient and catalytic electron donor.[9] Moreover, this mechanism 1 is also in line with the fact that monomeric Cu-Aβ is sufficient to catalyze H_2O_2 formation and a dimeric Cu center is not needed.[30,31]

Our findings do not only concern Cu-Aβ, they could have a general impact on a large variety of Cu-peptide complexes and their reactivity concerning $O_2^{\bullet-}$. A multitude of Cu complexes are able to catalyze the production of H_2O_2 from O_2 in the presence of a reducing agent, and as shown here, detection via the classical compounds cyt c, NBT or hydroethidine may fail, despite the production of $O_2^{\bullet-}$, due to the concomitant presence of ascorbate/ascorbyl. This failure was overcome by the use of electrochemical reduction of Cu-Aβ. Apart from the absence of ascorbate in the medium, it is possible that other parameters favor the detection of $O_2^{\bullet-}$.

In a biological setting, the present work extends the potential role of Cu-Aβ as a catalyst in the context of oxidative stress. Thus, Cu-Aβ is not only able to catalyze the production of H_2O_2 and $HO^{\bullet}$, but also $O_2^{\bullet-}$. The capacity to catalyze $O_2^{\bullet-}$ can be an important parameter, not only for mechanistic insights, but also for biological consequences. The production of $O_2^{\bullet-}$ can lead to other species in particular peroxynitrite, after reaction with $NO^{\bullet}$. Peroxynitrite is very deleterious and indeed several publications report on the role of peroxynitrite in neurodegenerative diseases.[32] Additionally, SOD upregulation as an early event in AD progression has been reported to be a compensatory effect against overproduction of $O_2^{\bullet-}$. Later during the progress of AD, SOD downregulation in the brain has been found. AD model mice with SOD deficiency showed increase Aβ oligomerization and memory impairment.[33] These reports underscore the implication of $O_2^{\bullet-}$ in AD and our results suggest that Cu-Aβ should be considered as a potential source of this overproduction of $O_2^{\bullet-}$ in AD. Thus SOD or SOD-mimicking compounds could be efficient in fighting against Cu-Aβ-catalyzed $O_2^{\bullet-}$, and hence inhibit the production of the very aggressive peroxynitrite.

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