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Copper-amyloid-β complex may catalyze peroxynitrite production in brain: evidence from molecular modeling

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Abstract

Rationalization of the origin of peroxynitrite-related damages in the brain of Alzheimer's disease (AD) patients linking to functional hyperemia, inexplicable on the basis of the accepted hydrogen peroxide catalytic route, is here provided by molecular modeling. The present theoretical work indeed strongly supports the facile occurrence of an Aβ-catalyzed generation of peroxynitrite in the brain, alternative to the already accepted H_2O_2-route, whenever ascorbate, dioxygen and nitric oxide are present near Cu-Aβ complexes without the necessity of generating short-lived superoxide ions.

The proposed route requires nitric oxide and dioxygen to be simultaneously present at sufficiently high concentrations near Cu-Aβ complexes, requirement which is frequently fulfilled in brain during functional hyperemia. Conversely, hydrogen peroxide would be produced during resting phases.

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1.Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders especially among the elderly [1]. The increasing life expectancy in modern societies is expected to amplify AD frequency in the near future so as to pose severe societal and economical problems. Nonetheless, more than 100 years after its discovery, the primary origin of AD still remains unclear and its diagnosis difficult to establish. For example, persons with severe cognitive decline may be unambiguously declared as AD patients only after post-mortem examination of their brains reveals clear signs of apoptosis connected with the presence of neurofibrillary tangles (NFT) and amyloid deposits [2]. NFT are aggregates of filaments of β-folded tau protein exhibiting hyperphosphorylation and oxidative modification [3]. Amyloid plaques, or "senile plaques", are insoluble aggregates of amyloid β (Aβ), a peptide composed of 39 to 43 amino acids [4]. Although there are direct correlations between these distinctive features and AD the mechanism(s) linking them to the degeneration and apoptosis of neurons is(are) not fully characterized [5].

A lot of experimental evidence indicates that oxidative stress is highly increased in the brains of AD patients near Aβ and NFTs [6-9]. Cells exhibit abnormally high amounts of oxidatively-modified proteins, lipids and DNA. Plaques and NFTs associated with the presence of redox-active metals such as copper [8,10,11] seem to induce severe oxidative stress conditions (amyloid hypothesis) [8,12]. Copper chelatants contribute to dissolving amyloid plaques [13] suggesting that copper ions are essential components of senile plaques [14-16]. Actually, Aβ coordinates copper(II) ions with an affinity as small as picomolar [17,18]. Furthermore, Aβ-copper complexes have been shown recently to catalyze the reduction of dioxygen into hydrogen peroxide [19,20].

Though ascorbate is generally presented as a main natural antioxidant (in fact, among the most abundant one in the brain, with concentrations ranging from 100-200 μM in the extracellular fluid, up to 10 mM in neurons [21,22]) it is also a strong reducing agent [23] prone to reduce Aβ-copper(II) complexes [24]. Cu(I) spontaneously adds dioxygen thereby opening a direct route to hydrogen peroxide and free radicals chains [25-26]. Hence, the copper/amyloid hypothesis offers an appealing mechanistic connection between amyloid plaques or NFTs and excessive oxidative stress in AD through the Aβ/copper-mediated formation of hydrogen peroxide (H_2O_2 route) [19,20,27].

Though attractive, such a view may be incomplete since it does not take into account that, at the concentrations of relevance, hydrogen peroxide is not a reactive species per se but needs

to be activated. This occurs via Fenton reaction to yield extremely reactive hydroxyl radicals able to react exergonically with almost any biological molecules [25,27,28]. Yet, this fierce reactivity impedes hydroxyl radicals to diffuse beyond a few angstroms from their source. Conversely, when not scavenged by catalases and peroxidases, hydrogen peroxide may act as a Trojan horse and diffuse to many cellular compartment and release hydroxyl radicals at whatever place where Fenton metal catalysts are present. To prevent this very fact, Fenton metal ions such as Fe(II) are not left free in living eukaryote cells but are tightly sequestrated in bionorganic proteic complexes, molecular clusters or by specific complex ligands. Hence, although certainly an important contributor, the H_2O_2-route seems hard to reconcile with every type of damages incurred by AD patients. For example, tyrosines are found to be nitrosylated [29-31] in the near vicinity of senile plaques, nitrosotyrosine concentrations being 8-times larger in the hippocampus and neocortical regions of AD brains than in age-matched populations. Similarly, lipid peroxidation [32] seems hardly reconcilable with the fact that free transition metals are not present in membranes. Conversely, such metabolites are compatible with the production of Reactive Nitrogen Species (RNS) in particular of peroxynitrite and its derivatives [14,33-46]. In this respect, it is noted that Cu/Zn superoxide dismutase, which prevents peroxynitrite toxicity, is over-expressed in AD patients though its overall activity seems decreased [47].

If peroxynitrite is formed in vitro through the diffusion-limited radical-radical coupling of superoxide ion and nitrogen monoxide [48], the aim of the present work is to demonstrate that, in brain, peroxynitrite may be formed without the necessity of generating short-lived superoxide ions. In particular we wish to proof that as soon as ascorbate, dioxygen and nitric oxide are simultaneously present near copper-amyloid β complexes, peroxynitrite may be catalytically produced by a route competitive to the H_2O_2 one.

Since, to the best of our knowledge, Aβ neurotoxicity has not been investigated in the presence of higher than nanomolar basal concentrations of nitrogen monoxide, in order to test and validate the above concept we will rely on quantum chemical calculations, rooted on Density Functional Theory, aimed at quantitatively assessing the mechanism and the feasibility, under physiological conditions, of a Cu-Aβ-driven catalytic cycle leading to peroxynitrite formation.

The paper is structured as follows: after a description of the computational protocol applied (section 2), the catalytic cycle related to the Cu-Aβ catalyzed peroxynitrite formation is

detailed in section 3. Finally, some general conclusions, also justifying the possible occurrence of these reactions under physiological conditions, are given in Section 4.

2.Computational details

All calculations were performed with the Gaussian 03 program [49]. A two-layers ONIOM approach [50] was used to model the Aβ-Cu system. A detail description of the high or low level parts as well as the chemical model used to represent the Aβ-Cu system is given in Section 3. The high-level layer (QM) was described at DFT level using the PBE0 [51] exchange-correlation functional and a double zeta quality basis set (lanl2dz), while the low-level layer was calculated at the MM level using the Universal Force Field. Ascorbate, molecular oxygen and nitric oxide were included in the high-level layer that is at DFT level. All structures were fully optimized and subsequent frequency calculations were carried out in order to confirm their nature as minima or transition states. Single-point energy calculations including solvent effects (aqueous environment) via a polarizable continuum model (C-PCM) [52] were successively performed.

In order to obtain a starting low-energy conformation of the Aβ-Cu complex to be used for the QM/MM calculations, classical molecular dynamics simulated annealing calculations were carried out using the AMBER force field. The whole reaction mechanism was analyzed considering that the peptide backbone conformation, although fully optimized for each intermediate analyzed, was preserved along the reaction path except around the copper center. This hypothesis, viz., that reactivity at the catalytic site was faster than any global conformational change of the peptide backbone, appears pertinent regarding the steric constraints provoked by chelation of the Cu(II) or Cu(I) center on the protein structure [18,53,54].

Finally, in order to better compare with previous calculations [55] based on the use of a minimal peptide model and relying on a different coordination sphere of the Cu atom, the catalytic cycle obtained with this minimal model is also reported in Supporting Information.

3. Aβ-copper catalyzed peroxynitrite formation : the catalytic cycle

The reactivity of the Aβ-copper complex with dioxygen has been extensively investigated in recent years owing to the involvement of both species in the amyloid H_2O_2-route [19,20,24,55,56]. Several reports have stressed the importance of the facile adjustability of the 3D Aβ structure which may then adapt to coordinate Cu(II) and Cu(I) centers [56-60]. Recent

EPR experiments [61-63] stressed the importance of residues Asp1, Ala2, His6, His13 and His14 in promoting such uncommon ability to meet the dissimilar requirements of Cu(II) and Cu(I) coordination shells. The presence, depending on pH, of two coordination modes, called component I and component II, has been suggested [58,63] with component I prevailing at pH 6.6 and component II at pH 8.6. Since only physiological pH ranges are of interest in this study, a copper-(II) coordination following component I (figure 1) was thus here considered for the theoretical modeling. In this case, two histidines, His6 and His13 (or His14[58,61]), the NH_2 terminus and the carbonyl group of Asp1 fill the four equatorial positions while the Asp1 carboxylate side chain fits in the axial position so as to offer a stable pyramidal coordination with a square planar base around copper(II) centers (as schematically depicted for species ***R0*** in figures 1-3).

Most experimental studies aimed to validate the H_2O_2-route relied on a commercially available truncated peptide, Aβ16, composed of the first 16 amino acids of the full protein. This highly soluble system is commonly accepted as a reliable experimental model of the full protein [60,63]. In the present theoretical work, a model of comprising the first 14 amino acids was used in order to accurately simulate the peptide sequence of relevance for copper chelation [18], the residues and molecules directly linked to the Cu atoms being treated at high level (DFT) while the rest being described at MM level, as depicted in figure 2 and supporting information.

The catalytic cycle yielding to the peroxynitrite formation computed using the Aβ14 model catalyst resulting from our calculation is schematically represented in figure 3 together with a sketch of the structures of all its relevant intermediates (***R1-R7***).

The computed relative free energies of all intermediates are reported in figure 4. Interestingly, these energies are below 17 kcal/mol, thus basically allowing this mechanism at physiological conditions of temperature.

Looking more in details the structure of the intermediate involved it can be noticed that complex ***R1*** follows from a rearrangement of ***R0*** which allows the ascorbate coordination reinforced by a hydrogen bond created with the Asp1 carboxylate. The strong affinity of Cu(II)/Aβ complexes for ascorbate, experimentally observed [64], is thus confirmed by the present calculations. The ***R1*** intermediate spontaneously evolves through a barrierless intramolecular reduction of the Cu(II) center by the ascorbate ligand associated with a proton transfer from the ascorbate hydroxyl group to the Asp1 carboxylate, which is thus released by the copper center to form the stable ***R2*** intermediate.

The computed spin density map associated to ***R2*** (reported in Supporting Information in Figure SI.1) clearly show how the spin density is fully localized on the ascorbate, thus proving that an electron is actually transferred from the ascorbate to the copper(II) center when going from ***R1*** to ***R2***.

The Cu(I) center thus formed could then react endothermically with dioxygen affording the formation of complex ***R3***. ***R3*** has two unpaired electrons and its spin density map (reported in Supporting Information in Figure SI.2) shows that its electronic structure can actually be represented as mesomeric form between the formal Cu(II)-($O_2^{\bullet -}$) and Cu(I)-($^3O_2$) ones.

Although the formation of *R3* is endothermic by 17.2 kcal/mol, this value does not appear incompatible with a bio-catalytic cycle [65,66] though its formation is the rate-determining step of the whole catalytic sequence.

More precisely, it is agreed that this very step is also essential to the, already reported in literature, H_2O_2-route, the species ***R3*** leading ultimately to the release of H_2O_2 [19,20,27].

Nonetheless, provided nitrogen monoxide is present at sufficient concentration, we can figure a facile departure from the previously reported catalytic H_2O_2-route through a sequence of steps initiated by the attachment of NO.

Indeed the present calculations show the possibility of an exothermic and barrierless attachment of NO to the unbound oxygen atom of ***R3***. This disrupts the H_2O_2-route by producing a Cu-coordinated peroxynitrite moiety (***R4***).

The structural flexibility of the Asp1 residue in ***R4*** allows the formation of a stabilizing hydrogen bond between its carboxylic acid terminal and the copper-bound oxygen atom of the peroxynitrite ligand (***R5***). A second slight conformational change allows protonation of the peroxynitrite ligand and its partial release, accompanied by re-coordination of the carboxylate onto the copper(II) center thus affording intermediate ***R6***. Interestingly, all these catalytic steps are exothermic. Peroxynitrous acid may then be released yielding complex ***R7***.

Complex ***R7*** can then undergo a facile reduction (or exchange) of its oxidized ascorbate ligand by a free ascorbate thus closing the catalytic cycle by regenerating the active catalyst ***R1***.

The present calculations thus proof that it is possible to conceive the formation of peroxynitrous acid as an alternative to the H_2O_2 route starting from the same catalyst.

4. Discussion and Conclusions

The present theoretical results offer strong evidence for the facile occurrence of an Aβ-catalyzed generation of peroxynitrite whenever ascorbate, dioxygen and nitric oxide are

present near Cu-Aβ complexes. The corresponding catalytic cycle (peroxynitrite-route, figure 3) is branched competitively onto that leading to H_2O_2 [20] at the level of intermediate ***R3*** (see SI). Hence, rather than disputing the presently accepted role of the H_2O_2-route in AD, the present mechanism helps to rationalize certain aspects of Aβ toxicity which could not be readily explained by the hydrogen peroxide hypothesis alone. Though, deviating from the H_2O_2-route into the ONOOH one requires that nitric oxide and dioxygen are simultaneously present at sufficiently high concentrations near Cu-Aβ complexes.

In this respect, it should be recalled that, in active brain areas, high fluxes of nitrogen monoxide are paired to high fluxes of dioxygen through the crucial and ubiquitous mechanism of functional hyperemia [67-70]. Active neurons claim the delivery of the high dioxygen doses required by their intense metabolism from nearby (<50 μm) blood capillaries by emitting high concentrations (in the half-micromolar range) of nitric oxide [69,70]. Hence, it is reasonable to consider that dioxygen and nitrogen monoxide are frequently present simultaneously at higher-than-normal concentrations near active neurons. Interestingly, the classical framework, the formation of peroxynitrite requires the simultaneous presence of nitric oxide and superoxide ion. While the latter is readily formed by the reduction of dioxygen, its usual reactivity under biological conditions provides H_2O_2 through its fast spontaneous and even faster SOD-catalyzed disproportionation [71]. Hence, superoxide ions cannot diffuse over long distances from their source while maintaining significant concentrations. Though, this is not a problem for formation of peroxynitrite within cells where superoxide ion is produced by NADPH-oxidases while NO-synthases simultaneously produce nitric oxide [72,73]. Thus, only a catalytic route of formation of peroxynitrite making use of nitrogen monoxide and dioxygen such as the one proposed in the present study, seems to be suitable under physiological conditions in brain.

Once formed, the peroxynitrite anion, ONO_2^-, is rather stable at low concentrations [34,36]. The pK_a of its protonated form is 6.8 [34] thus about half of it is in its acidic form (peroxynitrous acid) at physiological pH. The trans isomer [74] of the acidic form is lipophilic and may thus easily cross, or even rest in, bilipidic membranes [38,75] allowing a facile distribution of peroxynitrite into all cellular compartments and membranes. In aqueous environments at physiological pH the acid form decomposes through an overall bimolecular process to yield nitrite and/or nitrate ions [36]. Conversely, in apolar environments such as cell membranes or some protein sections, the protonated trans isomer may spontaneously decompose unimolecularly leading to a hydroxyl radical and NO_2 [43].

Hence, peroxynitrite may be a fierce precursor of radical chains leading to the peroxidation and/or nitrosylation of lipids and proteins. For example, peroxynitrite is reported to damage mitochondria by inducing permeability of their membrane [76-79] provoking a disruption of their oxidative stress balance and further leaking of radical species leading ultimately to apoptosis.

This corpus of experimental observations hints that peroxynitrite may be an essential precursor of several damages observed in the brain of AD patients which cannot be readily explained by the H_2O_2-route only hypothesis [14,29-32].

Following our results, bursts of peroxynitrite may be produced as a direct consequence of neuronal activity near clusters of Cu-Aβ complexes. Conversely, hydrogen peroxide would be produced during resting phases.

Therefore, this work provides a straightforward mechanistic rationale linking the origin of peroxynitrite-related damages in the brain of AD patients [29-31] to functional hyperemia.

The energetics of the Cu/Aβ-catalyzed peroxynitrite-route obviously rest on the assumption that the present Aβ14 model accurately represents the flexible copper-chelating functions of the peptidic backbone of the natural Aβ protein, but we believe that it is a proper approximation [18] and convincing enough to stimulate new experimental investigations in the presence of above than normal concentrations of nitrogen monoxide or of its precursors so as to ascertain directly whether peroxynitrite can be generated competitively with hydrogen peroxide during functional hyperemia events.

Supporting Information

Amino-acids sequence, Spin density map computed for intermediates ***R2*** and ***R3***, complete catalytic cycle obtained with a small model, complete reference 49.

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Figure captions

Figure 1. Schematic representation of Copper coordination sphere in Cu(II)-Aβ complex (His stands for histidine).

Figure 2. Structure of complex ***R1*** as optimized within the framework of ONIOM model. The structure of interest for this study, computed at DFT level, is highlighted.

Figure 3. Catalytic cycle of Aβ/Copper-catalyzed formation of peroxynitrous acid with indication of the optimized structures of intermediates ***R1-R7*** (Asc stands for ascorbic acid).

Figure 4. Relative Gibbs energies of the intermediates involved in the catalytic cycle of Aβ/Copper catalyzed formation of peroxynitrous acid within the framework of ONIOM model.

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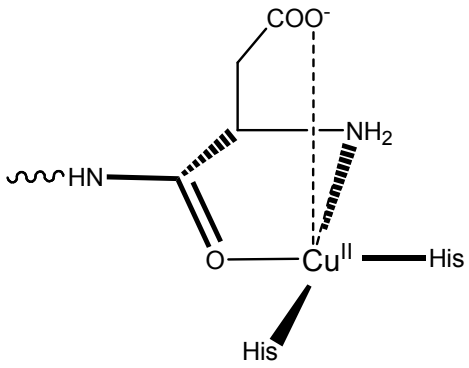

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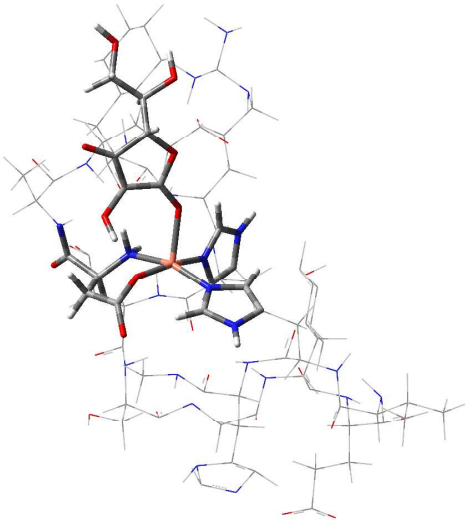

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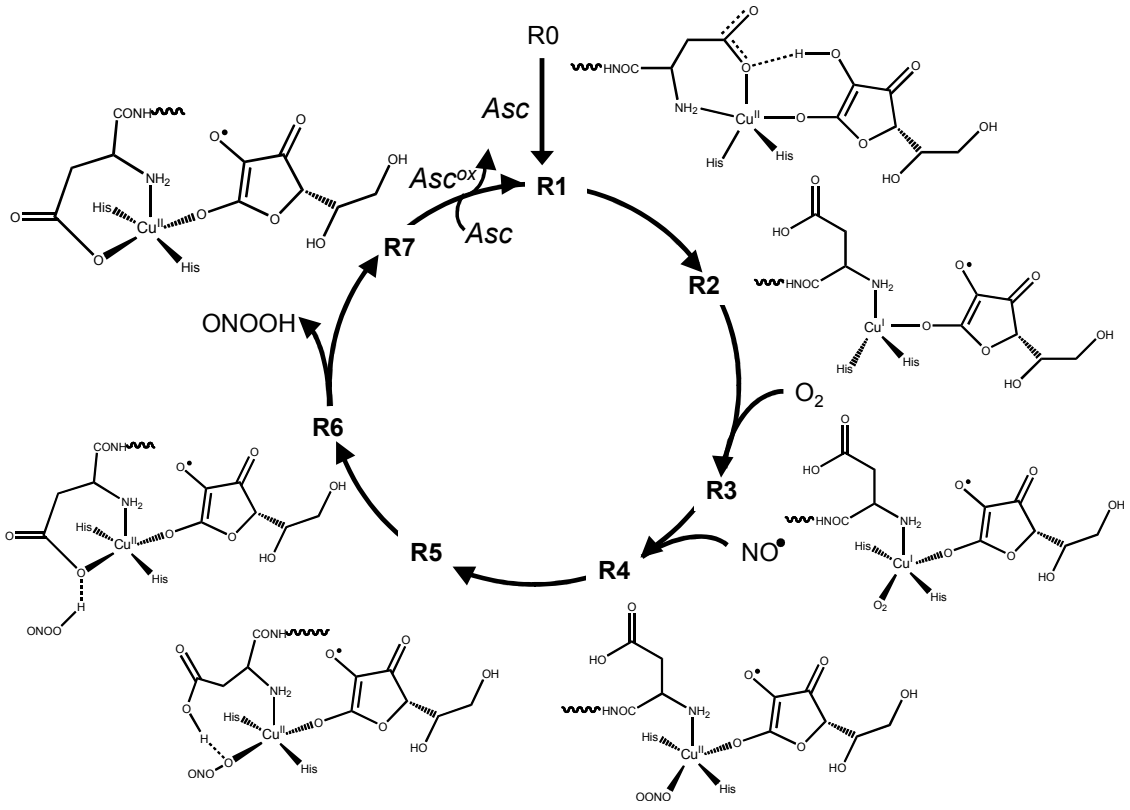

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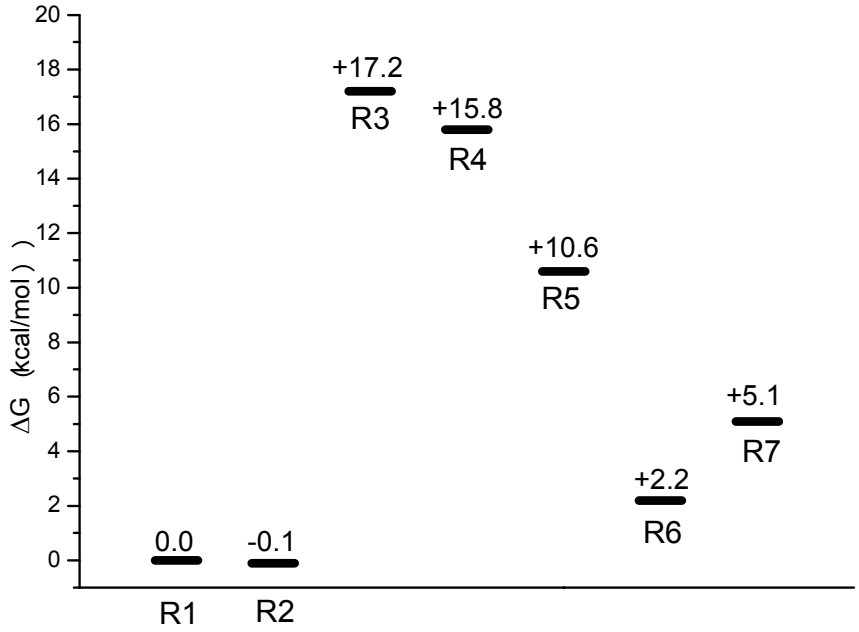

Table of contents entry

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