Peptide Oxidation

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The Catalytically Active Copper-Amyloid-Beta State: Coordination Site Responsible for Reactive Oxygen Species Production**

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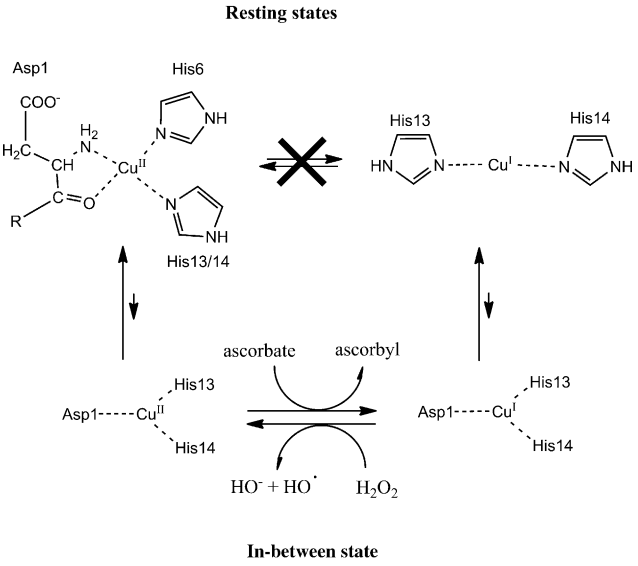
Amyloid plaques are a hallmark in the brain of Alzheimer's disease (AD) victims.[1] These plaques consist mainly of an aggregated peptide dubbed amyloid-β (Aβ),[2] which is also present in healthy brains in a soluble form.[3] It is thought that soluble oligomeric forms of Aβ are the most toxic species,[4] rather than more aggregated fibrils or protofibrils.[5] The presence of oxidative damage on neuronal lipids and proteins is evidence of a link between oxidative stress and AD (reviewed in Ref. [6]). Redox-active copper ions are highly accumulated in amyloid plaques, where they bind to Aβ. Cu-Aβ complexes are able to catalyze the production of reactive oxygen species (ROS), such as H_2O_2 and $HO^{\bullet}$, in vitro.[7] These reactions might contribute to the oxidative damage of diverse biomolecules observed in AD, including the peptide Aβ itself, which has been found to be oxidized in amyloid plaques in vivo.[8]

To catalyze the production of ROS, Cu has to cycle between the Cu^{I} and Cu^{II} redox states. Recently, a consensus about the most prevalent coordination spheres of Cu^{I} and Cu^{II} in Aβ has been reached (Scheme 1, top).[9] However, the interaction of Cu^{I} and Cu^{II} ions with Aβ is dynamic and there is a fast exchange between different ligands.[10] The high-affinity Cu^{II} binding sites are located in the N-terminal moiety of Aβ. At physiological pH, the predominant coordination sphere for Cu^{II} consists of the following four equatorial ligands: NH_2 of Asp1, the C=O from the Asp1-Ala2 peptide bond, N atoms of the imidazole ring of His6 and His13 (or His14 (in equilibrium in a 1:1 ratio); Scheme 1).[9,11] The Cu^{I} ion binds predominantly to Nδ of His13 and His14 in a linear fashion.[9,10b,12] These two coordination spheres differ quite a lot, suggesting a large structural rearrangement in the Cu coordination environment during the electron transfer.[13] Recent electrochemistry studies on the model Cu(Aβ1–16) confirmed that the redox reaction between these two prevalent coordination spheres is very inefficient. Hence, these states can be considered as the resting states of Cu^{I}(Aβ) and Cu^{II}(Aβ) complexes. They are in equilibrium with an in-between state that undergoes a fast redox reaction but represents only 0.1% of the species in solution.[14] The production of ROS is also based on efficient redox cycling between Cu^{I}(Aβ) and Cu^{II}(Aβ). The redox reaction occurs using a chemical reductant (here ascorbate) and oxidant (here H_2O_2 and O_2), that may coordinate to the Cu^{II} and Cu^{I} ions, respectively. Despite this difference, compared to the electrochemistry, it seems reasonable that ROS production proceeds through a similar, low-populated in-between state, because only such a state can undergo fast electron transfer (Scheme 1). So far, no structural information about this in-between state has been reported. One way to gain structural insight into it is to exploit the fact that only this state is redox competent. Metal-catalyzed oxidation (MCO) by the highly reactive $HO^{\bullet}$ produced by the Cu(Aβ)/ascorbate/O_2 or H_2O_2



Scheme 1. Top: resting states, that is, the most populated states of Cu^{II}(Aβ) (left) and Cu^{I}(Aβ) (right). The redox reaction between these states is sluggish owing to a large reorganization energy. Bottom: putative mechanism of $HO^{\bullet}$ production from H_2O_2 and ascorbate, through the efficient redox reaction of the in-between state.

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systems should occur predominantly within the in-between state. Thus, the resulting oxidative damage will allow for the identification of the ligands involved in metal-center coordination in the in-between state.

Herein, we use mass spectrometry coupled to high performance liquid chromatography (HPLC/MS) to identify the MCO of Cu(Aβ). Although this has been reported in the past (recently reviewed in Ref. [7c]), these reports aimed at mapping out the Cu^{II} binding site of Aβ and adapted the method accordingly.[15] For the in-between state, the following has to be considered: 1) only Cu-specific ROS production has to be taken into account, that is, non-specific background oxidation should be subtracted (we used radiation-induced oxidation to measure non-specific oxidative damage), 2) the very first site of oxidation has to be determined, because once a ligand becomes damaged, the Cu ion could move to another site. Therefore we studied the time dependence of the oxidative damage. With this unprecedented technique for Aβ, we were able to obtain information about the redox-competent in-between state of Cu(Aβ), responsible for ROS production.

HPLC/MS was used to characterize the Aβ oxidation in our model system consisting of Aβ1–28 (60 μM), Cu^{II} (50 μM) and ascorbate (500 μM). The truncated Aβ1–28 peptide (called Aβ28; sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNK) was used herein because it contains the metal-binding part Aβ1–16 used for the electrochemistry studies[14] and is also able to form the typical amyloid fibrils.[16] Cu^{II}(Aβ28) was found to react with ascorbate to produce ROS (in particular H_2O_2 and $HO^{\bullet}$[7b]) for about 15 minutes, as determined by assessing the ascorbate consumption (Supporting Information, Figure S1). Chromatograms of the tryptic peptides (peptides that have been digested by trypsin) of Aβ28 before and after oxidation for 15 minutes (m/z ratios are given in Table S1) showed that the peak intensity is lower for the non-oxidized peptides than for their oxidized counterpart (Figure S2). The peptide DAEFR was detected with mass shifts of −45, −89, and +16 Da, corresponding respectively to the decarboxylation/deamination of Asp1 and to the oxidative fragmentation of Asp1 by the diamide pathway[17] (both previously detected on shorter Aβ peptide models[15d,e]) and to the formal addition of one oxygen atom.

The other two tryptic peptides were also detected with a mass shift of +16 Da. This result was anticipated for the peptides DAEFR and HDSGYEVHHQK, because the copper binding sites are in Aβ1–16, but not for LVFFAEDVGSNK. The fragmentation spectra of LVFFAEDVGSNK + 16 (Figures S4,S5) contained almost a complete set of b/y ions and reliably revealed that Phe19 and Phe20 were the two oxidized residues (Figure 1). Oxidation of these Phe residues has not been reported by MCO studies on Aβ28/40.[15b] Similarly, the b and y ions obtained in the fragmentation spectra of HDSGYEVHHQK + 16 (Figures S6,S7) indicate that His13 and His14 were targeted by oxidation (conversion into 2-oxohistidine; Figure 1). In contrast, oxidation of His6 was not detected.

Poor information was gained for $DAEFR_{dd}$ (Figure S8), because only the monoprotonated ion was fragmented (the doubly protonated ion was not detected), and no fragmentation spectrum was obtained for $DAEFR_{ox}$ (mass shift −89 Da). High-resolution mass spectrometry coupled to liquid chromatography (LC–HRMS) allowed us to accurately determine the monoisotopic masses of these two oxidized peptides and to compare them with the calculated values (Figure S3); the difference was found to be as low as −3.0 ppm ($DAEFR_{dd}$) and −3.5 ppm ($DAEFR_{ox}$), thus validating the identification of these two products. The reaction mechanism of these oxidative cleavages, as proposed in Scheme S1, would proceed through an alkoxyl-radical pathway,[17] making the carbon at the α-position of the amino-acid residue sensitive to attack by ROS.

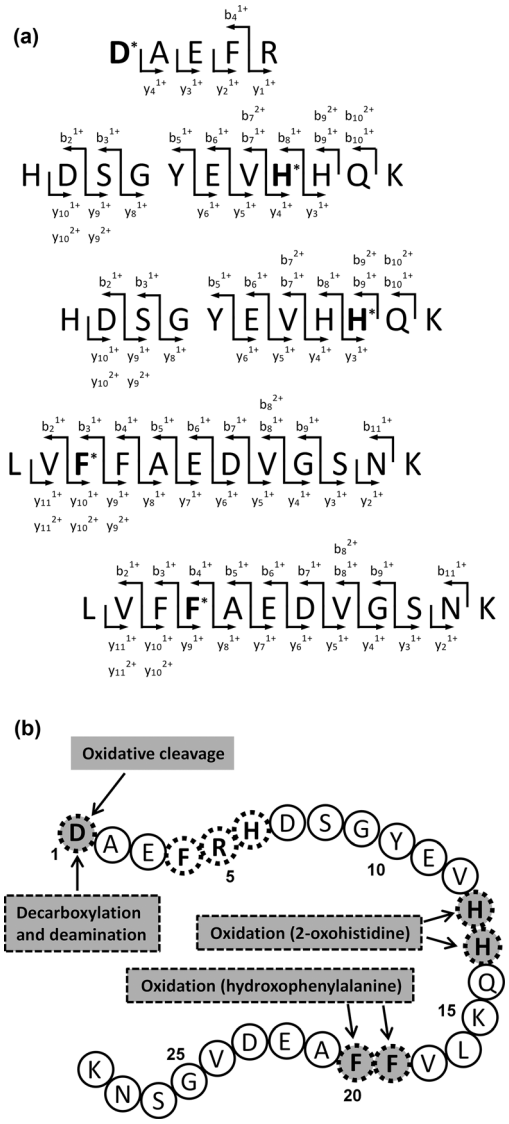

Figure 1. a) Fragmentation schemes of the Aβ28 tryptic peptides deduced from the ESI/MS-MS spectra (Figure S4–S8) of 2^+ ions (except DAEFR, monocharged), for Aβ28 (60 μM) with Cu^{II} (50 μM) and ascorbate (500 μM), after 15 min reaction time; confirmation of DAEFR oxidation is shown in Figure S3. b) Detected oxidized amino acid residues are highlighted on the sequence of Aβ28, along with the nature of the oxidative modification; gray background = MCO, dashed line = radio-induced oxidation.

These experiments allowed for the identification of the oxidized residues in Cu(Aβ28), to map the in-between state. However, oxidation of the ligands is likely to change the coordination of the Cu ion. Thus, we determined the first oxidation sites using time-dependent studies. The reaction of Aβ28 (60 μM), Cu^{II} (50 μM), and ascorbate (500 μM) was stopped each minute by decreasing the pH to 2. The N-terminal moiety of Aβ appears to be preferentially and highly oxidized by ROS (Figure 2a), relative to DAEFR oxidation,

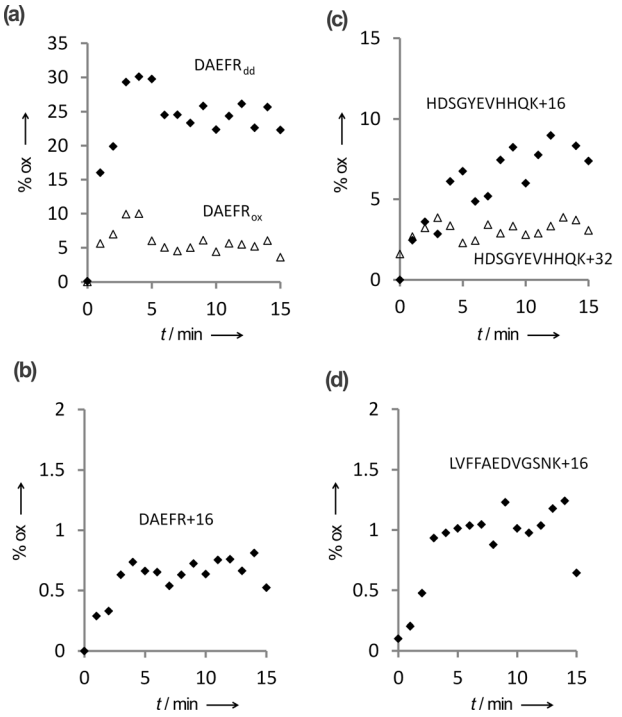

Figure 2. Oxidation of Aβ28 as a function of the reaction time. Area ratio between the oxidized and the equivalent non-oxidized (control, $t=0$) tryptic peptide of Aβ28 as a function of the reaction time; phosphate-buffered (100 mM, pH 7.4) solution of Aβ28 (60 μM) containing Cu^{II} (50 μM) and ascorbate (500 μM), digested by trypsin and analyzed by HPLC/HRMS. a) Decarboxylation and deamination of Asp1 (DAEFR$_{dd}$) and oxidative cleavage of Asp1 (DAEFR$_{ox}$); b) oxidation of DAEFR (DAEFR+16); c) oxidation of His13/His14 (HDSGYEVHHQK+16 and +32); d) oxidation of Phe19/Phe20 (LVFFAEDVGSNK+16). Mass tolerance set at 10 ppm; *m/z* ratios used for detection are specified in the Supporting Information, Table S1.

reaching 25–30% for DAEFR$_{dd}$ and 5–10% for DAEFR$_{ox}$. Interestingly, the curves of DAEFR$_{dd}$ and DAEFR$_{ox}$ production increase until reaching a plateau after approximately five minutes of reaction. The same behavior was observed for DAEFR+16 production (Figure 2b) and also for Phe19/Phe20 oxidation (Figure 2d). The yield (Table S2) is approximately 35-fold higher for Asp oxidation (DAEFR$_{dd}$ and DAEFR$_{ox}$) than for Phe oxidation, showing that Asp1 is preferentially targeted by the ROS. This suggests that Asp1 is a ligand in the in-between state. The decarboxylation and deamination of Asp1, driven by oxidation, would certainly change the coordination site of the Cu in this state (as well as in the resting state[9]).

The behavior of His13 and His14 oxidation look quite different because the curves of their oxidation (Figure 2c) increase continuously up to approximately 8% for single oxidation (+16). His13/14 are still coordinated to copper, at least in part, even after the change in the coordination mode of copper owing to Asp1 oxidation. In parallel, a non-negligible proportion of HDSGYEVHHQK+32 was detected and accounts for a third of the production of single His oxidation (yields 0.7 and 1.8, respectively; Table S2). Both His13 and His14 residues can be oxidized at the same time, and hence are thought to be ligands of the in-between state.

Amino acids have a different susceptibility to oxidation by HO• versus other ROS, which could bias the identification of the ligands. For instance, aromatic residues are more susceptible than aliphatic. This is exemplified by the oxidation of Phe19 and Phe20, reported above, residues that are likely not bound to Cu.

To address this point, radiation-induced ROS production was used to measure the non-specific oxidation. Thus, the difference between the MCO and radiation-induced oxidation should be purely owing to the metal-site-induced oxidation. So far only radio-induced azide radicals have been studied with Aβ.[18] Radio-induced oxidation of Aβ28 shows that the susceptibility of His and Phe to oxidation is higher than that of Asp1 (Figure 3). The average yield of HDSGYEVHHQK+16 and LVFFAEDVGSNK+16 is 1.6 times that of DAEFR$_{dd}$ and DAEFR+16. Decarboxylation and deamination of Asp1 (Figure S9) and oxidation of His13, His14, Phe19, and Phe20 (Figure S10,S13, respectively) are detected, as for MCO, while the oxidative cleavage of Asp1 was not observed. In addition, Phe4, Arg5, and His6 were also oxidized (Figure S14). In particular, oxidation of His6 is

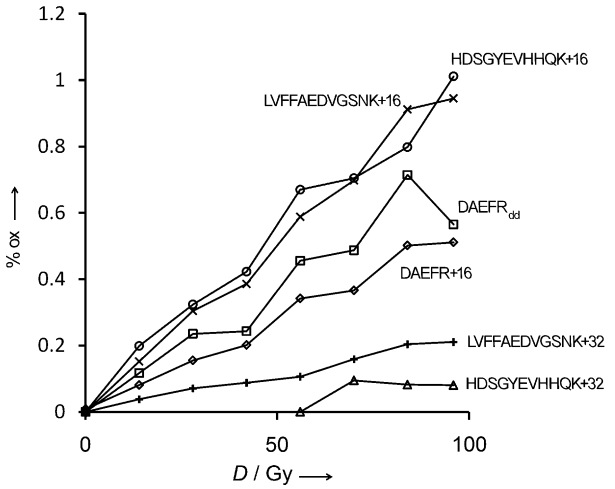

Figure 3. Radiation-induced oxidation of Aβ28. Area ratio between the oxidized and the equivalent non-oxidized (control, $t=0$) tryptic peptide of Aβ28 as a function of the radiation dose (D, in Gy); phosphate-buffered (100 mM, pH 7.4) solution of Aβ28 (60 μM) irradiated at 14, 28, 42, 56, 70, 84, and 98 Gy (positive control, non-irradiated), digested by trypsin and analyzed by HPLC/HRMS. DAEFR$_{dd}$ = decarboxylation and deamination of Asp1; +16 = single oxidation (formal addition of one oxygen atom), +32 = double oxidation (formal addition of molecular oxygen). Mass tolerance set at 10 ppm; *m/z* ratios used for detection are specified in Table S1.

intriguing because it was not observed by MCO of Cu-Aβ, clearly indicating that His6 does not take part in the ligand sphere of the in-between state.

In summary, this study suggests that Asp1, His13, and His14 are the main ligands in the state competent to efficiently undergo oxidation by H_2O_2 and reduction by ascorbate. Thus, the ligand sphere is neither identical with the main $Cu^{II}(A\beta)$, nor with the main $Cu^{I}(A\beta)$ coordination sphere. This put limits on the use of MCO to determine the main copper binding site for intrinsically disordered peptides (like Aβ, α-synuclein, and others). Rather, it is in line with the identification of the ligands of the most redox reactive state.

Additionally, to reach the in-between state from the resting $Cu^{II}(A\beta)$, His6 has to move out of the coordination sphere (Scheme 1). This is surprising as His6 is a main ligand in the resting $Cu^{II}(A\beta)$ and is also a minor ligand (in exchange with His13/14) of the resting $Cu^{I}(A\beta)$. Thus His6 seems to be a kind of gate keeper, which has to break its coordination bond with Cu to allow the start of the redox reaction. Indeed, a minor $Cu^{II}(A\beta)$ species with an uncoordinated His6, but bound His13 and 14 has been reported (named component 1c).[19] In contrast, to reach the in-between $Cu^{I}(A\beta)$, Asp1 has to coordinate to the Cu^{I} ion, which is anticipated to be the rate-limiting step.

We provide herein, for the first time, information about the coordination chemistry of the elusive in-between state of Cu(Aβ), that is able to undergo fast electron transfer and hence catalyzes ROS production. The transient character of this state explains why it was not detected in spectroscopic studies of $Cu^{I}(A\beta)$ and $Cu^{II}(A\beta)$.[9–12] This state might be of therapeutic interest, because its inhibition or neutralization could suppress Cu(Aβ)-based ROS production in vivo. Future work includes the study of the elusive in-between state in the full length Aβ and its different aggregation states. This is very important to establish the link between aggregation and ROS production, two key events in the etiology of AD.

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