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Amyloid-β and α-Synuclein Decrease the Level of Metal-Catalyzed Reactive Oxygen Species by Radical Scavenging and Redox Silencing

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Supporting Information

ABSTRACT: The formation of reactive oxygen species (ROS) is linked to the pathogenesis of neurodegenerative diseases. Here we have investigated the effect of soluble and aggregated amyloid-β (Aβ) and α-synuclein (αS), associated with Alzheimer’s and Parkinson’s diseases, respectively, on the Cu²⁺-catalyzed formation of ROS in vitro in the presence of a biological reductant. We find that the levels of ROS, and the rate by which ROS is generated, are significantly reduced when Cu²⁺ is bound to Aβ or αS, particularly when they are in their oligomeric or fibrillar forms. This effect is attributed to a combination of radical scavenging and redox silencing mechanisms. Our findings suggest that the increase in ROS associated with the accumulation of aggregated Aβ or αS does not result from a particularly ROS-active form of these peptides, but rather from either a local increase of Cu²⁺ and other ROS-active metal ions in the aggregates or as a downstream consequence of the formation of the pathological amyloid structures.

A hallmark of the two major neurodegenerative disorders, Alzheimer’s disease (AD) and Parkinson’s disease (PD), is the deposition within the brain of the amyloid β peptide (Aβ) and α-synuclein (αS), respectively.1 Despite the difference in the specific protein found to be the main component of the amyloid deposits in AD and PD, the formation of the pathological aggregates appears to occur via a common misfolding and self-assembly process.1 The cytotoxic species involved in both diseases appear to be the soluble oligomeric intermediates that form during the process of amyloid formation. Although the precise mechanism responsible for the toxicity of such species is not fully established, increasing evidence suggests that the neuronal cell loss in AD and PD is at least in part linked to excessive free radical generation.2

Aβ and αS bind metal ions, including Cu²⁺, that promote oligomerization and amyloid formation by both polypeptides3–7 and catalyze the formation of reactive oxygen species (ROS) that cause oxidative damage. In the brains of both AD and PD patients, increased oxidative damage, including protein, DNA, and RNA oxidation and lipid peroxidation, is observed relative to healthy controls.8–11 Impaired copper and iron homeostasis has also been associated with AD and PD, with elevated levels of both metals being found in the senile plaques from AD patients and in the Lewy bodies and cerebrospinal fluid of PD patients,9–11 which has stimulated interest in understanding the interaction of Aβ and αS with metal ions and its implications in AD and PD.

The coordination of Cu²⁺ to soluble Aβ and αS has been characterized in atomic detail. In Aβ, Cu²⁺ is primarily coordinated to Asp1, His6, His13, and His14 at physiological pH.12,13 In αS, a high affinity binding site has been identified, involving the first nine residues at the N-terminus.14,15 This binding site is, however, inactivated when the N-terminus is acetylated,16 but two low affinity binding sites in the vicinity of residues His50 and Asp121 bind Cu²⁺ in the acetylated form of αS found in vivo.16–18 It has been proposed that Cu²⁺ coordinated to Aβ and αS, in the presence of physiological reductants such as ascorbate, catalyzes the reduction of molecular oxygen to H₂O₂ and hydroxyl radicals (HO·) via Fenton chemistry (Figure 1). The coordination of Cu⁺ is

![Figure 1](https://example.com/figure1.png)
different from that of Cu$^{2+}$ for both Aβ and αS. In Aβ, Cu$^+$ is only coordinated to His13 and His14,13 and in αS, it is primarily coordinated to the side chains of Met1, Asp2, and Met5.19 Thus, the change in the oxidation state of Cu during the Fenton reaction cycle will induce structural changes in the protein—Cu complexes. The more ordered aggregated states of Aβ and αS may shift the energy difference between the Cu$^+$ and the Cu$^{2+}$ complexes relative to the flexible monomeric states and potentially influence the kinetics of ROS formation.

Here we have explored how the Cu$^{2+}$ interaction with different aggregated states of Aβ and αS affect ROS production in the presence of 100 μM ascorbate. We used a 2:1 protein/Cu$^{2+}$ molar ratio to avoid the presence of free Cu$^{2+}$ in solution. No detectable ROS production was observed in the samples without the addition of Cu$^{2+}$ and ascorbate (Figure S1). We measured the production of both H$_2$O$_2$ and HO$^*$ with colorimetric and fluorescence assays and also followed the consumption of ascorbate as a direct assay of ROS production. Aβ$^{40}$ and αS were studied, along with variants deficient in the ability to bind Cu$^{2+}$, namely, Aβ$^{40}[H6A/H13A/H14A]$, where the Cu$^{2+}$-coordinating histidine residues in Aβ were all substituted by alanine, and αSΔ2–9, where residues 2–9 in αS were deleted. Aggregated Aβ and αS fibrils were prepared both in the absence and presence of Cu$^{2+}$. In addition, αS oligomeric species with structural features that are intermediate between the intrinsically disordered monomeric protein and the highly organized mature fibrils were included in the analysis. These oligomeric forms of αS induce ROS production when internalized in healthy neuronal cells.21,22 The mechanism of αS-oligomer-induced ROS production has been linked to the presence of free metal ions in the culture media.23

The rates and the levels of ROS production were highly dependent on which form of Aβ or αS was present in the solution. In the absence of protein, all ascorbate was consumed within approximately 10 min (Figure 2A,B). The presence of Aβ$^{40}[H6A/H13A/H14A]$, which does not bind Cu$^{2+}$, had no effect on the ascorbate consumption rate (Figure S2A). Only very small effects on ascorbate consumption were seen on addition of monomeric and oligomeric αSΔ2–9 to the Cu$^{2+}$/ascorbate reaction mixture (Figure S2B). The rate of ascorbate consumption, however, decreased 2- to 3-fold when Cu$^{2+}$ was bound to soluble wt-Aβ$^{40}$ or wt-αS. The rate was reduced even more (5-fold) when Cu$^{2+}$ was bound to either oligomeric or fibrillar states of Aβ$^{40}$ and αS (Figures 2A,B and S3). The most pronounced effects were monitored for Aβ$^{40}$ and αS fibrils that were formed in the presence of Cu$^{2+}$, which produced ROS at a rate decreased nearly 20-fold relative to that for the same concentration of free metal ions in solution (Figures 2A,B and S3). Even for αSΔ2–9, the fibrils formed in the presence of Cu$^{2+}$ reduced the rate of ascorbate consumption more than any other αSΔ2–9 species (Figure S2).

The formation of H$_2$O$_2$ (Figure 2C,D) closely followed the consumption of ascorbate and reached the same level for all states of the proteins. In contrast, not only the rate but also the initial levels of HO$^*$ varied between the samples, with lower rates being associated with lower initial levels of the radical (Figure 2E,F). Fibrils of Aβ$^{40}$ completely abolished the generation of free HO$^*$ species that could react with 3-CCA. In addition, the fibrils of αS that were formed in the presence of Cu$^{2+}$ resulted in very low levels of free HO$^*$. Moreover, in the presence of Cu$^{2+}$, the oligomers of αS lowered the level of HO$^*$ to the same extent as that of fibrils of αS formed in the absence of Cu$^{2+}$ (compare Figure 2F blue and gray curves). Together, the data suggest that Cu$^{2+}$ is less accessible to the solvent when bound to the aggregated forms of Aβ$^{40}$ and αS than when bound to the monomeric state, and much less than when free in solution; hence, it is less able to react with ascorbate, resulting in slower ROS formation. Although all ascorbate was consumed and the same levels of H$_2$O$_2$ were produced at the end of the reaction under all conditions, significant differences in the amount of free HO$^*$ were observed, which correlates with the variations in the initial rate of ROS production by the different protein species. This observation suggests that the proteins act as efficient scavengers of HO$^*$ produced by Cu$^{2+}$—protein complexes.

To confirm that the proteins do indeed act as radical scavengers and to characterize the covalent modifications of Aβ$^{40}$ and αS induced by the oxidation, we monitored the time-dependent changes in molecular mass using MALDI-TOF MS (Figures 3 and S4). In the presence of Cu$^{2+}$ and ascorbate, we observed a low level of oxidation and cleavage of αS. The oxidative patterns for monomeric, oligomeric, and fibrillar states of αS are similar to each other, although the monomer demonstrates a slightly higher level of oxidation (Figure 3D). More oxidation and oxidation-driven cleavage of the polypeptide chain are evident for Aβ$^{40}$. Here, an intense peak in the mass spectrum corresponding to cleavage of the peptide backbone between the Cu$^{2+}$ coordination residues His13 and Met14,19 is observed, demonstrating that some of the residues that are not coordinated to Cu$^{2+}$ are modified.
The Cu concentration in all samples was 5 μM respectively. The Cu concentration in all samples was 5 μM. We demonstrated that Cu2+-catalyzed ROS formation is significantly reduced when the metal ion is bound to aggregated species, which also act as HO* scavengers. Although we and others have previously shown that certain amyloid aggregates such as those used in this study are able to induce more aberrant ROS production than are monomeric species when internalized in cells, the in vitro data presented here reveal that Cu2+ bound to Aβ or Aβ will decrease. As a consequence, ROS production mediated by Cu2+ will be decreased when Cu2+ is coordinated to Aβ and in particular when coordinated to Aβ fibrils. Furthermore, the decrease in redox activity in the aggregated state may reflect the fact that redox cycling of coordinated Cu+ and Cu2+ requires formation of a transient intermediate coordination state where the coordination sphere differs from the resting states of Aβ−Cu+/Cu2+. If the free energy barrier for formation of this transient state is increased in the aggregated species, e.g., as a result of a decrease in flexibility of the coordination sphere, then the redox activity of Cu2+ coordinated to aggregated Aβ or Aβ will decrease. Formation of the transient intermediate state may be further inhibited by the increase in the number of Cu2+ coordination modes in the aggregates compared to the soluble proteins.

Using two different amyloidogenic proteins, we have demonstrated that Cu2+-catalyzed ROS formation is significantly reduced when the metal ion is bound to aggregated species, which also act as HO* scavengers. Although we and others have previously shown that certain amyloid aggregates such as those used in this study are able to induce more aberrant ROS production than are monomeric species when internalized in cells, the in vitro data presented here reveal that this is likely not to be a consequence of direct ROS formation catalyzed by the aggregates but rather a downstream consequence of a primary effect of these aggregates on the cells. Nevertheless, some ROS is produced from Cu2+ bound to aggregates, and as a result of the high concentrations of amyloid species in plaques and Lewy bodies, ROS may increase locally in the regions of amyloid accumulation, although relative to Cu2+ that is freely diffusing or even bound to physiological forms of Aβ or Aβ, the aggregates will strongly attenuate the ROS formation.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b13577.

- Materials and methods and three supporting figures. (PDF)

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**Notes**

The authors declare no competing financial interest.

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