

Prion peptides are extremely sensitive to copper induced oxidative stress

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ABSTRACT: Copper(II) binding to prion peptides does not prevent Cu redox cycling and formation of reactive oxygen species (ROS) in the presence of reducing agents. The toxic effects of these species are exacerbated in the presence of catecholamines, indicating that dysfunction of catecholamine vesicular sequestration or recovery after synaptic release is a dangerous amplifier of Cu induced oxidative stress. Cu bound to prion peptides including the high affinity site involving histidines adjacent to the octarepeats exhibits marked catalytic activity toward dopamine and 4-methylcatechol. The resulting quinone oxidation products undergo parallel oligomerization and endogenous peptide modification yielding catechol adducts at the histidine binding ligands. These modifications add to the more common oxidation of Met and His residues produced by ROS. Derivatization of Cu-prion peptides is much faster than that undergone by Cu- β -amyloid and Cu- α -synuclein complexes in the same conditions.

INTRODUCTION

The cellular prion protein (PrP) is a membrane-anchored glycoprotein of still incompletely understood physiological role, reaching high levels in the nervous system, and capable of producing a misfolded isoform, the prion, identified as the causative agent of several neurodegenerative diseases.¹ The infectious nature of the toxic isoform involves conformational remodeling and aggregation of PrP in a template-directed process.² Although human prion diseases are rare, the pathogenic mechanisms in much more common neurodegenerative diseases, such as Parkinson and Alzheimer's disease, appear to be similar to prions.³ Therefore, both tau,⁴ α -synuclein⁵ and β -amyloid⁶ can undergo the same type of template conformational change as prions and then spread from cells to cells, at least within the brain.

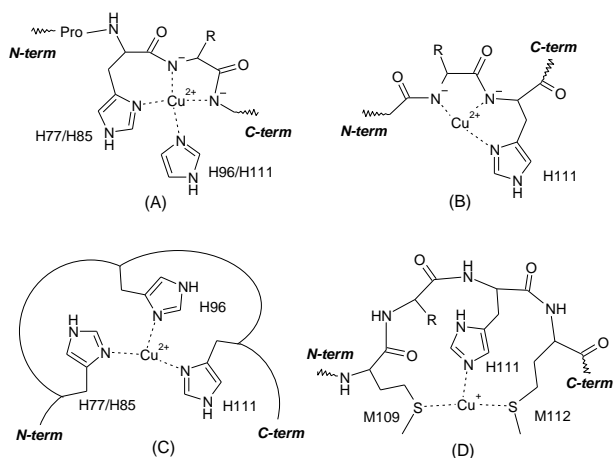
The role of PrP has been often associated with its Cu binding properties, with affinity in the nM range, or even lower;⁷ but the unique feature of this protein is its ability to bind up to six Cu²⁺ ions, depending on Cu²⁺ concentration. Copper plays an emerging role as a modulator of signal transduction and its release at synapses can modulate neurotransmitter receptors.⁸ In fact, Cu-PrP binding appears to modulate NMDA receptor activity,⁹ attenuating glutamate excito-toxicity.¹⁰ Interestingly, β -amyloid oligomers have been found to induce neurotoxicity by blocking Cu-PrP activity on NMDA receptor, probably

through Cu²⁺ chelation.⁹ Recent findings describe the truncated β -amyloid (4-42) as mostly responsible for Cu²⁺ binding, due to its high affinity.¹¹ In general, PrP appears to play a central role in neuroprotection and Cu-PrP complexes may be involved in cellular protection against oxidative stress.^{12,13} The proposal that Cu-PrP exerts superoxide dismutase activity¹⁴ has been challenged by subsequent studies.¹⁵ However, high levels of oxidized methionines have been observed in prions,¹⁶ and Cu-PrP was found to exert neuron protection through S-nitrosylation.¹⁷ Therefore, there is an urgent need to shed light upon the potential reactivity of Cu-PrP under oxidative stress conditions and what are the consequences of this reactivity on the integrity of PrP protein.

In this paper, we focused on the redox reactivity of Cu²⁺ bound to three *N*-acetylated and *C*-amidated PrP fragments, PrP₁₀₆₋₁₁₄, PrP₈₄₋₁₁₄ and PrP₇₆₋₁₁₄. These span the region where binding of PrP by β -amyloid oligomers occurs,¹⁸ which is key for the toxicity associated with Alzheimer's disease, and include the fragment outside the octarepeats (92-114),¹⁹ with the high affinity Cu site involving H96 and H111, and the larger fragments also containing one (84-114) or two (76-114) of the Cu sites within the four octarepeats.²⁰ Considering the complex pattern of Cu-PrP complexes that can be formed in various conditions,¹⁹ we focused on Cu-PrP fragments at low Cu loading in physiological conditions. The structure of the main Cu species is given in Chart 1. As reactive molecules capable

of inducing Cu redox cycling, we studied the neurotransmitter dopamine (DA) and the related, but structurally simpler, 4-methylcatechol (MC). Actually, a role of DA in promoting PrP aggregation,^{21a} dimerization and oxidative cleavage has been proposed.^{21b} In addition, the present investigation extends our comparative studies on the redox activity of Cu complexes with neuronal peptide fragments of β -amyloid²² and α -synuclein.²³

Chart 1. Schematic structures of the main species for Cu-PrP peptides in 1:1 ratio at physiological pH (A, B, and C, for Cu^{2+} , and D for Cu^+).



Structure (A) represents the main species for Cu^{2+} binding to PrP₇₆₋₁₁₄ and PrP₈₄₋₁₁₄ as deduced from potentiometric and spectroscopic studies.^{19,20} Cu^{2+} is bound to an octarepeat fragment (75-81 or 83-89), with a contribution by an additional distal His. Structure (B) is the main species for Cu^{2+} -PrP₁₀₆₋₁₁₄,^{19,20} note that H111 is outside the octarepeats; another ligand, that may be water, occupies the fourth coordination position. Structure (C) represents a hypothesized poly-His macrochelate complex involving Cu^{2+} binding to three or four His,¹⁹ this is a minor species at neutral pH but is appreciable at slightly acidic pH. Structure (D) shows the reduced Cu^+ -PrP species, as deduced from spectroscopic and theoretical studies;^{29b} it involves Met residues and H111 outside the octarepeats; also in this case a fourth donor ligand may be present.

RESULTS AND DISCUSSION

Oxidation of dopamine and 4-methylcatechol by Cu-PrP complexes. As shown by the plots of Figure 1 and 2, the rate of oxidation of DA (3 mM) by air oxygen and Cu^{2+} (25 μM), in HEPES buffer at pH 7.4, is strongly promoted by increasing amounts of PrP₇₆₋₁₁₄; on the contrary, the effect of PrP₁₀₆₋₁₁₄ is very low. The behavior of PrP₈₄₋₁₁₄ is more similar to that of PrP₇₆₋₁₁₄ but DA oxidation is appreciably lower (see Figure S1). It should be noted that the optical traces at 475 nm monitor the dopaminochrome intermediate (see Scheme 1),²⁴ but DA oxidation gives rise to a complex mixture of products, as shown by the broad absorption bands in the spectra of the reaction mixture (see Figure S2).

On the basis of previous thermodynamic studies^{19,25} it is possible to estimate the species distribution for the Cu-peptide complexes. The data obtained for Cu-PrP₇₆₋₁₁₄ show that Cu^{2+} is fully complexed (99.4 %) by the peptide already at 1:1 ratio (see Table S1 and Figure S3). For Cu-PrP₈₄₋₁₁₄ the situation is similar, with 93 % Cu bound at 1:1 Cu:peptide ratio (see Table S2 and Figure S4), whereas for Cu-PrP₁₀₆₋₁₁₄ at 1:1 ratio a significant Cu fraction is bound to the buffer, and only at 1:3 ratio most of Cu^{2+} is bound to the peptide (see Table S3 and Figure S5). For this reason, and also to take into account that oxidation experiments are carried out in the presence of more than 100 molar excess of catechol substrates, which are chelating ligands for Cu^{2+} , the kinetic traces were followed at increasing peptide/ Cu^{2+} ratios, to ensure that conditions of full occupancy of the peptide are approached. In any case, the trend displayed in DA oxidation by the complexes clearly indicates the reactivity order: $\text{Cu-PrP}_{76-114} \geq \text{Cu-PrP}_{84-114} \gg \text{Cu-PrP}_{106-114}$.

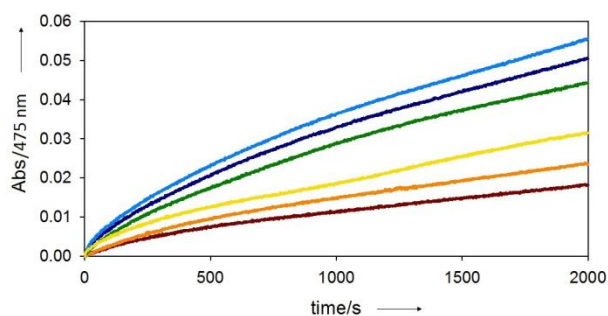


Figure 1. Kinetic profiles of DA (3 mM) oxidation with time (DA autoxidation in brown), in 50 mM HEPES buffer solution at pH 7.4 and 20 °C in the presence of Cu^{2+} (25 μM) (orange) and with addition of PrP₇₆₋₁₁₄ (6 μM , yellow; 25 μM , green; 50 μM , blue; 75 μM , light blue). DA oxidation was monitored through the absorption band of dopaminochrome at 475 nm.

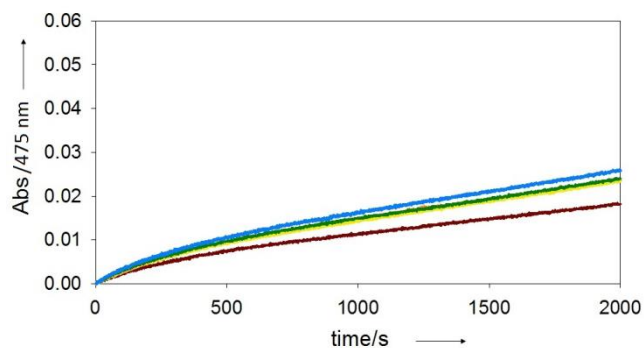
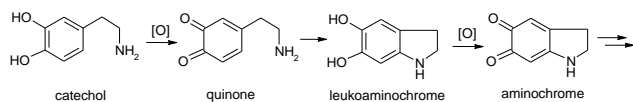


Figure 2. Kinetic profiles of DA (3 mM) oxidation with time (DA autoxidation in brown), in 50 mM HEPES buffer solution at pH 7.4 and 20 °C in the presence of Cu^{2+} (25 μM) (orange) and with addition of PrP₁₀₆₋₁₁₄ (25 μM , yellow; 50 μM , green; 75 μM , blue). DA oxidation was monitored through the absorption band of dopaminochrome at 475 nm.



Scheme 1. Initial steps of the dopamine oxidation pathway; the dopaminochrome intermediate is that with longer life and responsible for the broad absorption band between 450 and 500 nm. It undergoes further condensation and oxidative oligomerization reactions to produce insoluble melanic pigments.²⁴

A less discriminating behavior was observed in the oxidation of MC catalyzed by Cu-PrP peptides. This substrate has lower semiquinone/catechol redox potential and, lacking the amino group in the substituent chain, forms a more stable quinone with respect to DA.^{23a} The E° data for DA and MC redox couples are 0.53^{26a} and 0.46 V,^{26b} respectively, and should be compared with those of the Cu(II)/Cu(I) couples of Cu-PrP complexes. Here the literature values are more variable, ranging from 0.3 V, for the full length protein²⁷ to 0.57 V for PrP fragments including Met residues.^{15c} It should be pointed out, however, that the species ruling the reaction is not the copper(II)-PrP complex but the intermediate [Cu-PrP-catechol- O_2], as discussed below.

The kinetic profiles of MC (3 mM) oxidation by Cu^{2+} (25 μM), in a time interval where 4-methylquinone is the only product,^{22b} show a progressive increase of the rate with increasing equivalents of PrP₇₆₋₁₁₄ (Figure 3). The rate increase of MC oxidation promoted by PrP₈₄₋₁₁₄ (Figure S6) and PrP₁₀₆₋₁₁₄ is similar (Figure S7). It is interesting to note that the promoting effect by PrP peptides on Cu reactivity resembles that of β -amyloid,^{22b} whereas *N*-acetylated α -synuclein quenches Cu reactivity.²³

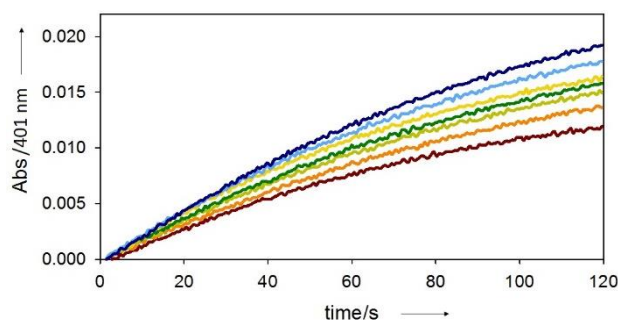


Figure 3. Kinetic profiles of MC (3 mM) oxidation with time, in 50 mM HEPES buffer solution at pH 7.4 and 20 °C in the presence of Cu^{2+} (25 μM) (brown) and with addition of PrP₇₆₋₁₁₄ (10 μM , orange; 20 μM , light green; 25 μM , green; 35 μM , yellow; 50 μM , light blue; 75 μM , blue).

As already characterized in a previous study,^{23b} at longer reaction time the maximum absorption band shifts to

480 nm upon formation of an adduct between excess MC and the quinone. In the presence of free copper the addition product is the main species even after 90 min reaction time (Figure S8 and Figure S9A), whereas in the presence of the Cu-PrP complex the reaction leads to further oligomeric products, as shown by the broadening of absorption between 400 and 800 nm (Figure S9B).

The reaction mechanism previously proposed for the oxidation of catechols by Cu- β -amyloid^{22b} and Cu- α -synuclein²⁸ peptide complexes can be extended to the present Cu-PrP peptides. It involves the following reaction steps:

1. $Cu^{2+} + PrP \rightleftharpoons [Cu^{2+}-PrP]$
2. $[Cu^{2+}-PrP] + catechol \rightarrow [Cu^{+}-PrP] + semiquinone^{+}$
3. $[Cu^{+}-PrP] + catechol \rightleftharpoons [Cu^{+}-PrP-catechol]$
4. $[Cu^{+}-PrP-catechol] + O_2 \rightarrow [Cu-PrP-catechol-O_2]$
5. $[Cu-PrP-catechol-O_2] \rightarrow [Cu^{2+}-PrP] + quinone$
6. $2 semiquinone^{+} \rightarrow catechol + quinone$

The rate-determining step of the mechanism outlined by reactions 1-5 is reaction 4 of the reduced form of Cu-PrP peptides with dioxygen, to generate the ternary complex indicated as [Cu-PrP-catechol- O_2]. This was probed performing the MC oxidation experiment by Cu-PrP₇₆₋₁₁₄ in a buffer solution saturated with pure oxygen (1 atm). The reaction rate indeed markedly increases with the increase of dioxygen concentration (Figure S10), showing that it is Cu^{+} , rather than Cu^{2+} , the species controlling the efficiency of the whole process. The reaction rate depends also on catechol concentration, with a hyperbolic behavior (Figure S11), indicating that substrate binding occurs as a pre-equilibrium step before the rate-determining binding of molecular oxygen. This shows that O_2 binds to [Cu⁺-PrP-catechol] adduct rather than [Cu⁺-PrP].

The structure of the Cu^{+} species bound to PrP peptides was recently characterized with various techniques indicating that under physiological conditions the ligands are provided by the two Met residues and H111.^{29b} However, upon redox cycling it is unlikely that Cu will undergo the structural changes corresponding to the more stable, resting Cu^{2+} and Cu^{+} forms (Chart 1), because this would involve large reorganization energy of the complex. The catalytically active form of Cu-PrP peptides 76-114 and 84-114 may correspond to the tris-histidine environment shown in structure C of Chart 1, involving H96 and H111 outside the octarepeats, which should be accessible to both Cu^{2+} and Cu^{+} species. The third histidine is one contained in the octarepeats, His85 for PrP₈₄₋₁₁₄ and either His85 or His77 for PrP₇₆₋₁₁₄.^{19c} In the case of Cu-PrP₁₀₆₋₁₁₄ the situation must be obviously different, and the coordination set of the redox cycling copper can only correspond to structure D of Chart 1. Binding to the methionines stabilizes Cu(I) and clearly reduces its reactivity to O_2 and the catalytic activity of this Cu-PrP complex. Binding of the catechol to copper(I) may also affect the coordinative environment of the metal ion.

A similar situation where the proposed redox cycling Cu active species is different from the resting form of the Cu complexes was recently proposed for Cu- β -amyloid.³⁰ In the present case, a clear advantage in catalytic efficiency would be associated with the reduced CuHis₃⁺ form, whose coordination set resembles the typical structural motif present in many copper enzymes activating dioxygen.³¹

We should also mention that an alternative pathway, operative for catechol oxidation promoted by free Cu²⁺, can in principle occur competitively, to some extent, for the Cu-PrP complexes. This mechanism involves superoxide and hydroxyl radicals formed through the Fenton reaction:

7. Cu²⁺ + catechol \rightarrow Cu⁺ + semiquinone^{•+}
8. Cu⁺ + O₂ \rightarrow Cu²⁺ + O₂⁻
9. 2 O₂⁻ + 2H⁺ \rightarrow O₂ + H₂O₂
10. H₂O₂ + Cu⁺ \rightarrow OH⁻ + OH[•] + Cu²⁺
6. 2 semiquinone^{•+} \rightarrow catechol + quinone

An indication of the relative importance of this mechanism for the catechol oxidation by Cu-PrP complexes could be obtained through the assay of hydrogen peroxide in the reaction solution, because this is the slowly reacting species and can accumulate. Thus, when assayed in parallel experiments, the amount of H₂O₂, detected by a peroxidase assay after quenching the oxidation of MC after 10 min, was 2.8 mol % with respect to initial MC for Cu²⁺ and 1.7 mol% for Cu-PrP₇₆₋₁₁₄ complex. It should be noted that the amount of hydrogen peroxide detected in the reaction of “free” Cu²⁺ is comparable to the MC oxidized after the same time (see Figure 5 below). On the other hand, accumulated H₂O₂ is only about 10 % of the amount of MC oxidized with Cu-PrP₇₆₋₁₁₄.

We have also found that hydrogen peroxide can participate in catechol oxidation promoting a further, albeit less efficient, mechanism. As shown in Figure S12, addition of hydrogen peroxide to a solution of MC and copper(II) gives rise to an increase in the reaction rate and the effect is larger in the presence of PrP₇₆₋₁₁₄. Therefore, the low accumulation of H₂O₂ observed for Cu-PrP₇₆₋₁₁₄ is partly due to the better capacity of this complex to use the peroxide as oxidant.

Competitive endogenous PrP peptide oxidation. In a system which is not preorganized for dioxygen activation and contains kinetically labile copper, the intermediate Cu/O₂ species typically evolves through a Fenton reaction producing harmful ROS species (H₂O₂ and OH[•]), capable of performing non-selective oxidations. The reactivity of these species can be directed in particular towards the peptide, that can explain the protecting, albeit suicidal, role exerted by PrP and β -amyloid.²² The presence of catecholic neurotransmitters further complicates the pattern of reactions occurring in the system, because their quinone oxidation products bear potential reactivity against nucleophilic amino acid side chains, as we have recently shown for α -synuclein^{23a} and β -amyloid peptides.^{22b} We have therefore investigated the possible com-

petition between catechol oxidation and endogenous PrP peptide modification. DA is not the ideal catechol for these experiments, because its oxidative polymerization gives rise to a complex mixture of oligomeric, and intractable, products. The competition between catechol oxidation and peptide modification was therefore investigated using MC.

In these experiments, solutions of copper (25 μ M) and PrP peptides (25 μ M) were incubated in the presence of MC (3 mM) and the mixture was analysed by LC/MS at different reaction times. The MS/MS spectra of the ion series obtained upon peptide fragmentation allow identifying the modification sites. The data show that all PrP peptides undergo severe modification upon Cu redox cycling, even at relatively short reaction times, in spite of the presence of more than 100 molar excess of catechol. For instance, in the case of PrP₇₆₋₁₁₄, 14 % peptide underwent modification after 10 min reaction time and almost 30 % after 35 min (Table 1 and Figure 4). The main modifications correspond to: (i) oxidation (+16 Da mass increment) of one of the histidines in the octarepeat region (H77, H85) or the external H96 (R_t 32'), and one of the two methionines (M₁₀₉, M₁₁₂) or, less likely, H₁₁₁ (R_t 29'); (ii) the formation of a covalent adduct (+120 Da) between one His residue outside the octarepeat region (H96, H₁₁₁) and 4-methylquinone (MQ) (Scheme S1); (iii) oxidation of one octarepeat His in addition to the former His(96/111)-MQ adduct (+136 Da); and (iv) formation of two His-MQ adducts at H96 and H77/H85, and oxidation of the other octarepeat His (H85/H77) (+256 Da). These derivatizations are worth noting considering that they occur at His residues we propose as the copper binding ligands for the redox cycling Cu-PrP₇₆₋₁₁₄ species or at Met residues close to them (Chart 1).

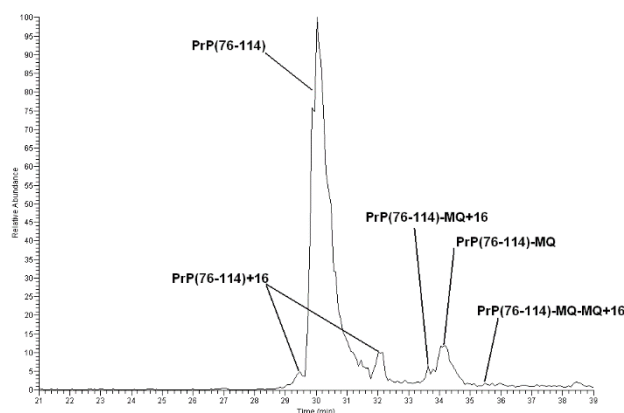


Figure 4. HPLC-MS elution profile of peptides produced upon oxidation of MC (3 mM) by Cu-PrP₇₆₋₁₁₄ (25 μ M) after 35 min reaction time in 50 mM Hepes buffer at pH 7.4 and 20 °C. The assignment of the peaks is given, with MQ indicating a mass increment of 120 Da and corresponding to the formation of a covalent adduct of the peptide with 4-methylquinone.

Table 1. Modifications of PrP₇₆₋₁₁₄ detected by LC/MS analysis, upon oxidation of MC (3 mM) by Cu-PrP₇₆₋₁₁₄ (25 μ M) in 50 mM Hepes buffer at pH 7.4 and 20 $^{\circ}$ C.

Peptide	PrP ₇₆₋₁₁₄ (%)	+16 His (%)	+16 Met (%)	+120 His (%)	+136 His (%)	+256 His (%)
Time (min)	(R _t 30')	(R _t 32')	(R _t 29')	(R _t 34')	(R _t 33')	(R _t 35')
1	96.8	0.3	1.4	0.9	0.5	0.1
10	86.2	2.1	3.0	6.0	2.2	0.5
20	81.1	3.9	3.3	6.7	3.4	1.6
35	71.1	5.5	4.4	10.4	5.6	3.0
60	65.1	6.8	3.8	10.8	8.6	4.9
90	20.3	16.9	4.0	12.5	27.1	19.2
110	17.7	17.5	6.9	15.0	27.0	15.9

The pattern of modifications undergone by Cu-PrP₈₄₋₁₁₄ in the same conditions is similar (Figure S13 and Table S4) and confirm the propensity of His and Met residues of PrP peptides to intercept ROS and in the first case reactive quinone species. The peptide modification pattern for PrP₁₀₆₋₁₁₄ shows more extensive oxidative damage at Met residues (see Figure S14 and Table S5), in agreement with the different structure of the redox cycling Cu(II)/Cu(I) species. In summary, the data from the competitive peptide modification studies confirm the hypothesis on the structure of the catalytically active species of Cu-PrP complexes suggested previously.

It is important to analyze in more quantitative terms the competition between catechol oxidation and endogenous peptide modification processes. To this end, the reaction mixtures resulting from MC oxidation in the same conditions described above were analyzed by NMR spectroscopy, monitoring the decrease of MC concentration with time. The spectrum of the reaction mixture was recorded after 5 min and every 30 min reaction time, following, in parallel, MC oxidation by Cu²⁺ and Cu²⁺-PrP₇₆₋₁₁₄ (Figure 5). The decrease in intensity of the MC signal at 6.55-6.75 ppm was used to monitor the progress of the reaction, with the Hepes buffer signal at 3.7 ppm serving as internal standard (Figure S15). Representative spectra (Figure S16) and numerical data on the decrease of MC concentration in solution with time (Table S6) are given in the Supporting Information.

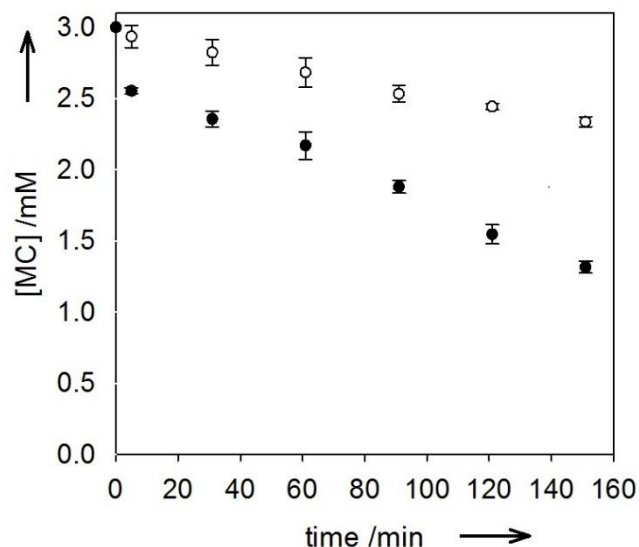


Figure 5. Consumption of MC (3 mM) evaluated by NMR spectroscopy, in 5 mM Hepes deuterated buffer solution at pH 7.4 and 20 $^{\circ}$ C in the presence of Cu²⁺ (25 μ M) (open circles) and Cu²⁺-PrP₇₆₋₁₁₄ (25 μ M) (full circles).

It can be noted that the rate of MC consumption is higher in the initial phase and then decreases as the peptide modification becomes more significant. However, the results are in agreement with the kinetic plots shown in Figure 2 and Figure S8, showing that the presence of prion peptide increases the reactivity of copper(II) in the oxidation of MC (and much more so for DA, see Figure 1).

The data obtained in these experiments can be compared with those obtained in the HPLC-MS/MS analysis (Figure 4 and Table 1). After 90 min reaction time in the presence of Cu²⁺ and MC, 80% of PrP₇₆₋₁₁₄ (20 μ M) undergoes modification with concomitant consumption of 1.10 mM MC, indicating approximately a ratio of 1 molecule peptide modified every 50 molecules MC reacted. Therefore, even an easy to oxidize external substrate does not protect the peptide from oxidation. In addition, as a consistent fraction of peptide undergoes modification by MC/MQ residues, this indicates that part of the reactive species formed during catalysis promoted by copper has the endogenous peptide as target.

SOD-like reactivity of Cu-PrP complexes. We also measured the SOD-like activity of Cu²⁺-PrP peptide complexes in comparison to the activity of free Cu²⁺ in solution. The activity was evaluated through the direct assay in which O₂⁻ is supplied as KO₂-crown ether complex, as we reported previously for Cu- β -amyloid^{22a} and Cu- α -synuclein^{23a} peptide complexes. As shown in Figure S17, neither PrP₇₆₋₁₁₄, nor PrP₈₄₋₁₁₄ and PrP₁₀₆₋₁₁₄ increase the SOD activity of free Cu²⁺, and this result supports the conclusions based on different SOD assays.¹⁵

CONCLUSIONS

We have shown that redox cycling of Cu-PrP complexes induced by reactive catechols, such as catecholamine neurotransmitters, produces a much more complex pat-

tern of peptide modifications than that resulting from simple reduction/reoxidation of Cu in the presence of reducing agents, that has been studied so far.²⁹ This is important in view of the emerging role of copper as neurotransmission modulator,³² and in particular its involvement in the control of neurotransmitter receptor activity.⁹ The present results emphasize the need of a strict homeostatic control of copper upon its release and recycle within the synaptic cleft. This control cannot be based only on high affinity binding of Cu²⁺ to proteins and peptides. In fact, the induction of ROS and more extensive post-translational modifications upon interaction of Cu with catecholamines occur even though copper may be bound to β -amyloid^{22b} or PrP. Although, it is worth emphasizing that the type of oxidative modifications occurring in hours on Cu- β -amyloid take only minutes in the case of Cu-PrP peptides in the same conditions. Apparently, only binding to *N*-acetylated α -synuclein²³ seems to be protective against the aberrant modifications produced on neuronal peptides as a result of Cu redox reactivity. Quinone modification, so far recognized to affect specific proteins involved in Parkinson's disease, such as α -synuclein³³ and parkin,³⁴ occurs easily on PrP peptides, and also on β -amyloid,^{22b} and may represent a more general mechanism occurring in neurodegenerative diseases, as a result of Cu-induced oxidative stress. As a matter of fact, dopaminergic neuron loss has been very recently reported to occur in Alzheimer's disease patients in brain regions different from the *substantia nigra*, which typically affects Parkinsonian subjects.³⁵ The effects of DA induced ROS on PrP were previously recognized on neuronal cells,^{21a} and Cu-loaded recombinant PrP₂₃₋₂₃₁ (Cu₅-PrP).^{21b} The treatment produced aggregation,^{21a} and protein carbonyls and fragmentation of the protein.^{21b} Other reports described ROS induced cleavage of PrP induced by copper(II) and reductants,³⁶ but in all these studies specific modifications were not investigated.

The (patho)physiological relevance of the interaction of DA (and more generally brain catecholamines), Cu and PrP is related to the simultaneous presence of the three partners upon extracellular DA release in glutamatergic synapses, particularly from degenerating DA neurons. Similar processes may be induced by other catecholamines. Simple protein/peptide modifications corresponding to the addition of a quinone molecule (or a few molecules) have not been identified (and probably have never been searched either) but are unlikely to be detected in the materials, e.g. amyloid deposits and plaques, obtained from diseased brains, because these materials contain derivatizations produced over very long time periods, typically several years, and therefore the initially formed peptide-quinone addition products will be further processed and oligomerized. However, the experimental signature of the involvement of quinone species derived from catecholamine neurotransmitters in protein modification is provided by neuromelanins, which are complex, insoluble substances consisting of covalently bound melanin, protein and lipid components.³⁷ Neuromelanin granules, in fact, have been detected in all major brain regions.³⁸ The

synapse clefts and glutamatergic terminals are then the plausible sites where neurotransmitters, metal ions, and amyloidogenic peptide fragments from APP and prion proteins can meet and interact.³⁹

MATERIALS and METHODS

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 μ m, 250 \times 10 mm). Mass spectra and LC-MS/MS data were obtained with a LCQ ADV MAX ion-trap mass spectrometer, with an ESI ion source. The system was run in automated LC-MS/MS mode and using a surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a Phenomenex Jupiter 4U Proteo column (4 μ m, 150 \times 2.0 mm). 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. NMR spectra were recorded on a Bruker AVANCE 400 spectrometer.

Peptide synthesis.

The two peptides PrP₈₄₋₁₁₄ (Ac-PHGGGWGQGGGTHSQWNKPSKPKTNMKHMAG-NH₂, mw 3297.65) and PrP₇₆₋₁₁₄ (Ac-PHGGGWGQPHGGWGQGGGTHSQWNKPSKPKTNMKHMAG-NH₂, mw 4074.45) were synthesized according to literature procedures.^{19ab}

PrP₁₀₆₋₁₁₄ (Ac-KTNMKHMAG-NH₂, mw 1057.5) peptide was synthesized using the standard fluorenyl methoxycarbonyl (Fmoc) solid-phase synthesis in DMF. Rink-amide resin MBHA (substitution 0.64 mmol/g) was used as polymeric support, which yielded the peptide amidated at the C-terminus.

After deprotection of the resin by treating the support twice, for 3 min and 7 min, with 20 ml of 20 % (v:v) piperidine in DMF, the first amino acid (2 mol equiv. vs. resin sites), was added in the presence of 2 equiv. of *N*-hydroxybenzotriazole (HOBt), 2 equiv. of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and \approx 2 equiv. of *N,N*-diisopropylethylamine (DIEA). 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; resin was washed by DMF, dichloromethane and isopropanol. 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, cold diethyl ether was added to precipitate the peptide and the mixture was filtered; 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 CH₃CN over 40 min (flow rate of 3 ml/min, loop 2 ml), as eluent. PrP₁₀₆₋₁₁₄ showed a retention time of \approx 21-22 min. The product was then lyophilized and stored at -30 °C until use. The identity of the peptide was confirmed by Electrospray ionization mass spectrometry (Thermo-Finnigan). ESI-MS data (direct injection, MeOH, positive-ion mode, capillary temperature 200 °C): *m/z* 1058 (PrP₁₀₆₋₁₁₄⁺), 530 (PrP₁₀₆₋₁₁₄²⁺), 358 (PrP₁₀₆₋₁₁₄³⁺).

Kinetics of oxidation of catecholic substrates.

Dopamine oxidation. The catalytic oxidation of DA by Cu²⁺ was studied at 20 °C in 50 mM Hepes buffer at pH 7.4, saturated with atmospheric oxygen. The reaction was monitored by UV-Visible spectroscopy through the absorption band of dopaminochrome at 475 nm. Firstly, DA autoxidation experiment and kinetic profile of the reaction catalyzed by Cu²⁺ was evaluated; the effect of PrP peptides on the copper-catalysed DA oxidation was studied by adding the peptide in variable stoichiometry (0-4 equiv. vs Cu²⁺) to the reaction solution. This experiments were carried out by adding copper(II) nitrate (25 μ M) as the last reagent to the solution containing DA (3 mM) and prion peptide (25-75 μ M). All measurements were performed in triplicate.

4-Methylcatechol oxidation. The catalytic oxidation of MC by Cu²⁺ was studied at 20 °C in 50 mM Hepes buffer at pH 7.4, saturated with atmospheric oxygen. The reaction was monitored by UV-visible spectroscopy through the absorption band of 4-methylquinone at 401 nm ($\epsilon = 1550 \text{ M}^{-1}\text{cm}^{-1}$). Copper(II) nitrate (25 μ M) and substrate (3 mM) were kept constant and PrP peptides were added at 0-75 μ M concentrations. All measurements were performed in triplicate. MC oxidation experiments were also performed under oxygen saturating conditions, obtained by bubbling extensively with dioxygen (1 atm) the buffer solution. The effect of substrate concentration was investigated by keeping constant the amounts of copper(II) (25 μ M) and PrP (25 μ M) and varying MC concentration from 0.15 to 4.0 mM.

Detection of hydrogen peroxide in the catalytic reactions.

The detection of hydrogen peroxide during the catalytic reaction was carried out by a peroxidase assay, as previously described,⁴⁰ with some minor modifications. The development of the characteristic I₃⁻ band (λ_{max} 353 nm; $\epsilon = 26000 \text{ M}^{-1} \text{ cm}^{-1}$), upon reaction with I⁻, was monitored. The oxidation reaction of MC in the presence of Cu²⁺ or Cu²⁺-PrP₇₆₋₁₁₄ complex was carried out as in the kinetic experiments. The reaction was quenched after 10 min with addition of H₂SO₄ to pH 2 and an equal volume of water was added. The quinone formed was extracted three

times using dichloromethane (removal of the quinone is necessary since it can oxidize iodide). The water layer, and one-half volume of a solution of KI (0.5 M) in water and horseradish peroxidase (about 200 nM) were added.

In the presence of hydrogen peroxide, a band due to the formation of I₃⁻ develops. Since atmospheric oxygen can oxidize I⁻, blank experiments (without catalyst or without catechol) were also performed. For the catalytic reactions performed in the presence of Hepes buffer or MC alone, almost negligible H₂O₂ accumulation was found. Instead, when the reaction was performed in the presence of copper(II) salt or Cu²⁺-PrP₇₆₋₁₁₄ complex, the assay revealed a concentration hydrogen peroxide corresponding to 2.8 % and 1.7 % of the initial catechol concentration, respectively.

Effect of hydrogen peroxide on the kinetics of MC oxidation.

In these experiments, the reactivity of free copper(II) (25 μ M) in MC (3 mM) oxidation in the presence of hydrogen peroxide (1 mM) was compared to that of copper(II) (25 μ M) and PrP₇₆₋₁₁₄ (25 μ M), in 50 mM Hepes buffer solution at pH 7.4 and 20 °C. The presence of H₂O₂ was found to increase slightly the rate of formation of MQ in both cases (Figure S12).

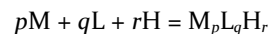
Identification and characterization of modified peptides by HPLC-ESI/MS.

The competitive peptide modification was studied by HPLC-ESI/MS, preparing samples in the presence of copper(II) nitrate (25 μ M), PrP (25 μ M) and MC (3 mM) in Hepes buffer (50 mM) pH 7.4. HPLC-ESI/MS analysis were performed at different reaction times. LC-MS data were obtained by using a LCQ ADV MAX ion-trap mass spectrometer. The elution of PrP₈₄₋₁₁₄ and PrP₇₆₋₁₁₄ was carried out 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; elution started with 98% solvent A for 5 min followed by a linear gradient from 98 to 55% A in 65 min. The elution of PrP₁₀₆₋₁₁₄ was performed by using pure distilled water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B).

Computed species distribution diagrams of copper(II)-PrP peptide complexes.

The species distribution as a function of the pH were obtained using the computer program Hyss.⁴¹

Species distribution diagrams of copper(II)-PrP₇₆₋₁₁₄, copper(II)-PrP₈₄₋₁₁₄, and copper(II)-PrP₁₀₆₋₁₁₄ systems are calculated according to the stability constants reported in potentiometric studies.^{19a,b,25} The overall stability constant refers to the following equilibrium:



and is expressed by the equation:

$$\beta_{pqr} = \frac{[M_p L_q H_r]}{[M]^p \cdot [L]^q \cdot [H]^r}$$

where β_{pqr} , $[M_p L_q H_r]$, $[M]$, $[L]$ and $[H]$ are the overall stability constants, the free concentrations of the complex, the metal ion, and the proton, respectively. Copper(II) hydrolysis constants and Cu-Hepes (Cu-B) stability constant are from ref. 42 and 43, respectively.

NMR quantification of oxidized catechol.

The consumption of MC was monitored recording proton NMR spectra of a solution containing Hepes buffer (5 mM) at pH 7.4 and MC (3 mM) in deuterium oxide at 20 °C. Integrated area of the peak at 3.7 ppm of Hepes buffer was used as internal standard for the determination of MC concentration, calculated from the area of the signal of proton C (Figure S14) at 6.55–6.60 ppm. The initial spectrum in the presence of Hepes and MC serves as time zero point of Table S6 and Figure 4. The acquisition was repeated after the addition of copper(II) nitrate (25 μ M) and Cu²⁺-PrP₇₆₋₁₁₄ complex (25 μ M). The initial data were obtained after 5 min, which is the time required to acquire the spectrum. The acquisition was repeated in the same conditions every 30 min until 150 min reaction time.

In order to overcome the low availability of dioxygen in the NMR tube, the reaction was carried out in an open vial and transferred to the NMR tube prior to each spectral recording. A blank experiment performed entirely in the NMR tube confirmed that the reaction rate of MC oxidation was much slower due to slow diffusion of dioxygen to replace its consumption in the solution.

Superoxide dismutase activity.

The SOD-like activity of [Cu²⁺-PrP] complex was compared to activity of free Cu²⁺ in solution. The activity was evaluated through the direct assay in which O₂⁻ is directly generated by dissolution of KO₂ salt (28 mM) in a solution of 18-crown-6 ether (80 mM) in anhydrous DMSO and detected by the reaction with nitro blue tetrazolium (NBT) (1.25 mM) in phosphate buffer, which forms methyl formazan (MF⁺), characterized by an intense absorption band at 560 nm.⁴⁴ After sonication, the solution becomes light yellow. UV-visible readings were performed in 50 mM phosphate buffer at pH 7.4 with the following final concentrations: 0.2 mM NBT, variable amounts of copper(II) (0–10 μ M) and [Cu²⁺-PrP] complex (0–10 μ M, with a 1:1 ratio of Cu/peptide), 0.4 mM KO₂ from the 18-crown-6 ether solution in DMSO. All measurements were performed in triplicate.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting figures, tables, kinetic experiments, copper-peptide species distributions, NMR and MS spectra, SOD activity experiments (PDF).

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Notes

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SYNOPSIS TOC (Word Style "SN_Synopsis_TOC").

Copper bound to prion peptides at the high affinity site adjacent to the octarepeats exhibits marked catalytic activity toward dopamine and 4-methylcatechol. Cu redox cycling and reactive quinones produce an extensive array of peptide oxidation and modification, yielding catechol adducts at the histidine binding ligands. Prion peptides appear to be much more sensitive to oxidative stress than β -amyloid and α -synuclein.

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