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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 Cu^{2+}-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 Cu^{2+} 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 Cu^{2+} 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 Cu^{2+}, that promote oligomerization and amyloid formation by both polypeptides[3−5] 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.[6−8] 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 Cu^{2+} to soluble Aβ and αS has been characterized in atomic detail. In Aβ, Cu^{2+} 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 Cu^{2+} in the acetylated form of αS found *in vivo*.[16−18] It has been proposed that Cu^{2+} coordinated to Aβ and αS, in the presence of physiological reductants such as ascorbate, catalyzes the reduction of molecular oxygen to H_2O_2 and hydroxyl radicals ($HO^•$) via Fenton chemistry (Figure 1). The coordination of Cu^+ is

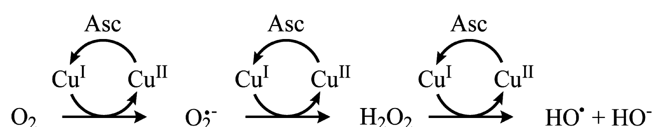

Figure 1. Fenton reaction cycle for the production of ROS from molecular oxygen and ascorbate (Asc); see ref 20

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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_2O_2 and $HO^{\bullet}$ 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 of 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[21] 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_2O_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 final levels of $HO^{\bullet}$ varied between the samples, with lower rates being associated with lower final levels of the radical (Figure 2E,F). Fibrils of Aβ_{40} completely abolished the generation of free $HO^{\bullet}$ 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 $HO^{\bullet}$. Moreover, in the presence of Cu^{2+}, the oligomers of αS lowered the level of $HO^{\bullet}$ 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

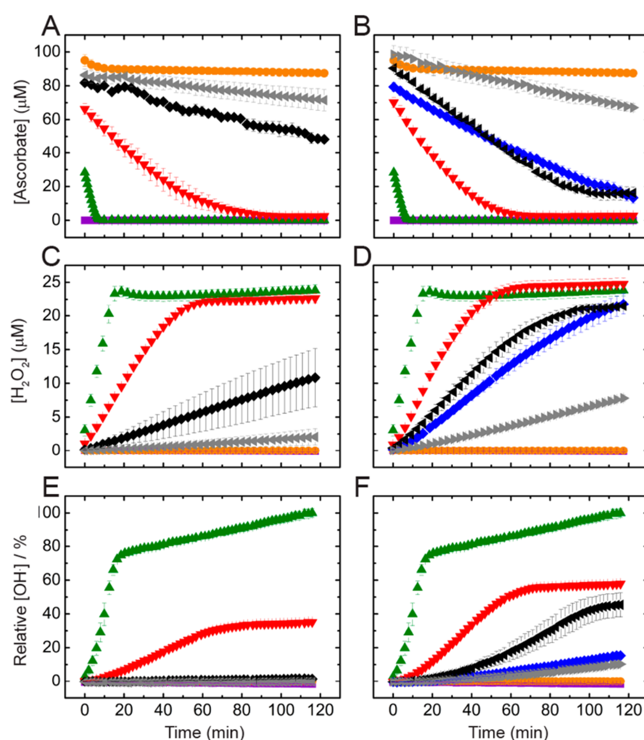

Figure 2. Generation of ROS in the presence of Aβ_{40} and αS. (A and B) Consumption of ascorbate, measured by the decrease in absorbance at 265 nm, for (A) Aβ_{40} and (B) αS. (C and D) Generation of H_2O_2, measured by the increase in resorufin fluorescence at 590 nm, for (C) Aβ_{40} and (D) αS. (E and F) The formation of $HO^{\bullet}$ was measured by the increase in fluorescence at 450 nm upon oxidation of 3-CCA for (E) Aβ_{40} and (F) αS. Seven different conditions were employed (red, monomeric protein; black, fibrils formed in the absence of Cu^{2+}; gray, fibrils formed in the presence of Cu^{2+}; blue, oligomers (αS only); purple, Cu^{2+} alone; orange, ascorbate alone; and green, Cu^{2+} and ascorbate alone). In all assays, the concentrations of protein, Cu^{2+}, and ascorbate were 10, 5, and 100 μM, respectively.

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_2O_2 were produced at the end of the reaction under all conditions, significant differences in the amount of free $HO^{\bullet}$ 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 $HO^{\bullet}$ 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

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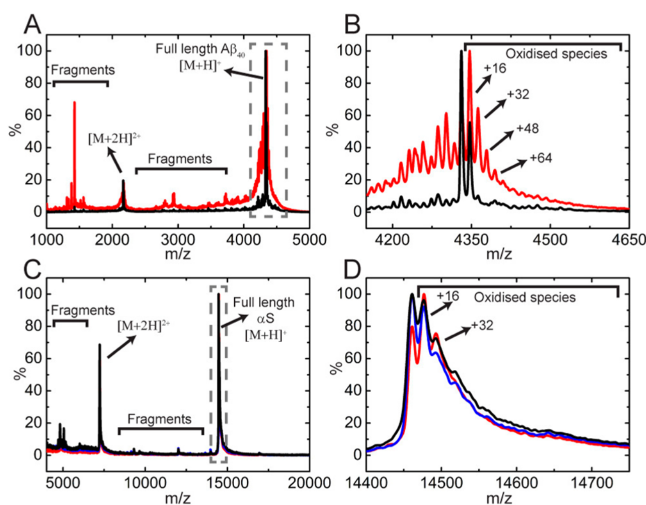

Figure 3. Oxidation effects detected by mass spectrometry. Samples were measured after 30 min incubation in the presence of 100 μM ascorbate. (A and B) Monomers (red) and fibrils (black) of Aβ_{40}. (C and D) Monomers (red), oligomers (blue), and fibrils (black) of αS. Panels B and D are expansions of the main peaks in panel A and C, respectively. The Cu concentration in all samples was 5 μM.

His14 appears within the first 30 min of the redox reaction (Figure 3B), in agreement with previous observations.[24,25] More oxidation is observed for monomeric Aβ_{40} than for αS, consistent with the results from the 3-CCA assay, which show that the level of free HO$^\bullet$ is lower in the Aβ_{40} samples than in the corresponding αS samples and that Aβ thus more readily reacts with HO$^\bullet$ (compare Figure 2E,F). Our results, therefore, indicate that Aβ_{40} is more efficient than αS as an HO$^\bullet$ scavenger.

The slower ROS formation and lower free HO$^\bullet$ levels in the presence of fibrils cannot be fully explained by the fibrils acting as more efficient ROS scavengers than the soluble species. If this were the case, then we would not expect the ascorbate consumption to be slowed down. Furthermore, we observe more oxidized species in the monomeric samples than in the fibrillar samples. The effect of the Aβ_{40} and αS fibrils, and αS oligomers, is rather to sequester Cu^{2+} from the solution and decrease the rate of Cu redox cycling.

Aβ_{40} and αS have previously both been suggested to act as pro-oxidants,[14,20,26] and Aβ_{40} has also been suggested to act as an antioxidant.[27,28] The main argument for αS and Aβ_{40} acting as pro-oxidants is that higher levels of free radicals are produced when Cu^{2+} is bound to these proteins than when Cu^{2+} is bound to other peptides or proteins. In contrast, the main argument for Aβ_{40} acting as an antioxidant is that less free radicals are produced by Cu^{2+} bound to Aβ_{40} relative to those from free Cu^{2+}. Here we have shown that both Aβ_{40} and αS, when bound to Cu^{2+}, reduce the ROS levels as compared to free Cu^{2+}. This reduction is likely to be related to the binding of Aβ_{40} and αS to Cu^{2+} because the ROS levels of Cu^{2+} in the presence of monomeric Aβ_{40}[H6A/H13A/H14A] and αSΔ2−9 are very similar to those in the presence of free Cu^{2+} (Figure S2). We have also shown that the oligomeric and fibrillar samples of Aβ_{40} and αS are much more efficient than soluble species at reducing the HO$^\bullet$ levels in solution.

Our data show that Aβ_{40} and αS both serve as HO$^\bullet$ scavengers, because they reduce the amount of free HO$^\bullet$ in the solution, and that the binding of Cu^{2+} to the proteins decreases ROS production. These effects become more prominent when Cu^{2+} is bound to the β-sheet-rich conformations of the aggregates in both Aβ_{40} and αS. It is likely that the compact structure of the aggregates prevents accessibility of oxygen and ascorbate to Cu^{2+} and, therefore, suppresses electron transfer from the metal ions to oxygen molecules. This notion is supported by EPR and ESEEM data that suggest that the preferred Cu^{2+} coordination mode in both soluble and aggregated Aβ_{40} is unfavorable for Cu^{+}/Cu^{2+} redox cycling.[29] As a consequence, ROS production mediated by Cu^{2+} will be decreased when Cu^{2+} is coordinated to Aβ_{40} and in particular when coordinated to Aβ_{40} fibrils. Furthermore, the decrease in redox activity in the aggregated state may reflect the fact that redox cycling of coordinated Cu^{+} and Cu^{2+} requires formation of a transient intermediate coordination state where the coordination sphere differs from the resting states of Aβ–$Cu^{+/2+}$.[12] 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 Cu^{2+} coordinated to aggregated Aβ_{40} or αS will decrease.[30] Formation of the transient intermediate state may be further inhibited by the increase in the number of Cu^{2+} coordination modes in the aggregates compared to the soluble proteins.[31]

Using two different amyloidogenic proteins, we have demonstrated that Cu^{2+}-catalyzed ROS formation is significantly reduced when the metal ion is bound to aggregated species, which also act as HO$^\bullet$ 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,[22,23,32] 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 Cu^{2+} 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 Cu^{2+} that is freely diffusing or even bound to physiological forms of Aβ_{40} or αS, 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

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■ REFERENCES

(1) Chiti, F.; Dobson, C. M. *Annu. Rev. Biochem.* **2006**, *75*, 333.
(2) Barnham, K. J.; Bush, A. I. *Chem. Soc. Rev.* **2014**, *43*, 6727.
(3) Riederer, P.; Sofic, E.; Rausch, W. D.; Schmidt, B.; Reynolds, G. P.; Jellinger, K.; Youdim, M. B. *J. Neurochem.* **1989**, *52*, 515.
(4) Pedersen, J. T.; Ostergaard, J.; Rozlosnik, N.; Gammelgaard, B.; Heegaard, N. H. H. *J. Biol. Chem.* **2011**, *286*, 26952.
(5) Drew, S. C. *Chem. - Eur. J.* **2015**, *21*, 7111.
(6) Sultana, R.; Butterfield, D. A. *J. Alzheimers Dis* **2010**, *19*, 341.
(7) Dexter, D. T.; Carter, C. J.; Wells, F. R.; Javoy-Agid, F.; Agid, Y.; Lees, A.; Jenner, P.; Marsden, C. D. *J. Neurochem.* **1989**, *52*, 381.
(8) Zhang, J.; Perry, G.; Smith, M. A.; Robertson, D.; Olson, S. J.; Graham, D. G.; Montine, T. J. *Am. J. Pathol.* **1999**, *154*, 1423.
(9) Lovell, M. A.; Robertson, J. D.; Teesdale, W. J.; Campbell, J. L.; Markesbery, W. R. *J. Neurol. Sci.* **1998**, *158*, 47.
(10) Castellani, R. J.; Siedlak, S. L.; Perry, G.; Smith, M. A. *Acta Neuropathol.* **2000**, *100*, 111.
(11) Barnham, K. J.; Bush, A. I. *Curr. Opin. Chem. Biol.* **2008**, *12*, 222.
(12) Cassagnes, L.-E.; Hervé, V.; Nepveu, F.; Hureau, C.; Faller, P.; Collin, F. *Angew. Chem., Int. Ed.* **2013**, *52*, 11110.
(13) Hureau, C. *Coord. Chem. Rev.* **2012**, *256*, 2164.
(14) Davies, P.; Wang, X.; Sarell, C. J.; Drewett, A.; Marken, F.; Viles, J. H.; Brown, D. R. *Biochemistry* **2011**, *50*, 37.
(15) Drew, S. C.; Ling Leong, S.; Pham, C. L. L.; Tew, D. J.; Masters, C. L.; Miles, L. A.; Cappai, R.; Barnham, K. J. *J. Am. Chem. Soc.* **2008**, *130*, 7766.
(16) Moriarty, G. M.; Minetti, C. A. S. A.; Remeta, D. P.; Baum, J. *Biochemistry* **2014**, *53*, 2815.
(17) Binolfi, A.; Lamberto, G. R.; Duran, R.; Quintanar, L.; Bertoncini, C. W.; Souza, J. M.; Cerveñansky, C.; Zweckstetter, M.; Griesinger, C.; Fernández, C. O. *J. Am. Chem. Soc.* **2008**, *130*, 11801.
(18) Dudzik, C. G.; Walter, E. D.; Millhauser, G. L. *Biochemistry* **2011**, *50*, 1771.
(19) De Ricco, R.; Valensin, D.; Dell'Acqua, S.; Casella, L.; Gaggelli, E.; Valensin, G.; Bubacco, L.; Mangani, S. *Inorg. Chem.* **2015**, *54*, 265.
(20) Guilloreau, L.; Combalbert, S.; Sournia-Saquet, A.; Mazarguil, H.; Faller, P. *ChemBioChem* **2007**, *8*, 1317.
(21) Chen, S. W.; Drakulic, S.; Deas, E.; Ouberai, M.; Aprile, F. A.; Arranz, R.; Ness, S.; Roodveldt, C.; Guilliams, T.; De Genst, E. J.; Klenerman, D.; Wood, N. W.; Knowles, T. P. J.; Alfonso, C.; Rivas, G.; Abramov, A. Y.; Valpuesta, J. M.; Dobson, C. M.; Cremades, N. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E1994.
(22) Cremades, N.; Cohen, S. I. A.; Deas, E.; Abramov, A. Y.; Chen, A. Y.; Orte, A.; Sandal, M.; Clarke, R. W.; Dunne, P.; Aprile, F. A.; Bertoncini, C. W.; Wood, N. W.; Knowles, T. P. J.; Dobson, C. M.; Klenerman, D. *Cell* **2012**, *149*, 1048.
(23) Deas, E.; Cremades, N.; Angelova, P. R.; Ludtmann, M.; Yao, Z.; Chen, S.; Horrocks, M.; Banushi, B.; Little, D.; Devine, M.; Gissen, P.; Klenerman, D.; Dobson, C. M.; Wood, N. W.; Gandhi, S.; Abramov, A. Y. *Antioxid. Redox Signaling* **2016**, *24*, 376.
(24) Kowalik-Jankowska, T.; Ruta, M.; Wiśniewska, K.; Łankiewicz, L.; Dyba, M. *J. Inorg. Biochem.* **2004**, *98*, 940.
(25) Schöneich, C.; Williams, T. D. *Chem. Res. Toxicol.* **2002**, *15*, 717.
(26) Turnbull, S.; Tabner, B. J.; El-Agnaf, O. M.; Moore, S.; Davies, Y.; Allsop, D. *Free Radical Biol. Med.* **2001**, *30*, 1163.
(27) Baruch-Suchodolsky, R.; Fischer, B. *Biochemistry* **2009**, *48*, 4354.
(28) Nadal, R. C.; Rigby, S. E. J.; Viles, J. H. *Biochemistry* **2008**, *47*, 11653.
(29) Gunderson, W. A.; Hernández-Guzmán, J.; Karr, J. W.; Sun, L.; Szalai, V. A.; Warncke, K. *J. Am. Chem. Soc.* **2012**, *134*, 18330.
(30) Balland, V.; Hureau, C.; Savéant, J.-M. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 17113.
(31) Parthasarathy, S.; Long, F.; Miller, Y.; Xiao, Y.; McElheny, D.; Thurber, K.; Ma, B.; Nussinov, R.; Ishii, Y. *J. Am. Chem. Soc.* **2011**, *133*, 3390.
(32) Rosales-Corral, S.; Acuna-Castroviejo, D.; Tan, D. X.; López-Armas, G.; Cruz-Ramos, J.; Munoz, R.; Melnikov, V. G.; Manchester, L. C.; Reiter, R. J. *Oxid. Med. Cell. Longevity* **2012**, *2012*, 843649.

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