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Is ascorbate Dr Jekyll or Mr Hyde in the Cu(A β) mediated oxidative stress linked to Alzheimer's disease?†

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Abstract

Evaluation of the pro *versus* antioxidant activity of ascorbate regarding Cu(A β) induced reactive oxygen species production in the context of Alzheimer's disease shows that a protective activity can only be observed at high ascorbate concentration for exogenous molecules but not for the amyloid- β peptide itself.

The misregulation of reactive oxygen species (ROS) leading to oxidative stress is observed in multiple diseases, including neurodegenerative disorders like Alzheimer's or Parkinson's disease. Therefore, there is strong interest in the effect of anti-oxidants as they could have potential therapeutic benefits.¹ There are different families of antioxidants depending on their mode of action (sacrificial or catalytic scavengers of ROS produced, inhibitors of ROS production). Sacrificial scavengers work by capturing ROS and other free radical species *via* reduction or addition reactions.² Ascorbate belongs to this family. It is a main physiological reducing agent present at a high concentration, in particular in the brain (up to 10 mM in neurons).³ It can reduce radicals and convert them into non-radicals, like for instance HO \cdot to HO $^-$, or O $_2^{\cdot-}$ to H $_2$ O $_2$. This is part of its antioxidant activity but transferring an electron to a non-radical species can also produce radicals. This happens when an electron is transferred to O $_2$ or H $_2$ O $_2$, leading to O $_2^{\cdot-}$ or to the very reactive HO \cdot radical. However, these reactions are not efficient,⁴ unless they are catalyzed. This can be performed by redox active metal ions like Cu(I/II) and Fe(II/III) which can catalyze very efficiently the transfer of a single electron. This is for instance the case in the well-known Fenton reaction leading to the formation of HO \cdot from dioxygen and a reducing agent like ascorbate. Actually, redox active metal ions, mainly Fe(II/III), Cu(I/II) and Mn(II/III) play a crucial role *in vivo* that essentially depends on their coordination chemistry. This is illustrated by the role of Cu ions that can be catalytic scavengers of O $_2^{\cdot-}$ when bound inside superoxide dismutase (SOD) or are involved in the catalytic production of ROS when loosely bound (Scheme 1).⁴

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In the case of the reaction of loosely bound Cu ions with ascorbate and dioxygen, it is known that the concentration of ascorbate has an important impact on the production of HO[•]. In general, it was concluded that low concentrations of ascorbate are required for prooxidant conditions, while high concentrations are needed for antioxidant conditions.⁵ Thus there is a “cross-over” point, *i.e.* an ascorbate concentration at which the prooxidant ability of ascorbate is maximal. Such a value depends on the experimental conditions.⁵ This has been supported by experiments in different systems *in vitro* and *in cellulo*.⁶ *In vivo*, the prooxidant activity of ascorbate seems to be limited to conditions where Fe and Cu are aberrantly coordinated and hence able to catalyze the Fenton type reaction.⁷

In Alzheimer’s disease (AD), Cu ions are found in high concentrations (0.4 mM) in amyloid plaques, a hallmark of the disease.⁸ Cu is bound to the amyloid- β (A β) peptide in the extracellular plaques themselves but its coordination to the monomeric soluble form of A β is also proposed,⁹ as the Cu concentration can reach up to 10 μ M in certain synaptic clefts.^{10,11} The coordination sites of Cu(II) and Cu(I) lie in the first 16 amino acids of the 40 to 42 amino-acid residue peptide. They have been extensively studied and are well characterized (Fig. S1 and S2[†]), in relation to their redox ability.⁹ *In vitro*, Cu(A β) is able to catalyze the production of ROS in the presence of ascorbate, and it was proposed that these ROS can contribute to the oxidative stress observed in AD.^{12,13}

In the present work, we have investigated the impact of Cu coordination to the A β peptide on modulation of the pro *versus* antioxidant properties of ascorbate. This is of interest with regard to therapeutic approaches in AD based on oxidative stress regulation. Coumarin-3-carboxylate (CCA) was used as a prototypical target mimicking the biological ones, to detect the deleterious radical HO[•]. CCA reacts with HO[•] and forms the fluorescent adduct 7-hydroxycoumarin-3-carboxylate (7-OH-CCA), which shows emission at 450 nm upon excitation at 390 nm. While the detection of HO[•] by CCA was reported to be sensitive and specific,¹⁴ there are some parameters to be considered in order to have a quantitative detection (at a particular pH, see the ESI for details: Fig. S3–S6[†]).

In Fig. 1, black lines show the time dependence of Cu-induced formation of 7-OH-CCA in line with similar literature data.^{15–18} Two main phases were observed: (i) a first fast process with an exponential growth profile and (ii) a second slow process with an almost linear increase. The origin of the latter one is not known, but it seems to be independent of the Cu-catalyzed HO[•] production from ascorbate and O₂ and thus irrelevant for the present study. Indeed, completion of the first process almost coincides with total ascorbate consumption (Fig. S7[†]) and thus there is not much ascorbate available when the second process occurs. Note that unless specified all the experiments described in the paper were performed with the C-terminally truncated peptide encompassing the first 16 amino-acid residues that correctly reproduce the binding properties of the full-length peptide.⁹

In the presence of 1.2 equivalents of A β ₁₆, the curves are different (Fig. 1, grey curves). The first phase takes more time, indicating that the formation of 7-OH-CCA is slower, in line with the lower catalytic activity of Cu(A β ₁₆) with respect to ROS formation, described already in the past.^{15–17} Also the plateau reached during the second phase is lower compared to the one obtained in the presence of Cu only. This is in line with the observation

that HO[•] reacts with the peptide Aβ itself thus precluding its reaction with CCA as previously observed.^{18–21} Indeed, the plateau corresponds to the part of HO[•] able to react with CCA, *i.e.* it reflects the part of HO[•] that would be able to damage biomolecules other than Aβ or ascorbate, like proteins, lipids or nucleic acids in a biological environment. In line with this comment, it is worth noting that the higher the ascorbate over the Aβ₁₆ ratio, the closer the plateau obtained for free Cu and Cu(Aβ) species. At a high concentration of ascorbate, the first phase is more complex in the presence of a peptide with a sigmoid-like trend in line with (i) the oxidation of the Aβ₁₆ peptide and (ii) the subsequent release of free Cu (inset in Fig. 1). This was verified by measuring the level of intact peptide by LC-MS (Fig. S8 and Table S1†).[§] Indeed the peptide was degraded at the end of the reaction. The degradation degree depends on the ascorbate concentration, but is complete above 1 mM (Fig. S9†).

In order to determine the *pro versus* antioxidant regime of ascorbate with both free Cu and Cu(Aβ₁₆), the ascorbate dependence of the 7-OH-CCA formation was measured and compared. Fig. 2[¶] shows the initial rate (calculated over the first five minutes) of the 7-OH-CCA formation at different ascorbate concentrations for Cu and Cu(Aβ₁₆). The initial rate can be considered as the most relevant reaction under steady state conditions of ascorbate and oxygen supposed to occur *in vivo*.

For free Cu, the shapes are similar for the three different Cu concentrations used and all show a very marked ascorbate concentration dependence. At low ascorbate concentration the initial rate of 7-OH-CCA formation increases with the ascorbate concentration. The maximal rate, which has previously been called the cross-over point,⁵ is observed around 0.7 mM in ascorbate. At higher ascorbate concentrations the initial rate of 7-OH-CCA formation decreases continuously, supporting an antioxidant property from 0.7 mM. This dependence can be explained due to the double effect of ascorbate: on one side, it promotes the production of HO[•] that further reacts with CCA (pro-oxidant part, Scheme 1) and on the other hand it scavenges HO[•] (antioxidant part).⁵ In the absence of ascorbate, no HO[•] is produced, as ascorbate is needed as an electron source to reduce dioxygen to HO[•]. At low concentration, HO[•] trapped by CCA increases with ascorbate concentrations, because ascorbate fuels the formation of HO[•], and does not scavenge HO[•] efficiently. At higher ascorbate concentrations, the initial rate does not increase anymore, because the ascorbate is no more the limiting species and the scavenging ability becomes more and more efficient. For these reasons, the initial rate of 7-OH-CCA formation decreases.

In the presence of Aβ₁₆, *i.e.* when HO[•] production is catalyzed by Cu(Aβ₁₆), the overall dependence is similar, with a cross-over point at 0.7 mM of ascorbate (Fig. 2, right panel). In general, the initial Cu(Aβ₁₆) induced formation of 7-OH-CCA formation is slower than without a peptide, in line with the more sluggish Cu(I/II) redox process of Cu when bound to Aβ.^{22–24} Note that only the very first points of the 7-OH-CCA formation curves were taken into account to avoid any issue due to the release of Cu at high ascorbate concentrations

[§]For technical reasons, the Aβ₂₈ peptide encompassing the first 28 amino-acid residues has been used in mass spectrometry experiments (see the ESI† for details).

[¶]Note that data shown in Fig. 2 and 3 are representative from a series of measurements performed under slightly different conditions, but for which the very same trends were observed.

(inset in Fig. 1). The rate of 7-OH-CCA formation is dependent on the Cu(A β ₁₆) concentration. This is in line with the slower rate of HO \cdot formation in the presence of A β ₁₆. The same trends were observed for the longer peptide encompassing the first 28 amino-acid residues and the full-length peptide (Fig. S10 \dagger).

Finally, the total amounts of 7-OH-CCA produced were measured. For doing this, the reaction was stopped after the total consumption of ascorbate (arrows in Fig. 1), and the intensities of the plateau were compared (Fig. 3) as a function of the initial ascorbate concentration and the Cu (resp. Cu(A β)) concentration.

For free Cu, the ascorbate concentration dependence follows a sort of saturation curve. Again this can be explained by the two properties of ascorbate that fuels the HO \cdot production and scavenges them. At low concentration the formation of 7-OH-CCA increases steadily, as under this condition the former property prevails. At increasing concentrations the scavenging ability progressively counter-balances the formation of HO \cdot and the highest ascorbate concentrations have no more effect on 7-OH-CCA formation. Note that a plateau is reached (and not the same shape curve as previously) because cumulative amounts of 7-OH-CCA (and not amounts of 7-OH-CCA at a given time) are measured in this case.

In the presence of A β a similar shape is obtained, but with much lower intensities. This is assigned to the scavenging ability of A β itself, which increases with the Cu(A β) concentration.

To probe the ability of ascorbate to protect the A β peptide, we have compared the degradation of the peptide after the same amount of HO \cdot was produced, but at different initial ascorbate concentrations, *i.e.* (i) at the end of the reaction with 1 mM ascorbate and (ii) after consumption of 1 mM ascorbate with the initial concentration of ascorbate at 10 mM (Fig. S11 \dagger). This should yield the same amount of HO \cdot produced, but in the second sample much more ascorbate was present to exert the antioxidant effect. Even under this condition, no rescue in A β degradation was observed (Table S2 \dagger). This indicated that although ascorbate can scavenge HO \cdot before it reacts with CCA, it is much less efficient in intercepting HO \cdot when reacting with the A β peptide itself. This could be explained by the fact that A β is linked to Cu ions and hence HO \cdot are produced in the vicinity of the peptide. In contrast, CCA is not bound to the metal centre and has to encounter HO \cdot by diffusion.

In the present communication, we have reported that ascorbate can have a pro- or an antioxidant effect concerning the initial rates of HO \cdot attack on biological targets, here modelled by the CCA compound. The approach proposed here in the AD context with Cu(A β) species as the ROS production catalyst, could apply to any other chemical system able to form ROS. This includes other Cu-amyloidogenic peptide species, as encountered in Parkinson's disease, prion diseases, *etc.*²⁵ In the present case, the "cross-over" point, *i.e.* the ascorbate concentration at which the rate of HO \cdot formation is maximal, was observed at about 0.7 mM for a range of 10–100 μ M Cu(A β). This means that below this concentration, ascorbate fuels the formation of HO \cdot that mainly reacts with CCA and the A β peptide. At higher concentrations, HO \cdot scavenging by ascorbate becomes more efficient and hence the rate of CCA oxidation slows down. Measuring the initial rate might be the most relevant

condition in a more biological environment, as systems tend to maintain a steady state in terms of ascorbate and dioxygen concentrations. Under such conditions, the “cross-over” point corresponds to the maximal rate of HO• escaping from Cu(Aβ), and hence to maximal damage to bio-molecules (proteins, DNA, lipids *etc.*) other than Aβ. At lower or higher ascorbate concentrations, less biomolecule damage would occur but for different reasons (weaker ROS production or higher scavenging activity, respectively). The situation for the Aβ peptide is different. Aβ scavenges the HO• due to its proximity with the place of ROS production and is oxidized regardless of ascorbate concentrations. Even very high ascorbate concentrations were not able to protect Aβ from oxidation, which is likely due to the proximity of the peptide residues to the HO• production site thus making it difficult for ascorbate to intercept the HO•. However, our *in vitro* studies could mainly be relevant for the surroundings of Cu(Aβ) complexes and the general situation *in vivo* under AD conditions could be more complex and the local prooxidant activity of ascorbate could be counteracted.

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Sacrificial antioxidants have been investigated as therapeutic compounds in several neurodegenerative diseases including AD.^{19,26} In contrast to ascorbate, such compounds are mainly HO• scavengers but can hardly induce the formation of ROS. In this context, our data indicate that ascorbate could be a “Dr Jekyll and Mr Hyde”²⁷ partner: (i) because ascorbate concentrations in the extracellular fluid in the brain are estimated to be 200–400 μM, the prooxidant regime is possible; (ii) in contrast, ascorbate concentrations in neurons are much higher (10 mM) leading to the antioxidant regime.³ However, even at much higher ascorbate concentration the anti-oxidant effect is incomplete as CCA can still trap HO•. This raises the question whether it is possible to reach high enough ascorbate or other sacrificial anti-oxidant¹ concentration *in vivo*, so that they could completely scavenge HO• produced by Cu(Aβ). Using compounds that can redox silence the Cu bound to Aβ (and hence stop ROS production in general) might be a more efficient strategy. Cu chelating agents that can bind and/or remove Cu ions from Aβ and redox silence them are among such compounds.^{28–31} Another possibility would be to use catalytic ROS scavengers, such as SOD mimics.^{32,33}

While high ascorbate concentrations could have an anti-oxidant effect for other biomolecules, they cannot prevent Aβ from oxidative damage. Whether this would be a beneficial or detrimental process depends on the toxicity of the oxidized Aβ, which is not clear yet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Di Domenico F, Barone E, Perluigi M, Butterfield DA. *Expert Rev Neurother.* 2015; 15:19. [PubMed: 25243342]
2. Sies H. *Eur J Biochem.* 1993; 215:213. [PubMed: 7688300]
3. Rice ME. *Trends Neurosci.* 2000; 23:209. [PubMed: 10782126]
4. Halliwell B. *J Neurochem.* 2006; 97:1634. [PubMed: 16805774]
5. Buettner GR, Jurkiewicz BA. *Radiat Res.* 1996; 145:532. [PubMed: 8619018]
6. Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J. *Nature.* 1998; 392:559. [PubMed: 9560150]
7. Carr A, Frei B. *FASEB J.* 1999; 13:1007. [PubMed: 10336883]
8. Miller LM, Wang Q, Telivala TP, Smith RJ, Lanzirotti Miklossy J. *J Struct Biol.* 2006; 155:30. [PubMed: 16325427]
9. Hureau C. *Coord Chem Rev.* 2012; 256:2164.
10. Hartter DE, Barnea A. *Synapses.* 1988; 2:412.
11. Kardos J, Kovács I, Hajós F, Kálmán M, Simonyi M. *Neurosci Lett.* 1989; 103:139. [PubMed: 2549468]
12. Bonda DJ, Wang X, Perry G, Nunomura A, Tabaton M, Zhu X, Smith MA. *Neuropharmacology.* 2010; 59:290. [PubMed: 20394761]
13. Smith DG, Cappai R, Barnham KJ. *Biochim Biophys Acta, Biomembr.* 2007; 1768:1976.
14. Manevich Y, Held KD, Biaglow JE. *Radiat Res.* 1997; 148:580. [PubMed: 9399704]
15. Hureau C, Faller P. *Biochimie.* 2009; 91:1212. [PubMed: 19332103]
16. Noël S, Perez F, Pedersen JT, Alies B, Ladeira S, Sayen S, Guillon E, Gras E, Hureau C. *J Inorg Biochem.* 2012; 117:322. [PubMed: 22819647]
17. Alies B, Sasaki I, Proux O, Sayen S, Guillon E, Faller P, Hureau C. *Chem Commun.* 2013; 49:1214.
18. Atrián-Blasco E, Cerrada E, Conte-Daban A, Testemale D, Faller P, Laguna M, Hureau C. *Metallomics.* 2015; 7:1229. [PubMed: 25926057]
19. Chassaing S, Collin F, Dorlet P, Gout J, Hureau C, Faller P. *Curr Top Med Chem.* 2013; 12:2573.
20. Kowalik-Jankowska T, Ruta M, Wi niewska K, Łankiewicz L, Dyba M. *J Inorg Biochem.* 2004; 98:940. [PubMed: 15149800]
21. Nadal RC, Rigby SEJ, Viles JH. *Biochemistry.* 2008; 47:11653. [PubMed: 18847222]
22. Balland V, Hureau C, Saveant J-M. *Proc Natl Acad Sci USA.* 2010; 107:17113. [PubMed: 20858730]
23. Cassagnes L-E, Hervé V, Nepveu F, Hureau C, Faller P, Collin F. *Angew Chem, Int Ed.* 2013; 52:11110.
24. Trujano-Ortiz LG, González FJ, Quintanar L. *Inorg Chem.* 2014; 54:4. [PubMed: 25521160]
25. Kozłowski H, Luczkowski M, Remelli M, Valensin D. *Coord Chem Rev.* 2012; 256:2129.
26. Persson T, Popescu BO, Cedazo-Minguez A. *Oxid Med Cell Longevity.* 2014; 2014
27. Stevenson, RL. *Strange case of Dr Jekyll and Mr Hyde.* Long-mans, Green, and Co; London: 1886.
28. Pithadia AS, Lim MH. *Curr Opin Chem Biol.* 2012; 16:67. [PubMed: 22366383]
29. Rodríguez-Rodríguez C, Telpoukhovskaia M, Orvig C. *Coord Chem Rev.* 2012; 256:2308.
30. Barnham KJ, Bush A. *Chem Soc Rev.* 2014; 43:627.
31. Savelieff MG, DeToma AS, Derrick JS, Lim MH. *Acc Chem Res.* 2014; 47:2475. [PubMed: 25080056]
32. Batinic-Haberle I, Tovmasyan A, Roberts ER, Vujaskovic Z, Leong KW, Spasojevic I. *Antioxid Redox Signaling.* 2014; 20:2372.
33. Batinic-Haberle I, Reboucas Julio S, Spasojevic I. *Antioxid Redox Signaling.* 2010; 13:877.

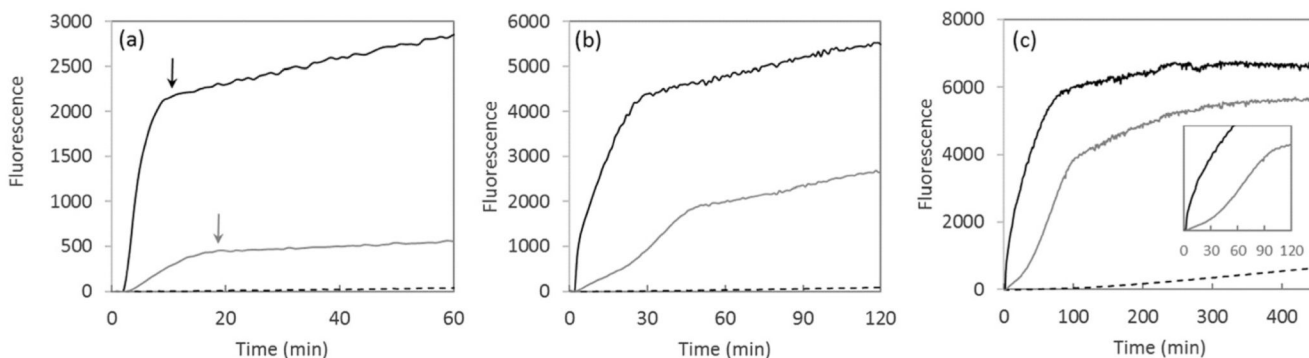


Fig. 1. Time dependence of HO[•] production measured by 7-OH-CCA fluorescence assay ($\lambda_{\text{excitation}} = 390 \text{ nm}$ and $\lambda_{\text{emission}} = 450 \text{ nm}$) for Cu (black curves), A β_{16} (dotted curves) and Cu-A β_{16} (grey curves) with ascorbate ((a) 0.5 mM, (b) 1 mM and (c) 5 mM). Phosphate-buffered solution (50 mM, pH 7.4) of CCA (0.5 mM), Cu (50 μM), either A β_{16} (60 μM) or no peptide. Experiments with only ascorbate give similar curves to A β_{16} with ascorbate (dotted curves). Arrows indicate the completion of ascorbate consumption as measured independently (Fig. S7[†]).

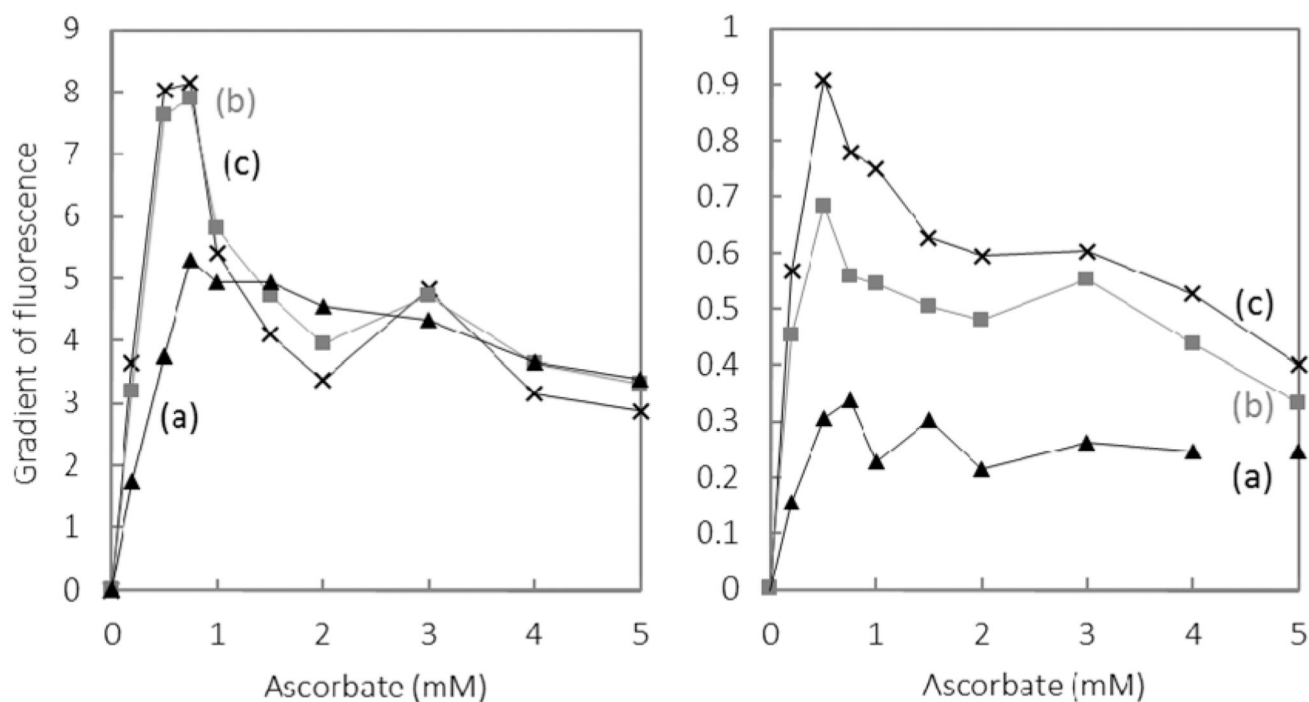
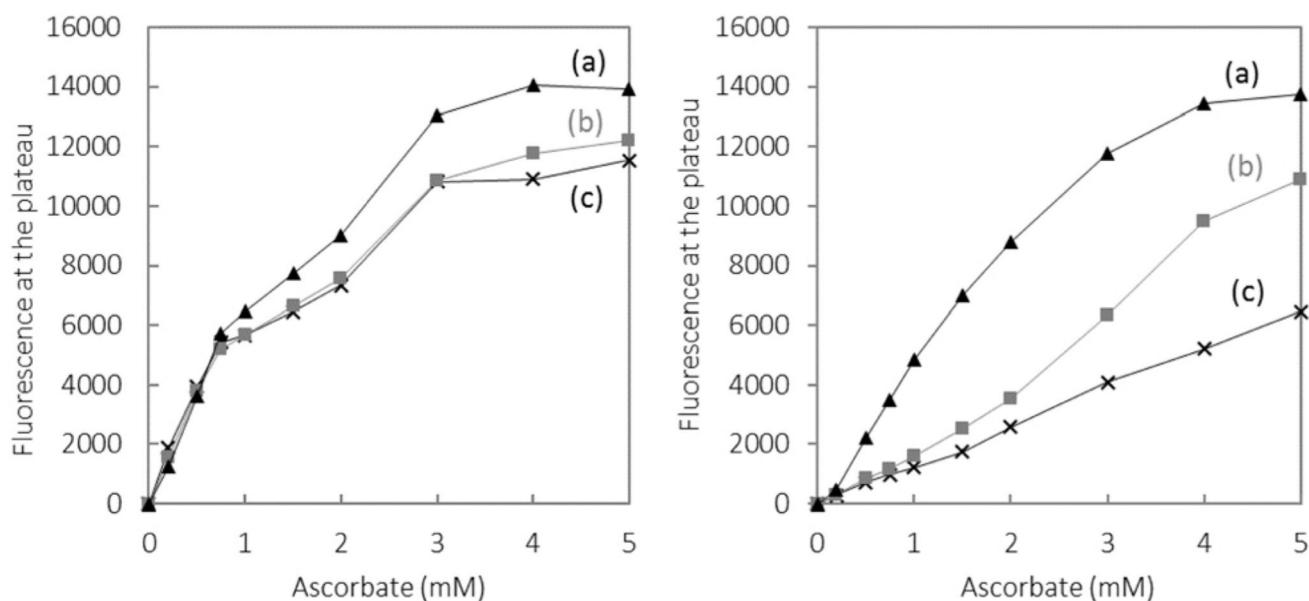
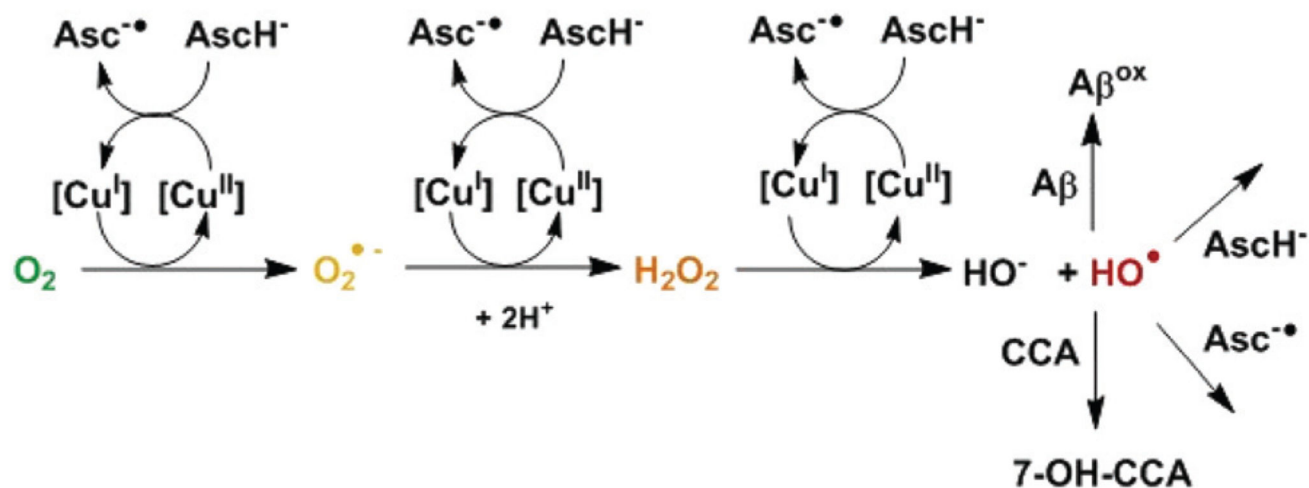


Fig. 2.

Initial kinetics of 7-OH-CCA fluorescence at 450 nm, reflecting the scavenging of HO[•] by CCA. Phosphate-buffered solution (50 mM, pH 7.4) of CCA (0.5 mM) with Cu (left panel, (a) 10 μM, (b) 50 μM, (c) 100 μM), or Cu-Aβ₁₆ (right panel, (a) 10–12 μM, (b) 50–60 μM, (c) 100–120 μM) and ascorbate (concentration between 0 and 5 mM).

**Fig. 3.**

Metal-catalyzed oxidation of CCA by HO[•]. Phosphate-buffered solution (50 mM, pH 7.4) of CCA (0.5 mM) with Cu (left panel, (a) 10 μM, (b) 50 μM, (c) 100 μM), or Cu-Aβ₁₆ (right panel, (a) 10–12 μM, (b) 50–60 μM, (c) 100–120 μM) and ascorbate (concentration between 0 and 5 mM). At the end of the ascorbate oxidation, POPSO buffer (pH 9.0, 0.4 M) is added to adjust the pH at 8.5. The fluorescence of 7-OH-CCA ($\lambda_{\text{excitation}} = 390 \text{ nm}$ and $\lambda_{\text{emission}} = 450 \text{ nm}$) is then measured.

**Scheme 1.**

Mechanism of HO^{\bullet} production from ascorbate and dioxygen catalyzed by copper. HO^{\bullet} can then react with different compounds, with CCA to form the fluorescent 7-OH-CCA, or with ascorbate/ascorbyl or with $A\beta$ in the case of Cu bound to $A\beta$. [Cu] corresponds to free or $A\beta$ -bound Cu.