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Copper-Aβ Peptides and Oxidation of Catecholic Substrates: Reactivity and Endogenous Peptide Damage

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Abstract: The oxidative reactivity of copper complexes with Aβ peptides 1–16 and 1–28 (Aβ16 and Aβ28) against dopamine and related catechols under physiological conditions has been investigated in parallel with the competitive oxidative modification undergone by the peptides. It was found that both Aβ16 and Aβ28 markedly increase the oxidative reactivity of copper(II) towards the catechol compounds, up to a molar ratio of about 4:1 of peptide/copper(II). Copper redox cycling during the catalytic activity induces the competitive modification of the peptide at selected amino acid residues. The main modifications consist of oxidation of His13/14 to 2-oxohistidine and Phe19/20 to *ortho*-tyrosine, and the formation of a covalent His6-catechol adduct. Competition by the endogenous peptide is rather efficient, as approximately one peptide molecule is oxidized every 10 molecules of 4-methylcatechol.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative condition that results in synaptic failure and neuronal death. Characteristic pathological hallmarks in the brains of those suffering from the disease include the presence of extracellular senile plaques, intracellular neurofibrillary tangles, and altered levels of neurotransmitters.[1] Amyloid plaques are composed of aggregated amyloid-β (Aβ) peptides, of 39 to 43 residues in length, that derive from a membrane-bound amyloid-β precursor protein (APP).[2] According to the amyloid cascade hypothesis,[3,4] an increase in Aβ production and its accumulation leads to the formation of oligomers, protofibrils and, in a sequence of steps, fibrils, the main constituent of amyloid plaques.[5] However, a number of studies suggest that small soluble, oligomeric forms of Aβ are the most toxic species, rather than more extensively aggregated fibrils or protofibrils.[3,5]

Metal ions have been shown to play a critical role in Aβ neurotoxicity, thus prompting an intense investigation into the formation of metal–Aβ complexes.[6] In fact, remarkably high concentrations of several transition-metal ions, mainly Cu^{2+}, Zn^{2+}, and Fe^{3+}, were found in amyloid deposits of AD-affected brains. In particular, the finding of high copper concentration (400 μM),[7] compared to the normal brain extracellular concentration of 0.2–1.7 μM,[8] suggests that its complexation with Aβ is linked to ROS-related neurotoxicity, Aβ aggregation, and formation of amyloid plaques.[9] In addition, both in vitro and in vivo studies revealed that copper (and zinc) may mediate the aggregation of Aβ peptides,[10] and treatment of AD brain extracts with metal chelators can dissolve these deposits.[11]

Aβ binds Cu^{2+} with high affinity ($K_d = 10^{-10}$–10^{-11} M)[12] in the hydrophilic N-terminal portion (residues 1–16, DAEFRHDSGYEVHHNK), in which His residues (His6, His13, and His14) are important for Cu^{2+} coordination and Cu^{2+}-mediated Aβ aggregation.[13] Aβ is a disordered peptide that can adopt a multitude of different coordination sites with similar energies and, indeed, two different Aβ coordination models for Cu^{2+} coexist around physiological pH.[14–16] In the first coordination mode, Cu^{2+} binding involves the $-NH_2$ terminal group, the adjacent carbonyl group function from the Asp1-Ala2 peptide bond, the imidazole ring of His6, and the imidazole ring of His13 or His14.[15] In the second coordination mode, copper is bound to the deprotonated Asp1-Ala2 amide bond, the N-terminal amine, the carbonyl group from the Ala2-Glu3 peptide bond, and an imidazole ring of either His6, His13, or His14.[15] Also, Cu^{+} plays an important role in the interaction with Aβ and, in fact, the brain is a rather reducing environment both in the intracellular and the extracellular media. Furthermore, cultured neurons are sensitive to Cu–Aβ complex-catalyzed production of ROS, which involves redox cycling between Cu^{+} and Cu^{2+}.[14] The electron-donation required for Aβ-mediated Cu^{2+} reduction can involve either internal Aβ amino acid side chains or an external reductant.[8] In the reduced form, copper binds only to His13 and His14 in a linear, two-coordinate structure.[17]

The exact role of copper binding to Aβ peptides is far from being completely elucidated and a controversy still exists regarding the pro-oxidant[18–20] versus antioxidant[21–24] effects of this complexation.

In a previous study,[21] we found that the formation of the Cu–Aβ complexes, both [Cu^{2+}–Aβ16] and [Cu^{2+}–Aβ28], reduced the ability of free copper to catalyze the oxidation of phenols and catechols. However, the experiments were performed in the presence of the chromophoric reagent 3-

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methyl-2-benzothiazolinone hydrazone (MBTH), as a trap for the quinone products,[19,25] but it was found that MBTH is not redox innocent and it also participates in the reaction as a reducing agent for copper(II).[26] The radical scavenging effect of Aβ in this system is in line with the previously reported hydroxyl radical scavenging property of the peptide.[22,23]

This evidence shows that the choice of experimental conditions is crucial in studies that aim at reproducing in vitro the physiopathological conditions of the cellular environment. In this context, among the potential external substrates the most important, in terms of redox reactivity, are neurotransmitter catecholamines, such as dopamine (DA), and their metabolites, due to their presence in many brain areas, and in particular in *substantia nigra* neurons.[27] These molecules bear a rich redox chemistry, potentially acting as one- and two-electron reductants, and their oxidation products are very electrophilic.[28] However, oxidation of catecholamines is a complex process because of the high reactivity of their primary quinone product,[28] which leads to rapid formation of insoluble melanic aggregates, thus making difficult the conduction and interpretation of kinetic experiments.[29] Therefore, in this paper, besides DA, we chose to investigate the oxidation of two catechol compounds lacking functional groups in the aromatic ring side chain, that is, 4-methylcatechol (MC) and 3,5-di-*tert*-butylcatechol (DTBC), as substrates for catecholase activity in biomimetic studies.[21,26] It is also important to take into account that another possible target of ROS generated by copper redox cycling is Aβ itself, since there is ample evidence of endogenous oxidative damage of the peptide by metal-catalyzed oxidation (MCO).[30–32] In general, the presence of oxidative damage on neuronal proteins is evidence of a link between oxidative stress and AD.[30] Herein, we report on the reactivity of the copper complexes with the N-terminal peptides Aβ16 (^{1}DAEFRHDSGYEVHHNK^{16}-NH_2) and Aβ28 (^{1}DAEFRHDSGYEVHHNKLVFFAEDVGSNK^{28}-NH_2) towards catecholic substrates and the competitive endogenous oxidative modification undergone by the peptide backbone.

Results and Discussion

The oxidation of DA by Cu^{2+} in 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) buffer at pH 7.4 was studied in the presence of increasing amounts of either Aβ16 or Aβ28, in parallel experiments. Dopamine oxidation was monitored by UV/vis spectroscopy through the development of the absorption band of dopaminochrome (DAC) at 475 nm. This product accumulates in the initial stages of the reaction.[29,33] After approximately 20 min of reaction time, other absorption bands at about 405 and 620 nm appear (Figure S1 C, Supporting Information) and a general increase of absorption over the whole wavelength range, due to the formation of melanin, is observed. Within about 1 h, formation of insoluble melanic products can be noted; therefore, we focused our attention on the first 30 min of reaction. Under these conditions, DA autoxidation is not negligible and needs to be subtracted from the kinetic profile. Figure 1 shows that DA (3 mM) oxidation catalyzed by Cu^{2+} (25 μM) is strongly promoted by the addition of

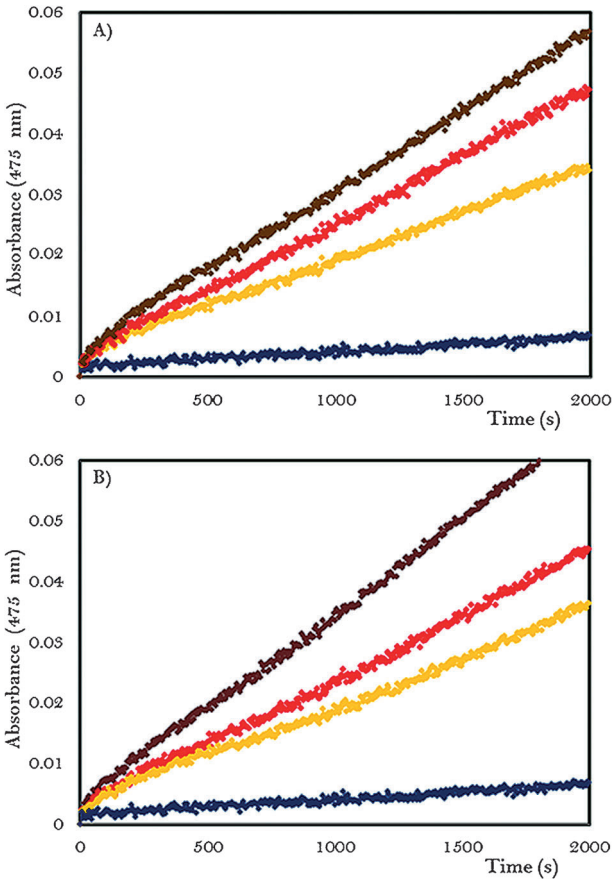

Figure 1. Kinetic profiles of DA (3 mM) oxidation with time, in 50 mM Hepes buffer solution at pH 7.4 and 25 °C in the presence of Cu^{2+} (25 μM) (blue) and with addition of A) Aβ16 (1 equiv: yellow; 2 equiv: red; 4 equiv: brown); B) Aβ28 (1 equiv: yellow; 2 equiv: red; 4 equiv: brown). In both cases, the absorption profiles were corrected for DA autoxidation under the same conditions.

either Aβ16 or Aβ28. In both cases, the DA oxidation rate increases up to a 4:1 Aβ/Cu^{2+} ratio, with a slightly stronger effect in the presence of Aβ28. The reaction rates increase by saturating the solution with dioxygen instead of air (Figure S2, Supporting Information), indicating that the rate-determining step involves O_2. The low [O_2] in Hepes buffer (240 μM) may be partly responsible for the slow reaction with oxygen. Notwithstanding the low concentration of O_2, which is substoichiometric with respect to DA, the peculiar kinetic profile is not associated to O_2 consumption, since only a very low fraction of DA is converted into DAC during the observed reaction times (Figure S3, Supporting Information).

Since DA oxidation is rather slow and occurs with formation of a mixture of products and precipitate, this substrate is not suitable to perform a more detailed kinetic study. Therefore, the oxidation of the more reactive MC (with a lower semiquinone/catechol redox potential)[26] can be conveniently studied as a model substrate of DA. Oxidation of MC (3 mM) promoted by Cu^{2+} (25 μM) and Aβ peptides at pH 7.4 (50 mM Hepes buffer) proceeds with a biphasic behavior where an initial step, concluded in about 100 s, is followed by a second linear phase (Figure 2). As for DA, also with MC the two-step kinetic profile

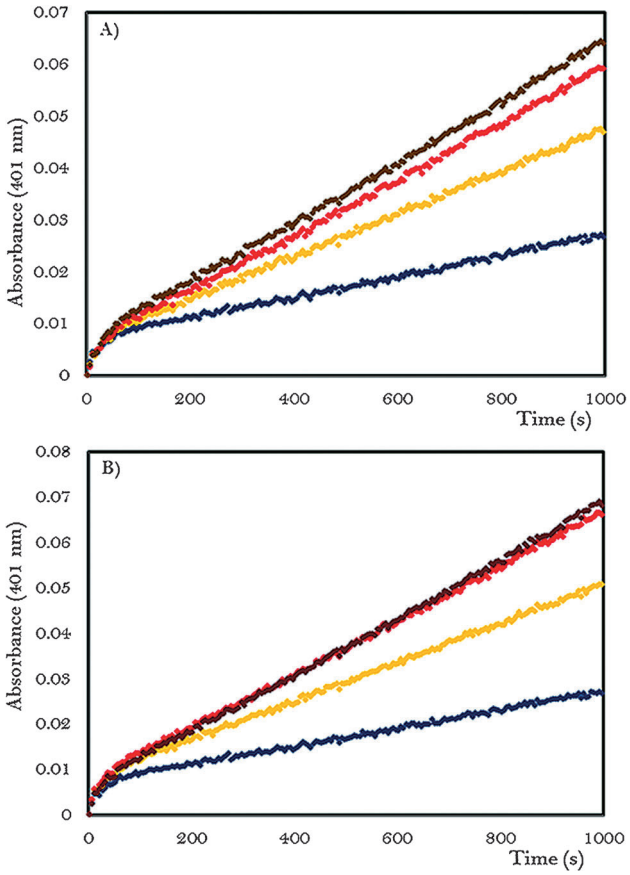

Figure 2. Kinetic profiles of MC (3 mM) oxidation with time, in 50 mM Hepes buffer solution at pH 7.4 and 25 °C in the presence of Cu^{2+} (25 μM) (blue) and with addition of A) Aβ16 (1 equiv: yellow; 2 equiv: red; 4 equiv: brown); B) Aβ28 (1 equiv: yellow; 2 equiv: red; 4 equiv: brown). In both cases, the absorption profiles were corrected for MC autoxidation under the same conditions.

is not associated to low $[O_2]$ in solution. The biphasic behavior can be noted also for DA oxidation but the presence of several absorbing species in solution makes the observation less clear. During the time course of MC oxidation, a shift of the developing absorption bands occurs from the initial value of 401 nm, due to the quinone (MQ, $\varepsilon = 1550\ M^{-1}cm^{-1}$), to higher wavelengths, which is related to the formation of the quinone addition product with catechol.[34] The rate of both steps increases by saturating the solution with dioxygen instead of air (data not shown), indicating that the slow step of the reaction in both phases involves the formation of a Cu/O_2 intermediate [Eq. (4)]. Moreover, by increasing the peptide concentration, the rate of both steps is accelerated reaching a saturation value at $A\beta/Cu^{2+}$ ratio of 2:1 or slightly above (Figure 2). Again, a slightly stronger effect is observed in the presence of Aβ28.

We can therefore propose the following mechanism, in which the rate-limiting step is the formation of some reactive copper–dioxygen intermediate [Eq. (4)]. This species is generically indicated as Cu–Aβ–substrate–O_2* because its nature has not been characterized yet.

$$Cu^{2+} + A\beta \rightleftharpoons Cu^{2+} - A\beta \quad (1)$$

$$Cu^{2+} - A\beta + MC \rightarrow Cu^{+} - A\beta + MC^{\bullet+} \quad (2)$$

$$Cu^{+} - A\beta + MC \rightleftharpoons Cu^{+} - A\beta - MC \quad (3)$$

$$Cu^{+} - A\beta\text{-}MC + O_2 \rightarrow Cu - A\beta - MC - O_2{}^{*} \quad (4)$$

$$Cu - A\beta - MC - O_2{}^{*} \rightarrow\rightarrow Cu^{2+} - A\beta + MC^{\bullet+} + O_2{}^{-} \quad (5)$$

According to the mechanism proposed, the biphasic behavior is not associated with a change in the rate-determining step of the reaction but in the product accumulated. The 4-methylcatechol semiquinone, $MC^{\bullet+}$, disproportionates to MC and MQ. In the presence of excess MC, an addition product to MQ is formed:

$$2\,MC^{\bullet+} \rightarrow MC + MQ \quad (6)$$

$$MC + MQ \rightarrow \text{addition products} \quad (7)$$

Therefore, in the initial phase of the reaction, MQ is formed and it accumulates in solution, but after about 100 s it is consumed by the formation of the addition products, according to Equation (7). These addition products absorb at higher wavelengths, giving rise to a reduction in the slope shown in Figure 2. This reaction path describes the biphasic kinetic trace observed. It is interesting to note that the reaction of Equation (2) proceeds in spite of the slightly larger one-electron standard reduction potential of the $MC^{\bullet+}$/MC couple[26] with respect to those of Cu^{2+}–Aβ/Cu^{+}–Aβ peptides.[35] This happens because the semiquinone species does not accumulate in solution but is consumed by the reaction in Equation (6). It is likely that also a Fenton-type mechanism, generating ROS, contributes to the catalytic oxidation of MC promoted by the copper complex of Aβ peptides.

DTBC is a widely used substrate in biomimetic studies of catecholase activity. Due to the low solubility of DTBC in water, the oxidation of this substrate is usually studied in organic solvents.[36] When the Cu^{2+}-mediated DTBC oxidation was carried out in a mixed methanol/aqueous Hepes buffer 50 mM (80:20 *v/v*) at pH 7.4, the reaction was completely quenched by the addition of two equivalents of Aβ16 (see Figure 3) or Aβ28 peptides (data not shown). This behavior was previously noted,[21] and suggests that solvent also plays an important role in the reaction. For this reason, we performed the DA and MC oxidation experiments in the same methanol/Hepes buffer mixture. In the case of the Cu^{2+}-promoted DA oxidation, the addition of Aβ16 increases the oxidation rate but to a minor extent with respect to aqueous buffer (Figure S1A, Supporting Information). It is also worth noting that the DA oligomerization process observed in aqueous buffer (Figure S1 C, Supporting Information) is suppressed in the mixed organic medium, in which only the absorption band of dopaminochrome at 475 nm can be observed (Figure S1 B, Supporting Information).

The Cu^{2+}-promoted MC oxidation in the methanol/Hepes buffer mixture exhibits similar behavior, as the reaction rate is progressively reduced when increasing amounts of Aβ16 are

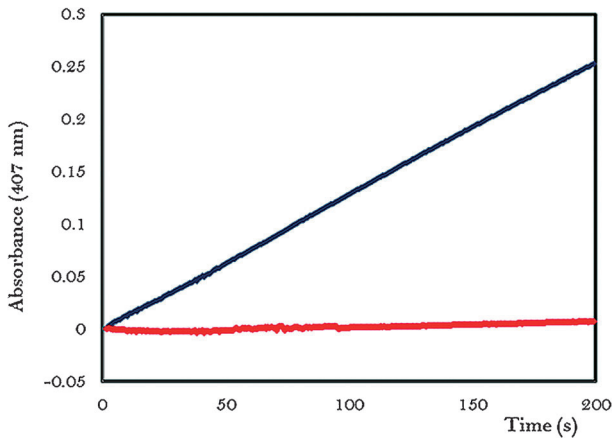

Figure 3. Kinetic traces of absorbance at 407 nm versus time for the oxidation of DTBC (3 mM) in MeOH-50 mM Hepes buffer (80:20 *v/v*) at pH 7.4 and 25 °C in the presence of free Cu^{2+} (25 μM) (blue trace), and Cu^{2+} (25 μM) and Aβ16 (50 μM) (red trace).

added (Figure S4, Supporting Information). Also in this case, the spectral profiles during the reactions are quite different in Hepes buffer versus methanol/Hepes buffer mixture. The presence of alcohol, in fact, favors the accumulation of MQ (absorption band at 401 nm, Figure S4B, Supporting Information), preventing the subsequent reactions observed in aqueous buffer (Figure S4C, Supporting Information). These results suggest that methanol may stabilize the copper(I) redox state in the presence of Aβ16. At the same time, a less polar solvent disfavors deprotonation of the catechol hydroxyl group and change of the redox potential of the species involved. The former effect prevents coordination of DTBC or MC to the [Cu^{+}–Aβ16] intermediate, shifting to the left the pre-equilibrium reaction in Equation (3) above. In addition, an increase of the substrate redox potential will make substrate oxidation more difficult. The spectra recorded over reaction time in a methanol/buffer mixture (Figures S1B and S4B, Supporting Information) compared to the same situation in the absence of methanol (Figures S1C and S4C, Supporting Information) seem to further confirm this hypothesis, because the reduced deprotonation of catechol in MeOH inhibits the nucleophilic attack to the quinone formed and thus the formation of condensation products is easily detectable in aqueous solvent.

In the case of DA, the catechol deprotonation is favored by the positive charge of the protonated amine group in the side chain at physiological pH ($pK_a \approx 9$).[37] Therefore, coordination of DA to [Cu^{+}–Aβ16] is favored. In addition, the oxidation of DA in MeOH is faster than in aqueous buffer, also because the concentration of dioxygen in the MeOH containing solvent increases (from 240 μM in aqueous buffer to 320 μM in the mixed solvent). In conclusion, the peptide stabilizes mostly the copper(I) form and the oxidation of the substrate greatly depends upon the conditions of the reaction.

The absorbance changes with time at 500 nm observed in the previous study using MBTH[21] show a biphasic behavior, with an initial fast phase followed by a slower phase, with a linear increase of the absorbance with time. The first step is stoichiometric with respect to copper concentration and its rate did not change by saturating the solution with pure oxygen. On the other hand, the second step was oxygen-concentration dependent and decreased by the addition of peptide. These results suggest that copper(II) is reduced by the catechol, according to the reaction in Equation (2), giving rise to the formation of MQ and thus to the MBTH–MQ adduct in the first, oxygen-independent phase. Then, it is also possible that MBTH competes with MC for coordination to [Cu^{+}–Aβ28] or [Cu^{+}–Aβ16] [Eq. (3)], decreasing the accumulation of the Cu^{+}–Aβ–MC adduct in the pre-equilibrium step and, consequently, the rate of the oxygen-dependent determining step of the reaction [Eq. (4)].

The reactivity described so far could have important consequences not only for oxidation of specific targets, such as DA and related catecholic substrates, but also for nonspecific oxidation reactions promoted by Cu^{I}–Aβ species generated during turnover. Unlike copper enzymes that activate oxygen for specific reactions,[38] Cu–Aβ complexes generate Cu/O_2 species capable of nonselective oxidations. This behavior is due to Fenton's reaction yielding harmful ROS that give rise to oxidative protein damage, through oxidation of amino acid residues, structural alteration, and loss of function.[8,30,31,39] These reactions contribute to the oxidative damage of several biomolecules observed in AD, including Aβ itself, which has been found to be oxidized in amyloid plaques in vivo.[40]

To identify the metal-catalyzed oxidation of Cu-Aβ in the reductive environment generated during catechol oxidation, we used LCMS analysis. Although DA is the most relevant among the catecholic substrates in this study, the LCMS analysis of the fragmentation pattern of modified peptides is very complicated due to the number of products generated by DA oxidation and the resulting adducts with Aβ. We therefore performed the analysis of Aβ modifications produced upon MC oxidation, following a protocol developed for the analysis of copper–α-synuclein peptide complexes.[26]

In these experiments, a solution of copper and Aβ peptides was incubated in the presence of MC under the same conditions as in the catalytic oxidations ([Aβ16] = 75 μM; [Cu^{2+}] = 25 μM; MC = 3 mM; in 50 mM Hepes buffer pH 7.4). After 90 min of incubation, only about half of the peptide remained unmodified (peak with $t_R = 21$ min), whereas the remaining part underwent a series of modifications to products with higher retention times (Figure 4).

In order to identify the oxidative modifications of Aβ16, we have to consider that aromatic amino acids are among the preferred targets for ROS attack.[41] In previous studies, it was reported that [Cu^{2+}–Aβ]/ascorbate/O_2 or [Cu^{2+}–Aβ]/H_2O_2 systems can induce histidine oxidation.[20,42–44] In our experiments, the most abundant modification (about 32%, see Table 1) corresponds to the peak with $t_r = 23.5$ min. The a, b (N-terminal fragments obtained upon peptide fragmentation at CH–CO and CO–NH bonds, respectively), and y (C-terminal fragments obtained upon peptide fragmentation at CO–NH bonds) ion series observed in the MS/MS spectra indicate that either one His residue or Phe4 undergo oxygen insertion (Aβ16 + 16), and

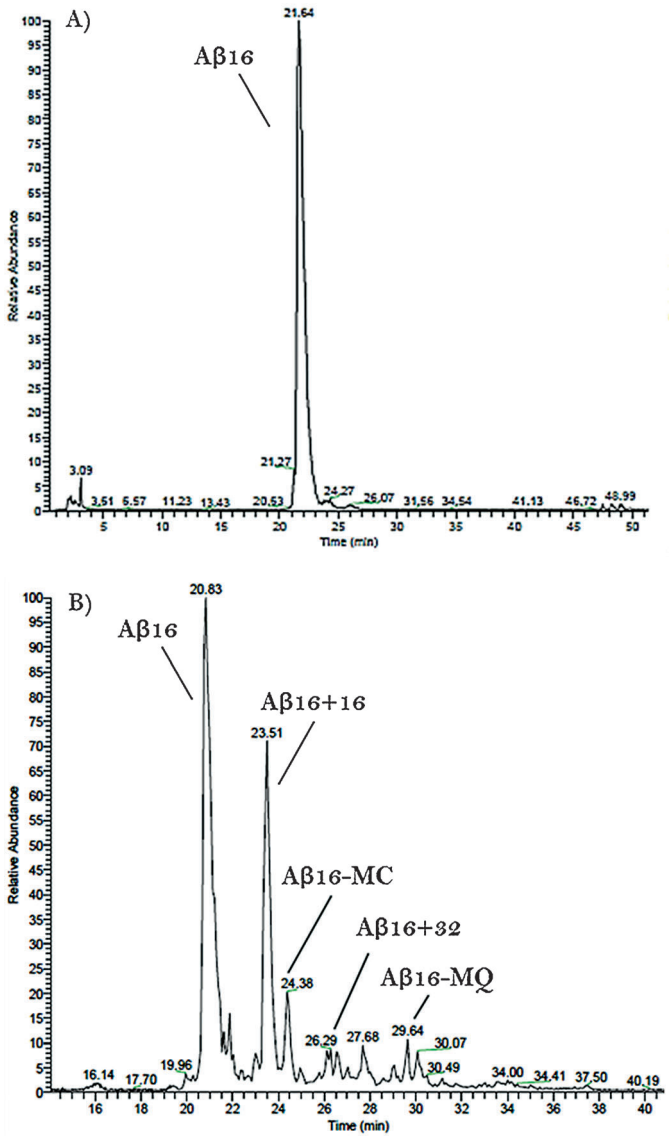

Figure 4. HPLC-MS elution profiles of Aβ16 (75 μM) in Hepes buffer (50 mM) pH 7.4 at 25 °C, in the presence of copper(II) (25 μM) and MC (3 mM) at the beginning (A), and after 90 min incubation time (B). The assignment of the peaks is shown.

Table 1. Modification of Aβ16 peptide (75 μM) detected by LCMS analysis, in the presence of 25 μM copper(II) nitrate and 3 mM MC in Hepes buffer (50 mM) pH 7.4 at 25 °C.

Incubation *t* [min]	Aβ16 [%]	Aβ16+16 [%]	Aβ16–MC [%]	Aβ16+32 [%]	Aβ16–MQ [%]
0	100	0	0	0	0
90	45	32	11	5	7
180	35	27	7	17	14
270	24	25	11	25	15
360	16	24	4	31	25

completely exclude oxidation of Tyr10. The formal addition of one oxygen atom deduced from MS data most likely corresponds to conversion of His to 2-oxohistidine (Scheme S1, Supporting Information)[45] or Phe to *ortho*-tyrosine,[40] respectively.

It is not possible to discern which His residue (6, 13, or 14) is involved in the oxidation, but other studies report that oxidation at the His6 residue was not detected in the fragments of Aβ peptides resulting from digestion with trypsin.[20] A mass increment of 32 Da was detected for the peak at 26.3 min (Aβ16+32) that corresponds to a modification of about 7% of the total peptide after 90 min of reaction time. MS/MS spectra indicate that this peak likely corresponds to simultaneous oxidation of Phe4 and one histidine residue (His13 or His14).

The peak at 24.4 min corresponds to the addition of MQ to one His residue (mass increment of 122 Da; Aβ16-MC); the fragmentation spectra indicate that the involved residue is probably His6.

This result is in agreement with the previous evidence that His6 is less prone to oxidation, whereas it is the main target of the reaction with quinones giving rise to Aβ16-MC adduct. The resulting peptide–catechol adduct is further oxidized by Cu^{2+}, thus explaining the peak at 29.6 min (Aβ16-MQ) with a 120 Da mass increment with respect to the starting peptide (Scheme S2, Supporting Information), the fragmentation spectra of which corroborate the selective derivatization at His6. Table 1 reports the variation over time of the percent modification of the native peptide.

As expected, the overall amount of oxidized peptide increases with time. The single oxidized derivative (Aβ16+16) reaches its maximum amount after 90 min, then this species is further oxidized forming Aβ16+32 species. In addition, the Aβ16–MC species is appreciably detected after 90 min, and then it is partly converted to Aβ16–MQ. As a blank experiment, Aβ16 was incubated in the presence of copper(II) but in the absence of MC; under these conditions, the peptide is not oxidized even after 4 h. However, a further control experiment interestingly shows that the incubation of Aβ16 with MC alone (Figure 5A) results in the development of the peaks corresponding to Aβ16-MC and Aβ16-MQ species (peaks at 25 and 28.3 min, respectively), albeit to lower extent with respect to the experiments with copper(II). Again, the formation of a covalent adduct between Aβ and MC or MQ is more favorable at His6. At longer incubation time a peak at 25.7 min clearly appears, corresponding to an Aβ16–MC species, in which addition of MQ occurs on His6 residue, and a further double oxidation occurs at Phe4 and either His13 or His14 (Aβ16-MC+32; 154 Da mass increment) (Figure 5B). It is also possible to detect a minor peak at 23 min, assignable to a singly oxidized Aβ16 species (Aβ16+16), the low intensity and proximity of which to the main Aβ16 peak preclude its quantification. These results indicate that catechol autoxidation in the presence of atmospheric oxygen at physiological pH can induce a slow Aβ16 oxidation even with the small impurities of redox active metals present in reagents and solvents. The dependence over time of these modifications is summarized in Table 2.

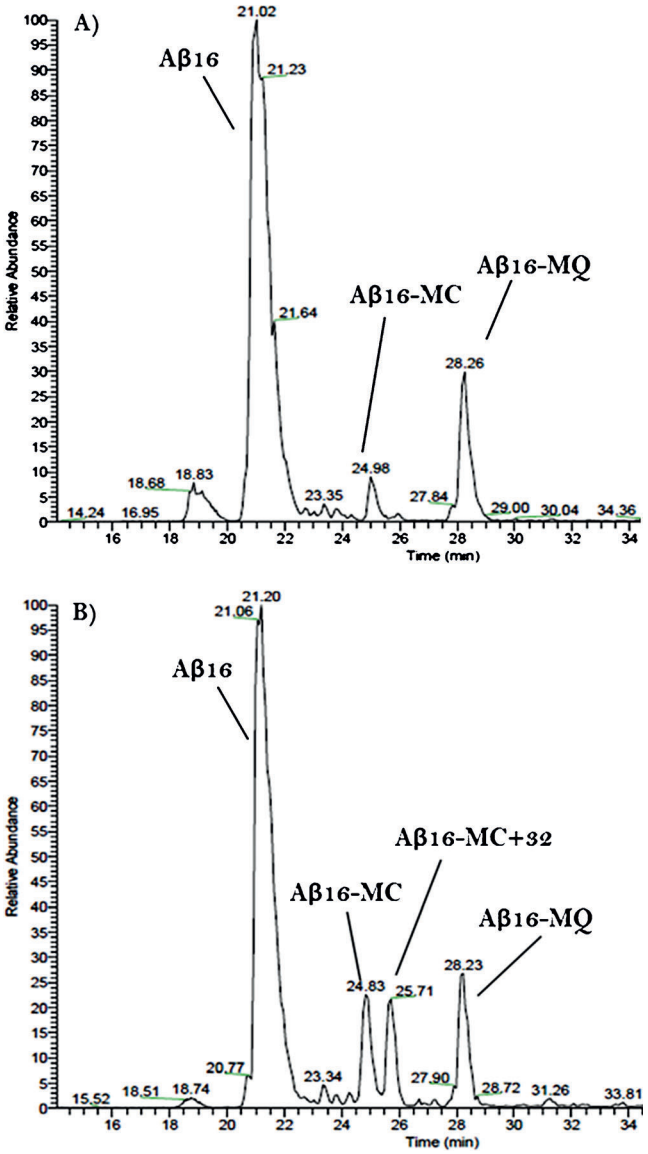

Figure 5. HPLC-MS elution profiles of Aβ16 (75 μM) in Hepes buffer (50 mM) pH 7.4 at 25 °C in the presence of MC after A) 90 min and B) 450 min reaction time. The assignment of the peaks is shown.

Table 2. Modification of Aβ16 peptide (75 μM) detected by LCMS analysis, in the presence of 3 mM MC in Hepes buffer (50 mM) pH 7.4 at 25 °C.

Incubation *t* [min]	Aβ16 [%]	Aβ16–MC [%]	Aβ16–MC +32 [%]	Aβ16–MQ [%]
0	100	0	0	0
90	83	4	0	13
180	76	6	3	15
270	73	7	5	15
360	74	8	6	12
450	71	10	8	11
540	67	11	11	11
630	64	12	14	10

It is worth noting that, when the same process is studied in a MeOH/Hepes buffer mixture, the modifications on Aβ16 occur to a lower extent. LCMS analysis of the reaction mixture containing Cu^{2+}, Aβ16, and MC showed the presence of four main peaks in different proportions, corresponding to modification of 20% of Aβ16 after 90 min, which is half of that obtained in aqueous buffer (Figure S5, Supporting Information). In particular, three principal peptide modifications were observed, which are characterized by mass changes of +16, −45, and +120 Da. MS/MS fragmentation spectra allowed the oxidation of one His or Phe4 residue (Aβ16+16), the decarboxylation/deamination of Asp1 (Aβ16-45), and the addition of MQ to one His residue (probably His6) with subsequent oxidation (Aβ16–MQ), respectively, to be identified. In particular, the peak at 25.6 min, corresponding to the decarboxylation/deamination of Asp1 to pyruvate, is a modification that was previously detected also on Aβ fragments of human and mouse β-amyloid.[24,44] The proposed mechanism for Asp1 oxidative cleavage, as reported in Scheme S3, Supporting Information, proceeds through an alkoxyl-radical pathway,[41] making the carbon atom at the α-position of the amino acid residue sensitive to attack by ROS. Table S1, Supporting Information, reports the percentages of peptide modification in MeOH/Hepes mixture over time. Under these conditions, peptide modifications due to the simple MC autoxidation in the absence of Cu^{2+} are negligible for incubation times lower than 8 h.

The modification of Aβ28 in Hepes buffer was also analyzed in order to compare its behavior with that of Aβ16 under the same reaction conditions. After 90 min incubation of Aβ28 with MC alone (Figure 6A), both Aβ28 and the ^{1}DAEFRHD^{7} fragment were detected with a mass increment of 120 Da (Aβ28–MQ, t_R = 40.2 min; 6%, and ^{1}DAEFRHD^{7}-MQ, t_R = 33.5 min; 3%, respectively) corresponding to the covalent linking of MQ to one His residue. Interestingly, the latter result confirms beyond any doubt that the addition of MQ occurs selectively at the His6 residue, as previously assumed also for Aβ16. In the same analysis other peptide fragments were identified, resulting from the spontaneous hydrolysis of the native peptide at physiological pH: ^{19}FFAEDVGSNK^{28} (t_R = 30.7 min; 1%), ^{15}QKLVFFAEDVGSNK^{28} (t_R = 38.3 min; 3%), and ^{17}LVFFAEDVGSNK^{28} (t_R = 41.4 min; 4%).

When the peptide was incubated for 90 min in the presence of copper(II) and MC (Figure 6B), more products of peptide hydrolysis were observed (Table 3). In this case, it was possible to identify a peak at 42.9 min linked to the oxidation of the native peptide at Phe residues 19 or 20 that also reports the covalent Aβ28-MQ adduct. The ion fragmentation analysis is compatible with MQ insertion into His6, a result also supported by the most abundant modification detected in the peak at 33.5 min (16%), which corresponds to the ^{1}DAEFRHD^{7}-MQ species, as mentioned before.

Additionally, it was possible to identify a peptide fragment at 47.6 min with a mass increase of +64 Da, in which His13, His14, Phe19, and Phe20 residues were oxidized at the same time to 2-oxohistidine and to *ortho*-tyrosine, respectively, in agreement with the results obtained by Cassagnes et al.[20]

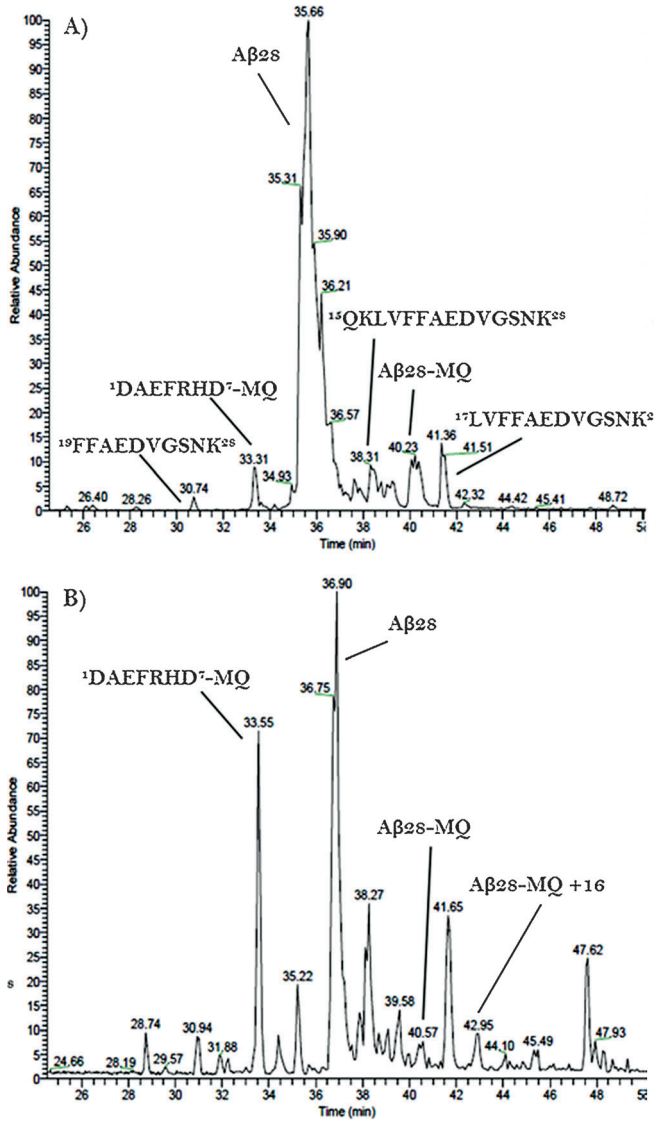

Figure 6. HPLC-MS elution profiles of Aβ28 (75 μM) incubated in the presence of A) MC, B) copper(II) and MC, after 90 min in Hepes buffer (50 mM) pH 7.4 at 25 °C. The assignment of the peaks for A is shown, whereas for B they are reported in Table 3.

Table 3. Modification of Aβ28 peptide (75 μM) detected by LCMS analysis, in the presence of 3 mM MC and 25 μM Cu^{2+} after 90 min in Hepes buffer (50 mM) pH 7.4 at 25 °C.

t_R [min]	Species	Amount [%]
28.7	^{1}DAEFRHDSGYEV^{12}	2
30.9	^{19}FFAEDVGSNK^{28}	2
33.5	^{1}DAEFRHD^{7}-MQ	16
34.4	^{11}EVHHQKLVFFAEDVGSNK^{28}	3
35.2	^{18}VFFAEDVGSNK^{28}	4
36.9	Aβ28	42
38.3	^{15}QKLVFFAEDVGSNK^{28}	11
40.6	Aβ28-MQ	2
41.6	^{17}LVFFAEDVGSNK^{28}	10
42.9	Aβ28-MQ + 16	3
47.6	^{8}SGYEVHHQKLVFF^{20} + 64	6

Regarding the possible oxidation of other amino acid residues, Berlett et al. reported that oxidation of the Tyr residue can form 3,4-dihydroxyphenylalanine,[41] with a 16 Da mass increment with respect to the peptide, but under our conditions we can exclude this amino acid oxidation in Aβ28 (as above reported for Aβ16). Furthermore, although there is evidence supporting metal-catalyzed damage to proteins with insertion of an oxygen atom at arginine and lysine residues,[46] MS/MS spectra analysis for the Aβ16 and Aβ28 fragments exclude with high probability the occurrence of such modifications.

It is apparent, therefore, that the principal targets of oxidative modifications are phenylalanine and histidine residues. In particular, His6, which is involved in Cu^{2+} coordination but not in Cu^{+} binding, is the main target for quinone addition. This process does not appear to be strictly copper-dependent, since it is also observed in the modification process derived from catechol autoxidation. On the other hand, ROS generated by Fenton's reactivity of Cu^{+}–Aβ complexes preferentially react with the imidazole rings that are directly bound to the metal site, that is, His13 and His14. Also Asp1, Phe4, Phe19, and Phe20 are targets of MCO, probably for their proximity to the metal site (Asp1) or chemical propensity to undergo oxidative reactions. In addition, under the experimental conditions described here, no evidence for the formation of Tyr–Tyr cross-linking has been found. This product was previously observed when Aβ peptide was incubated in the presence of H_2O_2 and copper,[47] or heme.[48] Furthermore, LCMS analysis reveals that Aβ28 peptide is more prone to hydrolysis when compared to Aβ16. A more detailed analysis regarding hydrolysis of Aβ28 and the role of metal ions in controlling this type of modification will be reported separately.

It is important to understand to which extent the oxidation of catechol competes or affects endogenous peptide modification processes. To this end, the reaction mixtures resulting from MC oxidation under the same conditions described above were analyzed by HPLC. In particular, in parallel experiments, solutions of MC (3 mM) in 5 mM Hepes buffer pH 7.4 were incubated for 90 min under the following conditions: a) MC alone (autoxidation); b) MC and copper(II) (25 μM); c) MC, copper(II) (25 μM), and Aβ16 (75 μM); d) MC, copper(II) (25 μM), and Aβ28 (75 μM). The analytical separation by HPLC of the reaction mixtures was performed after mixing and after 90 min incubation, using *p*-chlorophenol (3 mM retention time 9.3 min) as an internal standard (added just before injection in the HPLC).

The HPLC profiles are shown in Figures S6, S7, and S8, Supporting Information. The progress of MC oxidation with time is shown by the decrease in intensity of the peak at 5.9 min. The amount of MC oxidized by copper(II) or copper(II)/peptides could be obtained after subtraction of the contribution of MC autoxidation. After 90 min, a decrease in [MC] of 0.36, 0.47, and 0.55 mM was observed with free copper(II), CuAβ16, and CuAβ28, respectively. These data are in agreement with the results reported in Figure 2, showing that Aβ peptides increase the reactivity of copper(II) in the oxidation of MC and that the effect of Aβ28 is slightly higher with respect to that exerted by Aβ16. Moreover, the HPLC profiles shown in Figures S6 to S8,

Supporting Information, are in agreement with the formation of MQ and of addition products of MC (peaks at 7.2 and 7.6 min retention time, respectively), as indicated in Equation (7).

The data obtained in these experiments can be compared with those obtained in the HPLC-MS/MS analysis (Figures 4B and 5B and Tables 1 and 3). After 90 min incubation time in the presence of copper(II) and MC, 55% of Aβ16 (75 μM) undergoes modification with concomitant consumption of 0.47 mM MC, indicating approximately a ratio of one molecule peptide modified every 10 molecules MC reacted. It should be noted that, according to the mechanism proposed, the reaction proceeds with the formation of $MC^{\cdot+}$ and subsequent disproportionation to MC and MQ in solution. Then MQ undergoes addition by the nucleophiles in solution. Considering that a relevant fraction of the peptide is modified by the addition of MC/MQ, even if the MC is in excess, the data indicate that part of the reactive species formed during catalysis has the endogenous peptide as the target. Of particular relevance is the observation of oxidative modification of Aβ16 at His and Phe residues, as this reaction requires strongly reactive species. Thus, the easily oxidized MC substrate does not act as an efficient scavenger of the reactive species formed at the copper center and responsible for oxidative modification of the bound peptide.

A similar conclusion can be drawn for Aβ28. In this case, after 90 min incubation time in the presence of copper(II) (25 μM) and MC (3 mM), 58% of Aβ28 (75 μM) undergoes modification with the concomitant consumption of 0.55 mM MC. These data confirm that CuAβ28 is slightly more efficient than CuAβ16 in MC oxidation.

Conclusion

In this study, we have characterized two important aspects of the oxidative reactivity of copper–Aβ peptide complexes: the oxidation of exogenous catecholic substrates and the endogenous modifications induced by this reactivity on bound Aβ. Although this type of reactivity was considered in previous studies, this is the first time where the two effects have been evaluated simultaneously and quantitatively.

Both copper complexes with Aβ16 and Aβ28 peptides increase the rate of the catalytic oxidation of catecholic substrates, such as the neurotransmitter DA and its biomimetic analogues MC and DTBC. This work integrates previous data from our group[21] in which the catecholase reactivity was investigated in the presence of MBTH, which is indeed redox noninnocent and efficiently competes with the substrate in copper(II) reduction. From these data, it is evident that the experimental conditions and the choice of the substrate are crucial to reproduce more likely physiologic conditions.

A different situation was revealed by LCMS analysis of the modifications undergone by the two Aβ peptides. Compared to Aβ16, which principally undergoes oxidative modification, Aβ28 is also prone to hydrolysis processes. These data suggest that hydrolysis of Aβ peptides may play an important role in physiologic conditions, also considering the larger size of Aβ isoforms present in vivo (39 to 43 residues).

Moreover, we noticed that formation of catechol–Aβ covalent adducts is not negligible in the absence of copper. This behavior is due to catechol autoxidation in solution that generates reactive quinone species yielding addition products with Aβ nucleophilic residues. This behavior could be observed clearly only for MC, but it is probably present also for DA, that is a more relevant substrate for this kind of reaction. The situation in the presence of copper is different, since more oxidative reaction pathways are promoted. In this case, the formation of covalent Aβ–catechol adducts occurs to a lower extent, while peptide oxidation with insertion of oxygen atoms at His and Phe residues becomes more relevant.

The comparison of the efficiency in catechol oxidation versus Aβ modification indicates that the oxidative reactivity towards the peptide is relevant, showing that Cu–Aβ complexes may have an important role in vivo in radical scavenging. We can therefore deduce a dual role for Aβ peptides: on one hand the formation of Cu–Aβ complex exacerbates the Cu ability to promote oxidation of external substrates, and on the other hand, in this oxidative environment, Aβ peptides bear a strong tendency to undergo oxidation by Cu redox cycling. The latter aspect is in agreement with the hypothesis that Aβ peptides act as radical scavengers.[23]

Experimental Section

Materials and instrumentation

Protected amino acids, rink amide resin, and other reagents for peptide synthesis, were purchased from Novabiochem. All other chemicals were reagent grade from Sigma–Aldrich. Peptide purifications were performed on a Jasco HPLC instrument equipped with two PU-1580 pumps and a MD-1510 diode array detector (working range: 195–659 nm), using a Phenomenex Jupiter 4U Proteo semipreparative column (4 μm, 250×10 mm). Mass spectra and LCMS/MS data were obtained with a LCQ ADV MAX ion-trap mass spectrometer, with an ESI ion source. The system was run in automated LCMS/MS mode and using a surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a Phenomenex Jupiter 4u Proteo column (4 μm, 150×2.0 mm). MS/MS experiments by collision-induced dissociation (CID) were performed with an isolation width of 2 Th (*m/z*); the activation amplitude was around 35% of the ejection radiofrequency (RF) amplitude of the instrument. ESI-MS direct injection experiments were performed using a positive or negative ion mode, capillary temperature at 200 °C and MeOH as the eluent. For the analysis of peptide fragments, Bioworks 3.1 and Xcalibur 2.0.7 SP1 software were used (Thermo Finnigan, San Jose, CA (USA)). UV/Vis spectra and kinetic experiments were recorded on an Agilent 8453 diode array spectrophotometer, equipped with a thermostated, magnetically stirred optical cell. In the case of MC consumption, HPLC analysis was performed on a 1260 Infinity Agilent system using a XSelect® HSS C18 analytical column (2,5 μm, 4,6×50 mm).

Peptide synthesis

Aβ16 (^{1}DAEFRHDSGYEVHHNK^{16}-NH_2) and Aβ28 (^{1}DAEFRHDSGYEVHHNKLVFFAEDVGSNK^{28}-NH_2) peptides were syn-

thesized using the standard fluorenyl methoxycarbonyl (Fmoc) solid-phase synthesis in DMF. Rink-amide resin was used as a solid support, which yielded the peptides amidated at the C-terminus. For Aβ28 peptide, a low loading Rink-amide resin (substitution 0.36 mmol g^{-1}) was used. After deprotection of the resin with 20 mL of 20% (*v*/*v*) piperidine in DMF, the first amino acid (3 mol equiv vs. resin sites), was added in the presence of 3 equiv of *N*-hydroxybenzotriazole, 3 equiv of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, and 6 equiv of *N*,*N*-diisopropylethylamine. After 45 min, the same coupling procedure was repeated. After recoupling of each amino acid, a capping step was performed by using 20 mL of 4.7% acetic anhydride and 4% of pyridine in DMF. Deprotection of the Fmoc group was performed by treating the resin twice, for 3 and 7 min, respectively, with 15 mL of 20% piperidine in DMF. At the end of the synthesis, the protections of the side chains of the amino acids were removed with a solution of 95% trifluoroacetic acid (TFA, 25 mL for 1 g of resin), triisopropyl silane (2.5%), and water (2.5%), which serves also to release the peptide from the resin. After stirring for 3 h, the solution was concentrated under vacuum and cold diethyl ether was added to precipitate the peptide. The mixture was filtered and the precipitate washed with cold diethyl ether; then, it was dissolved in water and purified by HPLC, using a 0–100% linear gradient of 0.1% TFA in water to 0.1% TFA in CH_3CN over 40 min (flow rate of 3 mL min^{-1}, loop 2 mL), as the eluent. The product was then lyophilized, yielding a white solid, which was characterized by ESI-MS. ESI-MS data (direct injection, MeOH, positive-ion mode, capillary temperature 200 °C): *m*/*z*: 1955 ($A\beta16H^+$), 978 ($A\beta16H_2^{2+}$), 652 ($A\beta16H_3^{3+}$), 489.5 a.m.u ($A\beta16H_4^{4+}$); 1631 ($A\beta28H_2^{2+}$), 1088 ($A\beta28H_3^{3+}$), 816 ($A\beta28H_4^{4+}$), 653 a.m.u ($A\beta28H_5^{5+}$).

Kinetics of oxidation of catecholic substrates

a) Dopamine oxidation: The catalytic oxidation of DA by Cu^{2+} was studied at room temperature (25 °C) in 50 mM Hepes buffer at pH 7.4, saturated with atmospheric oxygen. The reaction was monitored by UV/Vis spectroscopy through the absorption band of dopaminochrome at 475 nm.[33] Firstly, a DA (3 mM) autoxidation experiment was evaluated; the kinetic trace obtained in the autoxidation experiment was subsequently subtracted from the kinetic profile of the reactions catalyzed by copper(II) and copper(II)–peptide complexes. In the former case, the experiments were carried out by adding copper(II) nitrate (25 μM) to the solution containing DA (3 mM). In the experiments with copper–peptide complexes, Aβ16 or Aβ28 were added at 25–100 μM concentrations, to a solution of DA (3 mM), followed by copper(II) nitrate (25 μM) as the last reagent. All measurements were performed at least in triplicate. In order to observe the dioxygen concentration effect on DA oxidation, the above-mentioned experiments were repeated saturating the solutions with pure molecular oxygen at 1 atm., instead of atmospheric oxygen.

b) 4-Methylcatechol oxidation: The catalytic oxidation of MC by Cu^{2+} was studied at room temperature (25 °C) in 50 mM Hepes buffer at pH 7.4, saturated with atmospheric oxygen. The reaction was monitored by UV/vis spectroscopy through the absorption band of 4-methylquinone at 401 nm ($\varepsilon = 1550\ M^{-1}cm^{-1}$),[26] which subsequently undergoes a shift to 480 nm due to the addition reaction to MQ by excess MC. Firstly, MC (3 mM) autoxidation was evaluated, and the corresponding kinetic trace was subsequently subtracted from the kinetic profile of the catalytic reactions. The latter reactions with free copper(II) and with copper(II)–Aβ complexes were performed under the same conditions used for DA oxidation. All kinetic experiments were performed at least in triplicate.

c) 3,5-Di-*tert*-butylcatechol oxidation: The catalytic oxidation of DTBC by Cu^{2+} was studied at room temperature (25 °C)), in a mixed solvent of 80% methanol and 20% 50 mM aqueous Hepes buffer at pH 7.4 (*v*/*v*), saturated with atmospheric oxygen. The reaction was monitored by UV/Vis spectroscopy through the absorption band of 3,5-di-*tert*-butylquinone at 407 nm ($\varepsilon = 1500\ M^{-1}cm^{-1}$).[26] The experiment was performed by adding copper(II) nitrate (25 μM) to the solution containing DTBC (3 mM). DTBC autoxidation is negligible under these conditions. In the experiment with copper(II)–Aβ complex, Aβ16 (50 μM) was added to a solution of DTBC (3 mM), followed by copper(II) nitrate (25 μM). The measurements were performed in triplicate.

Determination of the $[O_2]$ in the reaction media

The oxygen concentration in 50 mM aqueous Hepes buffer at pH 7.4 and in the mixed solvent of 80% methanol and 20% 50 mM aqueous Hepes buffer at pH 7.4 (*v*/*v*), saturated with atmospheric oxygen at 25 °C were determined with the classical Winkler method.[49]

Identification and characterization of oxidized peptides by HPLC-ESI-MS

Peptide modification was analyzed by HPLC-ESI-MS, performing experiments under the same conditions used for MC oxidation studies. Samples were prepared with copper(II) nitrate (25 μM), Aβ16 (75 μM) and MC (3 mM) in Hepes buffer (50 mM) pH 7.4 at different reaction times: 0, 90, 180, 270, 360 min. The experiment was repeated with MC 3 mM and Aβ16 (75 μM) in Hepes buffer (50 mM) pH 7.4, but in absence of copper, at the following reaction times: 0, 90, 180, 270, 360, 450, 540, 630 min. The effect of methanol as a co-solvent was evaluated by performing the analysis in the presence of copper(II) nitrate (25 μM), Aβ16 (75 μM) and MC (3 mM) in 80:20 (*v*/*v*) MeOH/HEPES buffer (50 mM) at different reaction times: 0, 90, 180, 270, 360 min. In the case of Aβ28, samples were prepared in the presence of copper(II) nitrate (25 μM), Aβ28 (75 μM), and MC (3 mM) in Hepes buffer (50 mM) pH 7.4 and analyzed after 90 min reaction time. LCMS and LCMS/MS data were obtained by using the LCQ ADV MAX ion-trap mass spectrometer, as described in the Materials and instrumentation Section. The elution was performed by using 0.1% HCOOH in distilled water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B), with a flow rate of 0.2 mL min^{-1}; elution started with 98% solvent A for 5 min followed by a linear gradient from 98 to 55% A in 65 min.

HPLC quantification of oxidized catechol

The consumption of MC was monitored by recording HPLC chromatograms (95–50% linear gradient of 0.1% TFA in water using 0.1% TFA in CH_3CN as an organic solvent over 12 min; flow rate of 1.4 mL min^{-1}; loop 10 μL; $\lambda_{obs} = 256$ nm) carrying out experiments under the same conditions used for MC oxidation studies and HPLC-ESI/MS analysis. A stock solution of MC in milliQ water was diluted in 5 mM Hepes buffer at pH 7.4 to 3 mM concentration and analyzed at 0 time and after 90 min of incubation. *p*-Chlorophenol (3 mM) was used as an internal standard, and it was added just before the HPLC injection. The same profiles were recorded with MC (3 mM) and Aβ16 or Aβ28 (75 μM) in the absence and presence of copper nitrate (25 μM) in 5 mM Hepes buffer at pH 7.4.

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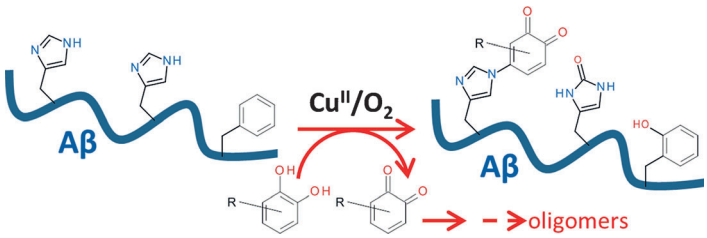

Competitive oxidative experiments with exogenous catechols show that copper complexes with both Aβ16 and Aβ28 peptides can significantly increase the oxidation rate of copper(II) towards the catechol compounds and promote endogenous peptide oxidation (see scheme). The main modifications consist of oxidation of His13/14 to 2-oxohistidine and Phe19/20 to *ortho*-tyrosine, and the formation of a covalent His6-catechol adduct.

Medicinal Chemistry

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Copper-Aβ Peptides and Oxidation of Catecholic Substrates: Reactivity and Endogenous Peptide Damage