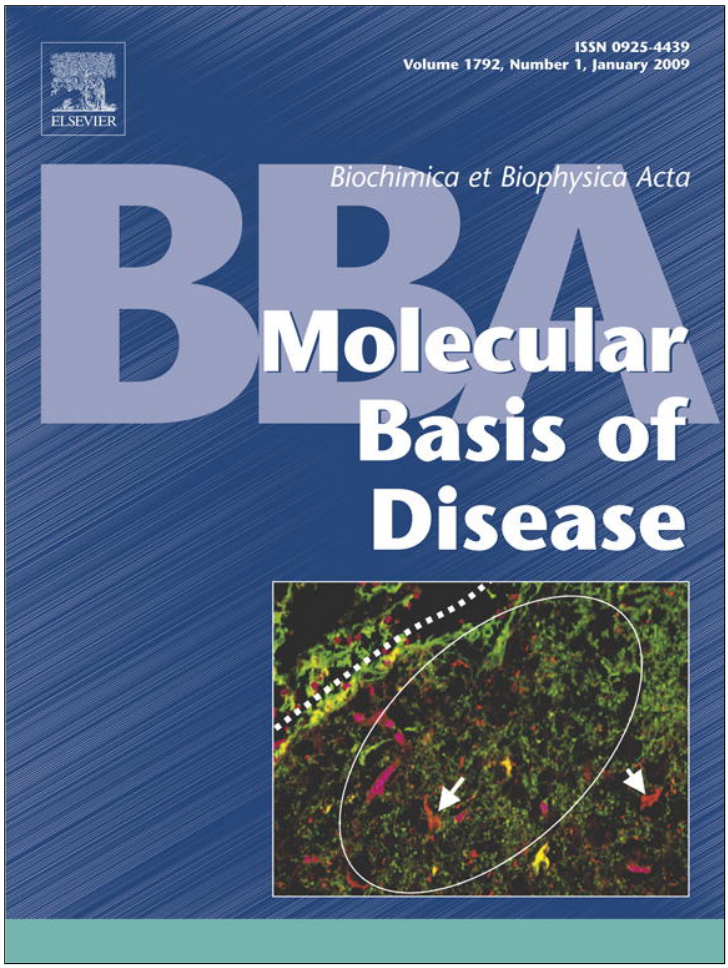

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Methionine does not reduce Cu(II)–β-amyloid!—Rectification of the roles of methionine-35 and reducing agents in metal-centered oxidation chemistry of Cu(II)–β-amyloid

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ABSTRACT

The potential risk of metal-centered oxidative catalysis has been overlooked in the research of the copper complexes of the Alzheimer's disease-related β-amyloid (Aβ) peptides. Cu^{2+} complexes of $A\beta_{1-40}$ and its 1–16 and 1–20 fragments have recently been shown to exhibit significant metal-centered oxidative activities toward several catecholamine neurotransmitters with and without H_2O_2 around neutral pH [G.F.Z. da Silva, L.-J. Ming, "Metallo-ROS" in Alzheimer's disease: metal-centered oxidation of neurotransmitters by Cu^{II}–β-amyloid and neuropathology of Alzheimer's disease, Angew. Chem. Int. Ed. 46 (2007) 3337–3341]. The results further support the metallo-Aβ-associated oxidative stress theory often considered to be connected to the neuropathology of the disease. The metal-centered oxidative catalysis of $CuA\beta_{1-16/20}$ challenges the long-standing proposed redox role of Met35 in Aβ because $A\beta_{1-16/20}$ do not contain a Met. External Met has been determined by kinetic, optical, and electron paramagnetic resonance methods to bind directly to the Cu^{2+} center of $CuA\beta_{1-40}$ and $CuA\beta_{1-20}$ with K_d = 2.8 mM and 11.3 μM, respectively, which reflects less accessibility of the metal center in the full-length $CuA\beta_{1-40}$. However, Met does not serve as a reducing agent for the Cu(II) which thus must amplify the observed oxidative catalysis of $CuA\beta_{1-20}$ *through a non-redox mechanism*. Conversely, the CuAβ-catalyzed oxidation reaction of dopamine is inhibited by bio-available reducing agents such as ascorbate (competitive K_{ic} = 66 μM) and glutathione (non-competitive, K_{inc} = 53 μM). These data indicate that the oxidation chemistry of metallo-Aβ is not initiated by Met35. The results yield further molecular and mechanistic insights into the roles of metallo-Aβ in this disease.

1. Introduction

Oxidative stress has been a key topic of research concerning cancer, aging, heart diseases, arthritis, diabetes, and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (AD) [1–6]. Mechanisms proposed for the neurodegeneration in the brain of AD patients generally focus on the β-amyloid peptide (Aβ), a proteolytic product of 40–42 amino acids of the ubiquitously distributed amyloid precursor protein (APP), and its complexes of redox-active metal ions [7,8]. The "Aβ cascade hypothesis" suggests that Aβ aggregates trigger a complex pathological cascade which leads to neurodegeneration in AD [9], including generation of H_2O_2 [10–12], free-radical induced oxidation [13–15] such as lipid peroxidation [16], and the involvement of Met-35 as a reducing agent [17–22] in the redox chemistry of metallo-Aβ. A central focus of the neuropathology of AD thus has been the effects of redox-active transition metal ions and reactive oxygen species (ROS), such as superoxide, hydroxyl free radical, and H_2O_2 [7,8]. Although physiological processes responsible for dealing with ROS can be up-regulated to tackle variations in oxidative stress [23,24], long-term effects of such chemical imbalance have been speculated [1–5]. Some AD treatment strategies have targeted the metal center in metallo-Aβ to prevent peptide aggregation and ROS generation [25–28] and the role of the metal therein was proposed [29,30]. However, comparatively little effort has been focused on the mechanism for the metal-centered chemistry, besides the production of H_2O_2, which renders direct substrate binding and oxidation by the metal center. If metal-mediated ROS generation is one of the leading causes of oxidative stress, the formation of ROS at the metal site as well as the reactivity of the "metallo-ROS" center [31] in CuAβ may

Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's diseases; APP, amyloid precursor protein; EPR, electronparamagnetic resonance; ESEEM, electron spin echo envelope modulation; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate; NMR, nuclear magnetic resonance; NQI, nuclear quadrupole interaction; ROS, reactive oxygen species

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contribute to the oxidative stress suspected to take place in AD [1–5]. Nevertheless, there are still debates on different views about the role of metalloamyloids even very recently [32].

Although A-beta accumulation is not likely to be the only factor involved in neuronal cell death, transgenic mouse models with human A-beta have provided significant insights into the neuropathology of AD and behaviors of AD patients, including Aβ aggregation and loss of memory [33–35]. Rodent Aβ has been shown to exhibit redox activity in vitro that was attributed to ROS generation via Met35 [36], even though the metal-binding residues were not fully conserved in rodent Aβ (i.e., His13→Arg). Since Aβ activity and aggregation in AD brains is sequence-specific and metal-dependent [7,8], it is a priority to establish the targets of redox activity that can contribute to the physiological and cognitive effects of AD. We have recently established that the Cu^{II} complexes of Aβ and its soluble fragments ($Aβ_{1-16}$ and $Aβ_{1-20}$) showed considerable activities toward the oxidation of phenol, polyphenol, catechol, and their derivatives to form *o*-quinones via a type-3 copper-centered mechanism [31,37,38]. Such reactivities can explain the mechanism for the formation of dityrosine in Aβ [39] via activation of the phenol side chain and also challenge the redox role of Met35 that is not present in the fragments.

Herein we present kinetic and spectroscopic investigations of the oxidation chemistry of $CuAβ_{1-20}$ and the influences of exogenous methionine and reducing agents on its oxidative activity. The results corroborate metal-centered oxidative stress and shed light on the mechanistic roles of Met-35 and reducing agents in the oxidation chemistry of CuAβ.

2. Materials and methods

The 1–20 fragment of Aβ was synthesized by the use of the Fmoc chemistry at the Peptide Center of the University of South Florida, and the identity of the peptide confirmed with a Bruker MALDI-TOF mass spectrometer. $Aβ_{1-40}$ was obtained from EZBiolab (Westfield, IN). The Cu(II) complexes of Aβ were prepared by addition of 1 equivalent of Cu(II) directly into the peptide solution under experimental conditions, wherein the $Aβ_{1-40}$ peptide was dissolved with sonication followed by removal of the undissolved residue with centrifugation. Dopamine, ascorbic acid, and glutathione (GSH) were obtained from Sigma-Aldrich (St. Louis, MO), 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) from Acros (Fairlawn, NJ), and H_2O_2, EDTA, L-methionine, and $Cu(NO_3)_2$ from Fisher Scientific (Suwanee, GA). All plastic ware and glassware were demetallized with EDTA and extensively rinsed with 18.0-MΩ deionized water.

The catechol oxidase assay toward dopamine was performed as previously reported [31,40]. Equal concentrations of dopamine and the *o*-quinone indicator MBTH were mixed in 100 mM HEPES at pH 7.00 in a final volume of 1.0 mL. The MBTH-adduct of *o*-quinone was monitored at 505 nm ($ε$=27,200 M^{-1} cm^{-1}) and 25 °C for 5–10 min with a Varian CARY50 Bio-UV–Vis spectrophotometer equipped with a CARY PCB-150 Water Peltier temperature regulation system, and the rates determined by the change in absorbance over time. The rates were fitted to appropriate rate laws, such as the Michaelis–Menten-like kinetics [41], and rate constants determined with non-linear regression. The effects by H_2O_2, ascorbate, GSH, and L-Met were determined similarly in the presence of different amounts of the corresponding reagent and the inhibition/dissociation constants determined accordingly. Binding of L-Met to $CuAβ_{1-20}$ was performed by direct titration of Met into $CuAβ_{1-20}$ in 100 mM HEPES at pH 7.0 and monitored with the CARY50 spectrophotometer. NMR measurements were performed on a Varian Unity-500 spectrometer.

EPR experiments of the samples under the conditions of the kinetic experiments were performed on a Bruker Elexsys E580 cw/pulsed X-band spectrometer. A typical cw EPR spectrum was obtained with a microwave frequency of 9.4 GHz, field modulation ~2 G, and time constant of 40–80 ms at ~5–6 K. The g and A tensors were obtained with numerical fittings using the "easyspin" toolbox for Matlab [42]. ESEEM (electron spin echo envelope modulation) spectra were recorded with the usual π/2-τ- π/2-T- π/2 pulse sequence and a π/2 pulse of 20 ns. A typical ESEEM spectrum consists of 1024 points taken at time intervals of 8 ns. The transient was first base-line corrected by subtracting the ordinary T_1 exponential decay function and any remaining constant baseline offset, then zero-appended to 2048 points. A Hamming window function was then applied to the time-domain spin-echo envelope, followed by Fourier transformation to afford the frequency-domain spectrum.

An ESEEM spectrum can reveal those nuclei having super-hyperfine coupling with the Cu^{II} center, including coordinated His side chains and water [43]. In the case of coordinated His side chains, the super-hyperfine coupling arises from the electron-nuclear interactions and the nuclear quadrupole interactions (NQI) of the remote non-coordinated nitrogen (^{14}N, I=1) on the imidazole ring of coordinated His side chains. At X-band, three zero-field nuclear quadrupole resonance lines $\nu_{\pm}$ and ν_o are observed which can be determined from the NQI lines in the ESEEM spectrum (Eqs. (1) and (2)), wherein e^2qQ is the quadrupole coupling constant and η the asymmetry parameter (η=0 for a complete axial symmetry and 1 for a pure rhombic symmetry).

$$\nu_{\pm} = 1/4(e^2qQ)(3\pm\eta) \quad (1)$$

$$\nu_o = 1/2(e^2qQ)\eta \quad (2)$$

The e^2qQ and η values can be obtained from the NQI lines in the ESEEM spectra and Eqs. (1) and (2), i.e., $(\nu_+ - \nu_o/2)/3 = {}^1/_4(e^2qQ)$. In the case of coordinated water, the super-hyperfine coupling can arise from deuterium atoms of deuterated water. The deuterium Zeeman interaction at X-band is much larger than the isotropic component of the electron-nuclear coupling. Thus, the deuterium peak in the ESEEM spectrum is found at the deuterium Zeeman frequency and split slightly by the electron-nuclear coupling.

3. Results

Dopamine is oxidized by $CuAβ_{1-20}$ in the absence of H_2O_2 with rate constants k_{cat}=0.0104 s^{-1} and K_m=0.89 mM (lowest trace ●, Fig. 1A), consistent with the metal-centered pre-equilibrium kinetics previously observed [31]. Addition of the amino acid L-Met to the reaction solution significantly increases the oxidative activity (Fig. 1A, B), which seems to also induce slight cooperativity, e.g., the activity profile with saturating amount of Met gives a Hill's coefficient θ=2.3 (trace □, Fig. 1A). The activity reaches a plateau at high [Met], indicating direct Met binding to $CuAβ_{1-20}$ in the presence of the substrate (S) dopamine. Fitting of the k_{cat} values as a function of [Met] to a simple equilibrium of [CuAβ-S + Met→Met-CuAβ-S] (which is followed by product formation) gives an affinity constant of 3900 M^{-1} (i.e., K_d=0.26 mM). Met follows a non-essential activation pattern toward dopamine oxidation by $CuAβ_{1-20}$ as shown in the Lineweaver–Burk plot (Fig. 1C), i.e., both $CuAβ_{1-20}$-S and Met-$CuAβ_{1-20}$-S complexes are active. From the data, the dissociation constants K_{Ac} and K_{Au} for the complexes Met-$CuAβ_{1-20}$ and Met-($CuAβ_{1-20}$-S) can be obtained to be 0.087 and 0.125 mM, respectively. The latter is close to the value obtained from the fitting of the activity with respect to Met binding to the pre-equilibrium complex ($CuAβ_{1-20}$-S) from the kinetic measurements.

The activity is further enhanced in the presence of H_2O_2. Note that both Met and H_2O_2 can activate dopamine oxidation and are not exclusive of each other, but showing a combined effect, i.e., the k_{cat} value of 0.180 s^{-1} in the presence of saturating amount of Met and H_2O_2 (Fig. 1D) is a combination of 0.088 s^{-1} for Met activation and 0.099 s^{-1} for H_2O_2 activation. This observation reflects plausible independent activation mechanisms of Met and H_2O_2. A much more

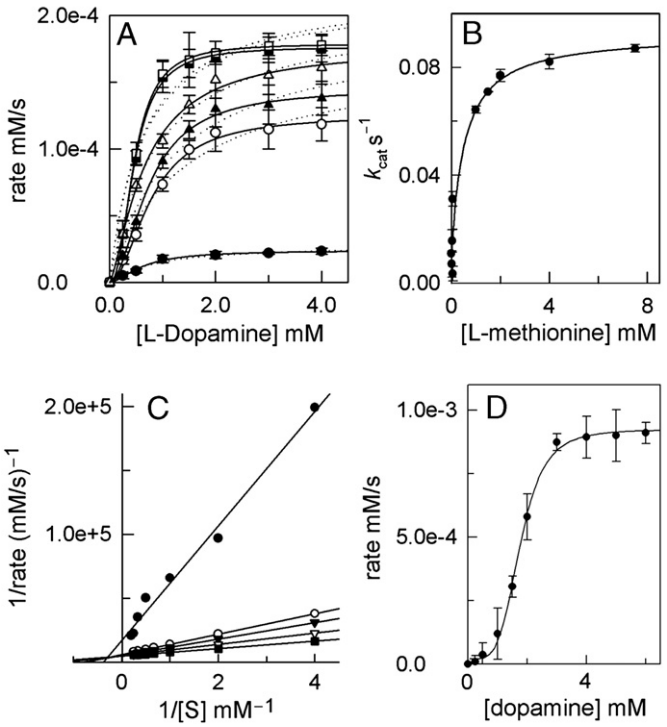

Fig. 1. (A) Rates of dopamine oxidation by 2.75 μM CuAβ$_{1-20}$ with [Met] of 0, 1.0, 2.0, 4.0, 7.5, and 10.0 mM (from bottom) in 100.0 mM HEPES at pH 7.0 and their fitting with the Michaelis–Menten equation (dotted traces) and with the Hill equation (solid traces), which yields the rate constants for the k_{cat}-vs.-[Met] plot in (B). (C) The Lineweaver–Burk plot which clearly shows a non-essential activation pattern of Met (0, 1.0, 2.0, 4.0, 7.5, and 10.0 mM from top) toward dopamine (S) oxidation under the same conditions as in (A). (D) Rate of dopamine oxidation by 5.0 μM CuAβ$_{1-20}$ in the presence of saturating amounts of L-Met and H_2O_2 as in (A).

significant cooperativity is observed in this case than the reaction without H_2O_2 (Fig. 1A), yielding θ=4.1.

As opposed to Met, the reducing agents ascorbic acid and GSH act as inhibitors toward dopamine oxidation by CuAβ$_{1-20}$ (Fig. 2), however, with different inhibition patterns. Herein, GSH was determined to be a non-competitive inhibitor with an inhibitor constant K_{inc}=53 μM; (Fig. 2A); whereas ascorbic acid was revealed to be a competitive inhibitor with an inhibition constant K_{ic}=66 μM (Fig. 2B).

Upon addition of Met, the electronic spectrum of CuAβ$_{1-20}$ exhibits an intense charge-transfer transition at 316 nm (Fig. 3). Such charge-transfer transition is not observed in the full-length CuAβ$_{1-40}$,

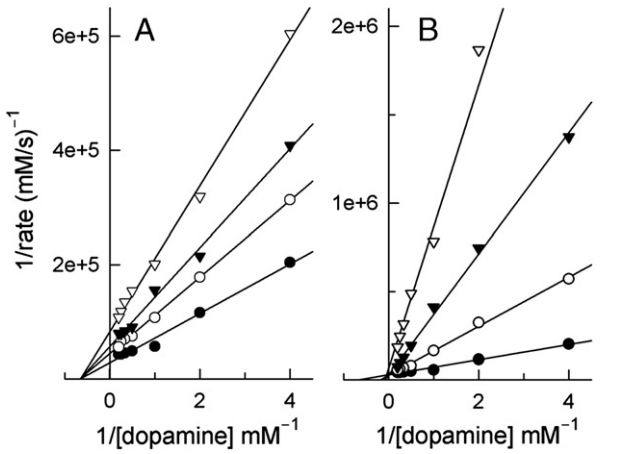

Fig. 2. (A) Glutathione (10.0 μM, 20.0 μM, and 40.0 μM from bottom) and (B) ascorbic acid inhibition (0.0, 0.12, 0.55, and 0.95 mM from bottom) toward dopamine oxidation by 2.75 μM Cu–Aβ$_{1-20}$ in 100.0 mM HEPES pH 7.0.

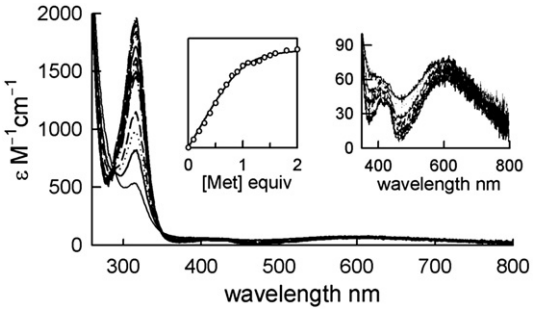

Fig. 3. Optical titration of L-methionine into 0.20 mM CuAβ in 100.0 mM HEPES pH 7.0. The insets show the d–d transition and fitting of the data to Met binding to CuAβ$_{1-20}$ with a stoichiometry of 1:1.

suggesting that Met35 does not interact with the Cu^{II} center. The d–d transition of CuAβ$_{1-20}$ at 600 nm is not significantly affected in the presence of saturating amounts of Met (Fig. 3; inset). The change in the intensity of the charge transfer transition as a function of [Met] can be fitted to the 1:1 binding pattern of [CuAβ$_{1-20}$ + Met→Met-CuAβ$_{1-20}$] to yield a dissociation constant K_d=11.3 μM (Fig. 3, inset).

The X-band EPR spectrum of CuAβ$_{1-20}$ can be well fitted to an axial magnetic environment with $g_{//}$=2.268, $g_{\perp}$=2.064, $A_{//}$=547, and $A_{\perp}$=51.1 MHz (Fig. 4A). These values are consistent with those reported for Cu^{II} complexes of Aβ and fragments [44], e.g., $g_{//}$= 2.265–2.269, $g_{\perp}$=2.059–2.062, and $A_{//}$=465–577 MHz. As opposed to the dramatic change in the electronic spectrum, the EPR spectrum changes only slightly to $g_{//}$=2.257, $g_{\perp}$=2.059, $A_{//}$=565, and $A_{\perp}$=35.8 MHz upon Met binding (Fig. 4A).

The X-band ESEEM spectrum of CuAβ$_{1-20}$ (solid trace; Fig. 4B) reveals three ^{14}N NQI lines at ν_o=0.33, ν_-=1.11 (shoulder), and ν_+=1.45 MHz (Eqs. (1) and (2)), the double-quantum transitions at ~4 Mz, and the combination lines at 2.33, 2.95, and 3.45 MHz. From which, an average e^2qQ value is obtained to be 1.71 MHz, a value found for a coordinated His [43], and a small η value of 0.39. The ν_- line can be better determined once the values of e^2qQ and η are determined from ν_o and ν_+. At least one coordinated water is revealed which attributes to the deuterium modulation at 2.29 MHz when the spectrum was acquired from a sample in D_2O buffer excited at 3391 G (dashed trace, Fig. 4B), consistent with ^{2}H resonance of 2.21 MHz at this field. This small discrepancy may be attributed to the presence of a small super-hyperfine coupling [43]. A spectrum for Met-bound CuAβ$_{1-20}$ in D_2O buffer was acquired under the same conditions (dotted trace, Fig. 4B) to investigate the status of the coordinated water upon Met binding. The ^{14}N NQI lines change only slightly, found at ν_o=0.15, ν_-=1.22 (shoulder), and ν_+=1.37 MHz which afford e^2qQ=1.73 MHz and a small η=0.17 (which seems too small that might be attributed to the large uncertainty of the small ν_o value). The deuterium line is observed at 2.19 MHz when excited at 3351 G, right

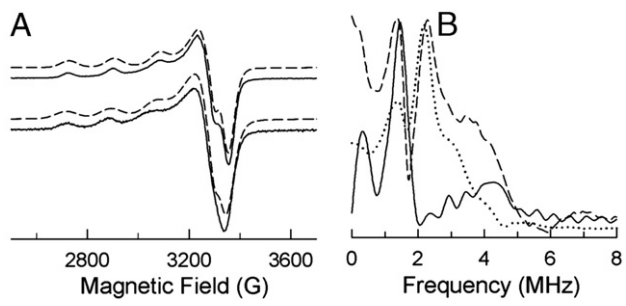

Fig. 4. (A) X-band EPR spectra of CuAβ$_{1-20}$ in the presence (top) and absence (bottom) of L-Met and the simulated spectra (dashed traces). (B) ESEEM spectra of 1.0 mM CuAβ$_{1-20}$ in 50.0 mM HEPES buffer in H_2O pH 7.0 (solid trace) and in D_2O (dashed trace) at pH(D) 7.0 and after addition of 8.0 (mM) Met (dotted trace).

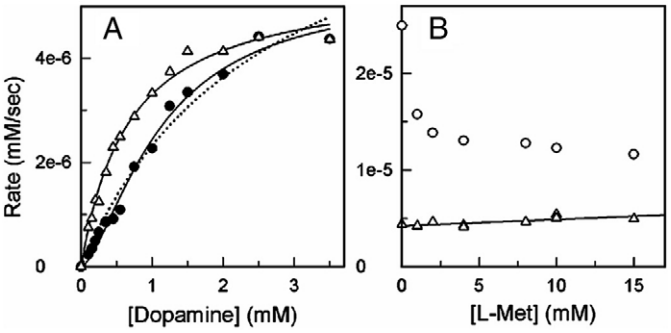

Fig. 5. (A) Oxidation of dopamine by 2.8 μM CuAβ$_{1-40}$ in the air in the presence (●) and absence (Δ) of 15 mM Met at pH 7.0 (HEPES 0.1 M). The solid traces are fittings to the Hill equation while the dotted trace is to the Michaelis–Menten equation. (B) The influence of Met on the oxidation of 5 mM dopamine by 2.8 μM CuAβ$_{1-40}$ in the presence (○) and absence (Δ) of 40 mM H_2O_2 at pH 7.0 (HEPES 0.1 M).

at the ^{2}H resonance frequency at this field which reflects a negligible super-hyperfine coupling.

The Cu(II) complexes of Aβ$_{1-40}$ and its fragments (1–16 and 1–20) have been demonstrated to exhibit type-3-copper-like oxidative activities in our previous studies; however, subtle differences among these CuAβ complexes of different lengths are still present, such as their different activities toward catechol- and phenol-containing substrates as well as interactions with and activations by lipid micelles to different extents [31,37,38]. Thus, it is also important to determine the influence of Met on Cu–Aβ$_{1-40}$ to reveal the similarities and differences from its 1–16 and 1–20 fragments. The oxidation of dopamine by Cu–Aβ$_{1-40}$ affords $k_{cat}=1.98\times10^{-3}$ s^{-1} and $K_m=0.68$ mM (Δ; Fig. 5A), comparing to $k_{cat}=0.0104$ s^{-1} and $K_m=0.89$ mM for dopamine oxidation by Cu–Aβ$_{1-20}$ under the same conditions (lowest trace ●, Fig. 1A). The presence of 15 mM Met does not significantly change the oxidative activity toward dopamine (●; Fig. 5A), affording $k_{cat}=2.9\times10^{-3}$ s^{-1} and $K_m=2.6$ mM (or fitted with the Hill equation to afford $k_{cat}=1.91\times10^{-3}$ s^{-1} and $K_m=1.2$ mM and a slight cooperativity with $\theta=1.54$). At most less than 15% increase in activity was observed at [dopamine]=5 mM and [Met]=15 mM (Δ; Fig. 5B). Conversely, the addition of Met decrease the enhancement effect of 40 mM H_2O_2 (○; Fig. 5B), affording an activity only about twice higher than that in the absence of H_2O_2 at [Met]=15 mM (Δ; Fig. 5B). The binding of Met to CuAβ$_{1-40}$ was monitored via electronic transitions, wherein the increase in intensity at ~300 nm as a shoulder reaches a saturation at >3 mM (Fig. 6) with a dissociation constant of 2.8 mM (inset, Fig. 6), analogous to the results in Met titration (○; Fig. 5B), which is much larger than that for Met binding to Cu–Aβ$_{1-20}$ (11.3 μM; inset plot in Fig. 3). Moreover, the d–d transition at ~620 nm is shifted to ~720 nm at [Met]>3 mM (inset, Fig. 6), different from the persistent d–d transition upon Met binding to Cu–Aβ$_{120}$.

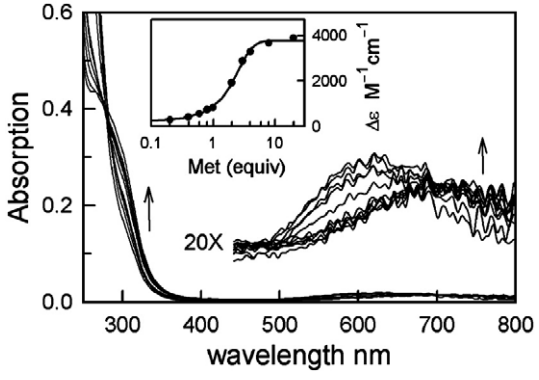

Fig. 6. Change in electronic spectrum of CuAβ$_{1-40}$ (0.14 mM in 0.1 M HEPES at pH 7.0) upon addition of L-Met and the plot of the change in the absorption of the shoulder at 300 nm therein (inset).

4. Discussion

Metabolic malfunctions of catecholamine neurotransmitters have been suggested to be related to the neuropathology of AD [45–48]. Of these neurotransmitters, dopamine is also known to directly link to the neurodegenerative Parkinson's disease [49] and has been proposed to be associated with adult neurogenesis in the subventricular zone [50,51]. We have recently shown that CuAβ can catalyze oxidation of catecholamine neurotransmitters, including dopamine [31], to generate neurotoxic quinone products [52–54]. The oxidation of catechol and phenol and their derivatives by the Cu^{II} complexes of Aβ$_{1-40}$, Aβ$_{1-20}$, and Aβ$_{1-16}$ in the presence or absence of H_2O_2 was demonstrated to be consistent with the mechanism of type-3 copper oxidases such as di-Cu catechol oxidase (Fig. 7) [31,37,38]. Therein, the catechol-containing substrate binds to a presumed transient di-Cu^{II} active center (step ***i***) and is oxidized to *o*-quinone via 2-electron transfer to afford a di-Cu^{I} active center (**C** and step ***ii***). The di-Cu^{I} center can bind O_2 (step ***iii***) to form a μ-η^2:η^2 peroxo-Cu$^{II}_2$ center shown in **D** (or its isoelectronic counterparts, oxy-di-Cu^{I}, μ-η^1:η^1-hydroxyperoxo-di-Cu^{II}, and bis-μ-oxo-di-Cu^{III}) as demonstrated in enzyme [55] and chemical model systems [56,57], which may bind and oxidize the substrate (steps ***iv*** and ***v***). The presence of a reducing agent such as ascorbic acid can thus facilitate the aerobic pathway to yield the di-Cu^{I} active center (**C**) ready for O_2 binding and H_2O_2 production (step ***vi***) [10,12]. Moreover, the inhibitory effects of ascorbic acid and GSH described herein point to a different mechanism for their action. The binding of ascorbic acid as a competitive inhibitor to the metal in CuAβ that prevents the oxidative catalysis is also consistent with the "antioxidant" role of CuAβ toward ascorbate oxidation previously suggested [58]. A short-cut route analogous to the "peroxide shunt" in heme-containing peroxidase [59] can take place to form the μ-peroxo-Cu$^{II}_2$ center upon H_2O_2 binding (step ***vii***), followed by substrate binding and oxidation (step ***iv*** and **E**). The ternary complex **E** can also be formed upon H_2O_2 binding to the intermediate **B** (step ***viii***), representing a random bi-substrate mechanism.

Met$_{35}$ in Aβ$_{1-40}$ has been suggested to serve as a reducing agent [17–22] responsible for the initiation of the redox cycling of the Cu^{II} center in CuAβ$_{1-40}$ which leads to H_2O_2 production (i.e., step ***vi*** in

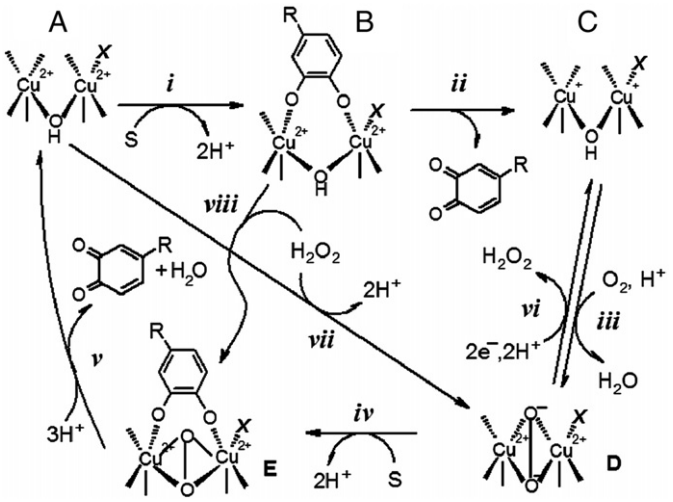

Fig. 7. One plausible mechanism for the catechol oxidase-like activity of CuAβ toward the oxidation of dopamine. The route ***vii***, ***vi***, and ***v*** represent a "peroxo shunt" pathway. X is an exogenous ligand such as a bound Met. A monodentate bridging peroxo/hydroxyperoxo binding mode may also be an appropriate intermediate at **D** and **E** to reduce the coordination crowding upon binding of Met, H_2O_2, and the substrate, wherein the proton of the hydroxyperoxo can be obtained from the catechol substrate.

Fig. 7) [10,12]. The oxidation of the thioether moiety of Met to its sulfoxide form in $A\beta_{1-40}$ has been proposed to be involved in aggregation, lipid peroxidation, and redox chemistry in association with the metal center [17–22]. In the case of the $CuA\beta_{1-20}$ complex, the activation profile of [Met] (Fig. 1) and the optical spectrum (Fig. 3) indicate direct Met binding to the metal in the complex which potentially can reduce the Cu^{II} center as proposed previously for Met35. In this case, the amount of di-Cu^{I} (**C**) would increase which in turn would increase the amount of the μ-peroxo-Cu^{II}_2 center upon O_2 binding (**D**; step ***iii***) and complete the catalytic cycle upon substrate binding (***iv*** and ***v***). Once $CuA\beta_{1-20}$ is reduced, it cannot follow the peroxide shunt pathway anymore. Our observation that H_2O_2 can still significantly enhance the activity in the presence of a saturating amount of Met (Fig. 1B) and has an additive effect with Met on the activity suggests that the Cu^{II} center is not significantly reduced by Met. Moreover, the difference in electrode potentials between Cu^{II}-Aβ and Met is 0.71 V [60], which gives a dramatic 68.5 kJ difference in Gibbs free energy that is equivalent to a negligibly small equilibrium constant of 9.8×10^{-13} for one-electron reduction of Cu^{II}-$A\beta_{1-20}$ by Met at 298 K. The binding of Met to Cu^{II}-$A\beta_{1-20}$ does not render Met oxidation within a couple of weeks under the experimental conditions based on its virtually unchanged methyl NMR signal (data not shown), further concluding the non-redox active role of Met. Thus, the observed Met35 oxidation in $CuA\beta_{1-40/42}$ is most likely to be attributed to other mechanisms which may not be observable under our experimental conditions and time frame, rather than being a reducing agent directly involved in the Cu-centered oxidation chemistry. In the case of the $CuA\beta_{1-40}$ complex (Figs. 5 and 6), the external Met does not significantly enhance the activity as in the case of $CuA\beta_{1-20}$ and exhibits a competitive manner with H_2O_2 as opposed to the additive pattern in the latter case. This observation suggests that the folding of the peptides chain in these two complexes may be different which renders the metal active center less accessible in the case of $CuA\beta_{1-40}$ which results in significant decrease in Met affinity and less room for the binding of both the external ligands, Met and H_2O_2. This hypothesis is consistent with the observation that $A\beta_{1-40}$ has a higher tendency to coagulate more than its short fragments upon metal binding.

The presence of the charge transfer band (Fig. 3) is indicative of direct Met binding to the Cu^{II} center. This charge transfer transition is consistent with a thio-to-Cu^{II} charge-transfer transition observed in Cu^{II}-methionine complexes [61]. The K_d value of 11.3 μM deviates from the K_{Ac} value obtained from the activity profile discussed above (87 μM), indicating that there are other factors that affect the Met binding to the Cu^{II} center when monitored with activity, such as the cooperativity discussed above (i.e., the formation of the reactive intermediate or synergy due to multiple substrates). The oxidation state of a Cu^{II} center in $CuA\beta_{1-20}$ upon Met binding can be concluded from the electronic and EPR spectra, wherein the detection of d–d transition at 600-nm (Fig. 3) and EPR features (Fig. 4A) indicate that Cu^{II} is not reduced by Met to initiate the oxidative reaction. Met35 was previously suggested to be a reducing agent which initiated the oxidation chemistry of $CuA\beta_{1-40}$ [17–22]. The observation herein is consistent with the kinetic results that a Met, internal or external, is not needed for the metal-centered oxidation reaction to take place (Fig. 1). In the case of $CuA\beta_{1-40}$, the lack of a Met-to-Cu(II) charge-transfer transition at ~300 nm in the absence of external Met clearly indicate the lack of interaction of the internal Met35 with the metal center. Moreover, the addition of external Met does not create a charge-transfer transition at ~300 nm, but only a shoulder concomitant with a change in the d–d transition. The results indicate binding of Met to the metal center of $CuA\beta_{1-40}$ does not change the oxidation state of the metal center while the small affinity constant of 360 M^{-1} (K_d=2.8 mM) once again suggests a less accessible metal center for external ligand binding.

The non-exclusive nature of Met and H_2O_2 binding to and additive activation toward $CuA\beta_{1-20}$ indicate that there are two pathways for the oxidation of dopamine in the presence of Met and H_2O_2, i.e., the pathways ***i–v*** and ***vii–iv–v*** with a bound Met (the "X" ligand in Fig. 5), wherein the enhancement of the activity with Met alone is possibly due to a non-redox mechanism that fine-tunes the reduction potential of the Cu^{II} to favor the oxidative catalysis. The sigmoidal activity profiles for dopamine oxidation in the presence of Met (Fig. 1A) and with both H_2O_2 and Met (Fig. 1D) reflect a possible presence of substrate-mediated cooperativity, such as the formation of the dinuclear active center (Fig. 5; **E**). Despite the lack of a Met and any redox-active amino acid, the fragments $CuA\beta_{1-16}$ and $CuA\beta_{1-20}$ exhibit significant metal-centered oxidative activity [31,37,38] which indicates that the redox role of Met35 might have been overstretched. Instead, it may simply be a substrate during the oxidative catalyses by metallo-Aβ. Moreover, the activity and optical studies of Met binding and influence on the metal center in $CuA\beta_{1-40}$ further suggest that Met35 may not necessarily be involved in the action of this Aβ complex and its smaller 1–16 and 1–20 fragments.

The catalytic pathway of $CuA\beta_{1-20}$ is altered under reduction conditions, wherein the reaction is locked into a H_2O_2-producing cycle (***iii*** and ***vi***, Fig. 5). The competitive inhibition of ascorbate toward the oxidation of dopamine by $CuA\beta_{1-20}$ may be because of possible chelation and reduction of the Cu^{II} center by ascorbate. GSH may bind to and reduce the Cu^{II} as a monodentate ligand which does not prevent substrate from binding to the Cu^{II} center to form the inactive inhibitor–CuAβ–substrate complex, thus exhibiting non-competitive pattern (Fig. 2A). The inhibitory effect of these reducing agents toward metal-centered catalysis is consistent with the proposed mechanism wherein H_2O_2 is generated (step ***vi***) [10,12].

The EPR spectrum of $CuA\beta_{1-20}$ can be attributed to a typical tetragonally distorted Cu^{II} center, i.e., an elongation along the z axis due to the Jahn–Teller effect. The EPR spectral features upon Met binding to $CuA\beta_{1-20}$ confirm the retaining of the d^9 electron configuration of the Cu^{II} center. The lack of $\Delta M_S=\pm2$ transition, i.e., between the $M_S=-1$ and +1 levels in an antiferromagnetically coupled di-Cu^{II} center [62,63] in the EPR spectrum at low field indicates magnetic coupling between the Cu^{II} centers does not exist. Thereby, the previously proposed [64] His-bridged dinuclear Cu,Zn-superoxide dismutase center for $CuA\beta_{1-28}$ is not present in the case herein. The dinuclear catalysis observed in the kinetic measurement must be a transient state during the reaction in the presence of the substrate. The small change in the EPR spectrum upon Met binding indicates the charge transfer transition must be due to the binding of the thioether of Met through the non-magnetic d_{z2} orbital at an axial position of $CuA\beta_{1-20}$. Otherwise, more significant changes in the g factor and the $A_{//}$ tensor would be observed as reported for the binding of thio-groups to Type-2 Cu proteins [65–69].

The ESEEM spectrum of $CuA\beta_{1-20}$ confirms the binding of Cu^{II} to $A\beta_{1-20}$ through His side chains via the magnetic $d_x2_{-y}2$ orbital in equatorial positions which gives rise to the quadrupole coupling with the remote non-coordinated ^{14}N on the coordinated His imidazole ring. There are at least two coordinated His side chains as reflected by the combination lines since a single coordinated His does not give rise to these lines [66,70]. Very small η values are observed for the amine-N of imidazole when H-bonding is scarce [71], which may be the case for the coordinated His in CuAβ. The detection of a deuterium line at 2.29 MHz suggests the presence of at least one coordinated water (as D_2O), presumably in an equatorial position bound to the Cu^{II} via the magnetic d_{x2-y2} orbital [43]. This signal is not much affected upon Met binding, suggesting Met binding to an axial position. The signal at 4.6 MHz seems to be attributable to the double-frequency peak of two or more coordinated water. The significant decrease in the intensity of this signal upon Met binding indicates that one or more weakly coupled deuterium atoms in the Cu^{II} coordination sphere are replaced upon Met binding.

Since the binding of Met to $CuA\beta_{1-20}$ does not reduce the Cu^{II} center, the enhancement in activity must be attributed to a change in the reduction potential of the Cu^{II} center. The axially coordinated Met

ligand in blue copper proteins has been suggested to play a role in controlling the reduction potential of the protein since mutation of this Met results in a significant change in the potential [72,73]. Similarly, the binding of Met to CuAβ_{1-20} may modulate the Cu^{II}/Cu^{I} potential to favor the oxidation catalysis, yet is not necessarily directly involved in the redox chemistry as a reducing agent. The oxidation of Met35 previously observed may result from the metal-centered oxygenation reaction discussed herein, i.e., Met binds to the metal center (the X in Fig. 5) and serves as a substrate via step ***iv*** and ***v*** without the catechol substrate. If occurred, this reaction would be very slow and beyond the detection limit under the conditions herein.

5. Concluding remarks

Although reducing agents can potentially inhibit the oxidation of neurotransmitters such as dopamine by CuAβ_{1-20}, yet not to the full extent under physiological conditions [31], their capability in the production of H_2O_2 may exacerbate the situation of oxidative stress. Oxidation of dopamine to yield quinone can result in polymerization of tau protein into fibrils [74,75] and covalent modification of dopamine transporter which directly affects dopamine uptake [76]. Since dopamine plays important roles in behavior and cognition, such as delay-period activity, memory, attention, mood change, pain processing, and motivation, as well as motor activity, long-term oxidation of dopamine may be expected to render physiological and psychological imbalance in the human brain. Moreover, the association of dopamine motor activity and coordination seems to be a common factor that may explain the Parkinsonian-like symptoms in AD patients. Hence, the acceleration of dopamine oxidation via metal-centered mechanism that can be modulated by small molecules such as reducing agents, H_2O_2, and/or Met and Met-containing peptides suggests a significant role of CuAβ complexes in the mechanism of oxidative stress in the brain of AD patients. The results herein further support the metallo-ROS-centered redox chemistry of CuAβ_{1-20} [31,37,38] and provide additional structural and mechanistic information for the oxidative stress in the brain of AD patients. The role of Met35 has been redefined and the dual mechanistic roles of reducing agents (i.e., inhibition of metal-centered oxidation and H_2O_2 generation) in the redox cycle of CuAβ_{1-20} are clearly shown. Although CuAβ_{1-40} and its fragments exhibit similar reactivities, subtle differences are still present which suggests further investigations and comparison are needed for both CuAβ_{1-40} and its fragments to gain a comprehensive understanding of the molecular and mechanistic basis for their roles in AD. The overall picture of AD neuropathology is likely to be composed of the pieces of information uncovered thus far, including generation of ROS, metal-dependent aggregation of Aβ, and the largely overlooked metal-centered degradation of biomolecules. Treatment and prevention strategies toward the oxidative stress in AD hence must address all of these pathways, including inhibitions toward H_2O_2 production and oxidative damage of neurotransmitters and other biomolecules by CuAβ. Further studies targeted toward the elucidation of the metallo-ROS species are being pursued to determine the species responsible for the oxidative activity of CuAβ_{1-20}.

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