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Alzheimer's Disease Related Copper(II)-β-Amyloid Peptide Exhibits Phenol Monooxygenase and Catechol Oxidase Activities**

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Over the past few years an enormous effort has been directed toward the investigation of the metal-dependent mechanisms that lead to the neuropathology of Alzheimer's disease (AD).[1] The self-assembled metallo-β-amyloid (Aβ) peptide fibrils are the hallmark of this disease[2] and have been attributed to Fe^{III}- and Cu^{II}-centered generation of H_2O_2 under reducing conditions. The latter, H_2O_2, has been postulated to be of significant importance in connection with neuropathy in AD.[3,4] However, an area of oversight has been the detailed chemical processes associated with the neuropathology of AD, besides the generally acclaimed assault by ROS (reactive oxygen species; e.g. H_2O_2).[5] Hence, a better understanding of metal-centered redox chemistry and the mechanism for the generation of ROS and their fate can provide insight into potential strategies for the prevention and treatment of AD.

Several examples of redox chemistry in biological systems are known to be associated with di- or multinuclear "Type-3" Cu oxidases,[6] which may be related to the redox activity of Cu^{II}Aβ.[1–4] A number of chemical model systems that target Type-3 copper centers have successfully been demonstrated to contain highly active isoelectronic copper–dioxygen species (i.e. Cu^{II}_2-μ-η^1:η^1-peroxo, Cu^{II}_2-μ-η^2:η^2-peroxo, and Cu^{III}_2-bis-μ-oxo), which are responsible for copper-dependent oxidation and hydroxylation reactions.[6–9] Despite extensive modeling studies, peptide mimics of these enzymes have apparently been excluded from the studies. Cu^{II}Aβ seems to fill the gap as it is a naturally occurring Cu–peptide complex demonstrated to exhibit oxygen-associated redox chemistry,[1–4] although details about its oxygen binding and activation mechanisms are lacking. Herein, we present results which bring together two distinct fields of research: Alzheimer's disease and Type-3 copper centers. The results elucidate that the Cu^{II} complex (CuAβ_{1-20}) of the icosapeptidyl metal-binding domain of Aβ (DAEFR[5]HDSGY[10]EVHHN[15]-KLVFF[20]) exhibits metal-centered redox chemistry that is consistent with the mechanisms of the Type-3 copper enzymes, namely, phenol monooxygenase (e.g. tyrosinase) and catechol oxidase.

The metal-centered redox chemistry of CuAβ_{1-20} was probed using catechol and the more-inert phenol as substrates.[10,11] The oxidation of catechol under aerobic conditions reached a plateau at low mM concentrations, and the saturation profile fits well to pre-equilibrium kinetics [Eqs. (1) and (2)][12] to afford the rate constant $k_{cat} = 0.154\ s^{-1}$, the dissociation constant $K'_{app} = 0.35$ mM (Figure 1a), and a significant second-order rate constant $k_{cat}/K'_{app} = 440\ M^{-1}s^{-1}$ (cat = catalytic, app = apparent). As the formation of quinone from catechol is a two-electron oxidative process, the reaction is expected to follow the two-electron dinuclear reaction pathway for catechol oxidase,[13] wherein the binding of catechol to the active-site dicopper(II) center results in the reduction of the center to yield dicopper(I) with concomitant production of *o*-quinone. The reduced dicopper(I) center can bind dioxygen to afford the active peroxo-bridged dicopper(II) center, which can further oxidize a subsequently bound substrate. H_2O_2 can also be generated in this reaction pathway from the peroxo-bridged dicopper(II) center in the presence of a reducing agent such as the substrate itself. This pathway for the production of H_2O_2 under reducing conditions is consistent with previous observations in AD studies.[3] The catechol oxidase like mechanism has also been observed in kinetic studies of several chemical model systems[14] and in the oxidation of polyphenols by CuAβ_{1-20}.[15] Note that a recent study based on density functional theory (DFT) pointed to mixed-valence Cu^{II}–Cu^{I} transition states,[16] which support the suggested reduction pathway for the Cu center.

We previously determined the Cu^{II}/Aβ_{1-20} stoichiometry as 1:1 for oxidative activity with three N_ε-coordinated imidazole histidine rings as metal-binding ligands.[15] As activity is an excellent probe for determining stoichiometry, gradual replacement of Cu^{II} centers in CuAβ_{1-20} with redox-inactive Zn^{II} centers can serve as a practical method for addressing the nature of the active metal center by virtually "silencing" the active sites through dilution with Zn^{II}. A quadratic correlation between the activity and the extent of Zn^{II} dilution should be observed for simple 1:1 metal binding if there is no cooperativity and/or interactions between the Cu^{II} centers at the active site with different Aβ strands. Conversely, a sigmoidal activity profile was observed as a function of the mole fraction of Cu^{II} in Aβ_{1-20} toward the oxidation of the catechol derivative 3,5-di-*tert*-butyl catechol (DTC, $k_{cat} = 0.411\ s^{-1}$ and $K'_{app} = 0.781$ mM) which can be fitted well to the Hill equation [Eq. (3)][17] with a Hill coefficient of $\theta = 2.40$ and $r^2 = 0.99$ (Figure 2a, solid line). The data clearly cannot be fitted well to a quadratic equation for 1:1 noncooperative binding mode ($r^2 = 0.91$; dashed line, Figure 2a). These results imply the possible presence of a cooperative dinuclear active Cu^{II} center during the catalytic

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[**] G.F.Z.S. dedicates this work to his parents for their unyielding support. The authors dedicate this work to the Kelley and Sotolongo families for their unfortunate experience with Alzheimer's disease. Our research on the structure and activity of metallopeptides was partially supported by the Research and Creative Award of the University of South Florida and the Petroleum Research Fund administrated by the American Chemical Society (PRF AC #40851-AC3). We thank John Kelley for the modification of the picture for the Table of Contents.

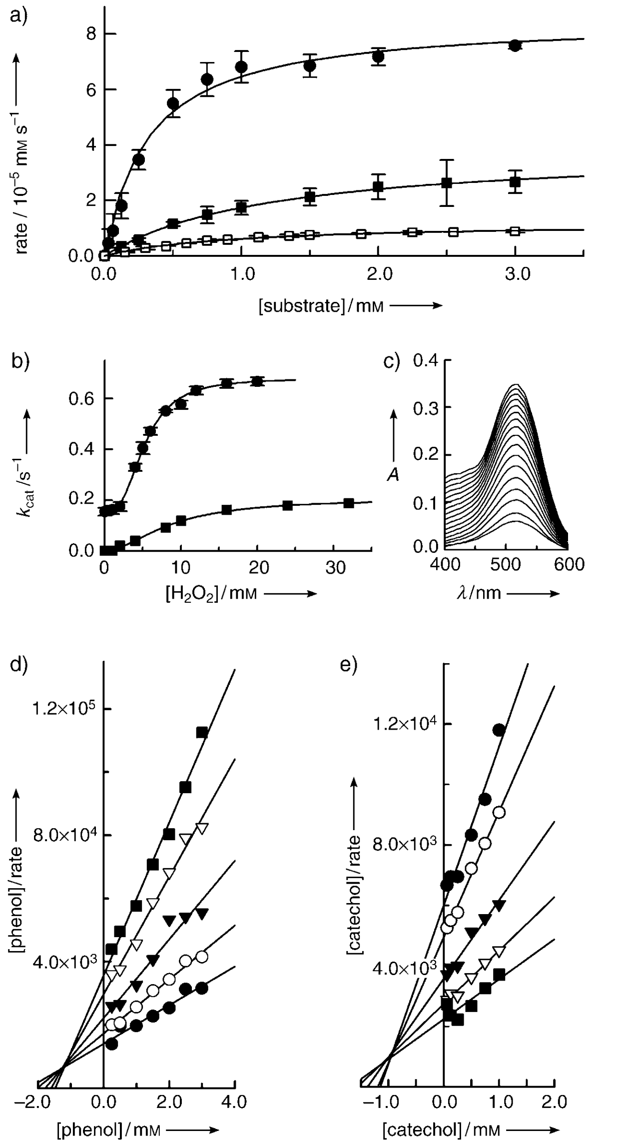

Figure 1. a) Saturation profile for the oxidation of phenol (■), deuterated phenol (□), and catechol (●) in the absence of H_2O_2. b) The effect of the concentration of H_2O_2 on the first-order rate constant k_{cat} toward the oxidation of phenol (■) and catechol (●). c) The production of *o*-quinone from phenol in the absence of H_2O_2 is monitored by the increase in the absorption as a result of the formation of its adduct with 3-methyl-2-benzothiazolinone hydrazone (MBTH). d,e) Hanes plot analysis of kinetic data from parts (a) and (b), with [H_2O_2] = 0, 5, 10, 20, and 30 mM from top to bottom.

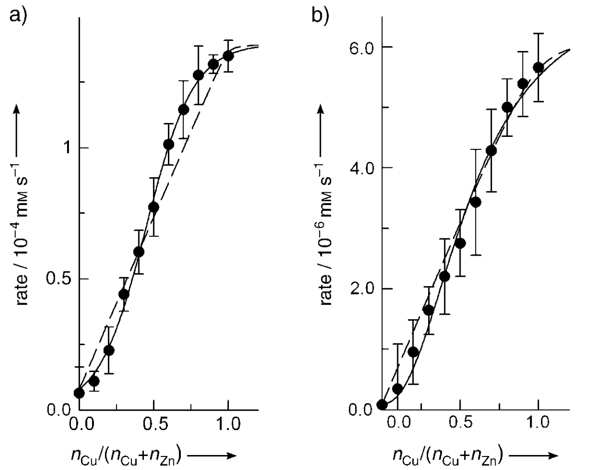

Figure 2. Oxidative activity of CuAβ_{1-20} toward the oxidation of a) DTC and b) phenol in the absence of H_2O_2 as a function of the mole fraction of Cu^{II} at a constant total concentration of Cu^{II} and Zn^{II} at pH 7.0 and 25 °C. The solid lines show the fit to the Hill equation, and the dotted lines are the fits to a quadratic binding pattern with a metal/ligand ratio of 1:1.

oxidation of catechol by CuAβ_{1-20}, consistent with the catalytic cycle of catechol oxidase.[13]

The presence of the reactive oxygen species H_2O_2 (25 mM) significantly enhances the turnover and catalytic efficiency of CuAβ_{1-20} toward catechol oxidation, yielding $k_{cat} = 0.531\ s^{-1}$ and $K'_{app} = 0.342$ mM, and a significant second-order rate constant $k_{cat}/K' = 1.51\times10^3\ M^{-1}s^{-1}$ from the Hanes plot [Eq. (4) and Figure 1e][18,19] for a random two-substrate reaction, in which the binding of the two substrates H_2O_2 and catechol are independent of each other. Note, the second-order rate constant for catechol oxidation is only about 20 times lower than that for catechol oxidase extracted from gypsywort.[20] This pathway is consistent with the so-called peroxide shunt in the action of catechol oxidase in the presence of H_2O_2 for which a Cu^{II}_2-μ-η^2:η^2-peroxo intermediate is proposed to be the active species.[6] The oxidation of catechol to form *o*-quinone in the absence and presence of H_2O_2 exhibits remarkable rate accelerations (3.25×10^5 and 1.12×10^6 fold, respectively) in terms of the first-order rate constant k_{cat} relative to that for aerobic autooxidation of catechol in the absence of CuAβ_{1-20} ($k_o = 4.74\times10^{-7}\ s^{-1}$).

Owing to their inertness, metal-centered hydroxylation of phenol and its derivatives, particularly polychlorophenols, poses some challenging tasks in chemical synthesis and environmental detoxification and remediation[21] and may provide further insight into the action of those metalloenzymes for the monooxygenation of arenes.[8,22] Besides the oxidation of catechol described above, phenol was hydroxylated and oxidized by CuAβ_{1-20} in the presence of a saturating amount of H_2O_2 (> 50.0 mM). The formation of *o*-quinone exhibited rate and dissociation constants of $k_{cat} = 0.213\ s^{-1}$ and $K'_{app} = 1.31$ mM, respectively, and $k_{cat}/K' = 457\ M^{-1}s^{-1}$ from the Hanes plot (Figure 1a and d). This result represents a remarkable 4.6×10^6-fold rate acceleration for the hydroxylation/oxidation of phenol to form *o*-quinone in terms of k_{cat} relative to that for aerobic autooxidation of phenol ($k_0 = 4.6\times10^{-8}\ s^{-1}$). This reaction is expected to take place according to a dinuclear mechanism similar to the hydroxylation and oxidation of tyrosine by the dicopper enzyme tyrosinase, in which the active center is believed to contain dinuclear μ-η^2:η^2-peroxo-Cu^{II}_2 species on the basis of spectroscopic studies.[8,16]

Cooperativity of H_2O_2 in the oxidation of both catechol and phenol in terms of k_{cat} is observed upon titration of H_2O_2 and is reflected in the sigmoidal activity profile with respect to the concentration of H_2O_2 (Figure 1b). The data from the oxidation of catechol and phenol by H_2O_2 fit well to the Hill equation [Eq. (3)], yielding Hill coefficients of $\theta = 2.23$ and 1.78, respectively. Moreover, fitting of the rates to a random two-substrate reaction mechanism yields corrected K' values of 2.07 and 2.10 for phenol and catechol, respectively, and a cooperativity index based on the ratio K'_{app}/K' of 1.70 for H_2O_2 in both phenol and catechol oxidation reactions. These values suggest a small cooperativity of H_2O_2 and the independent binding of H_2O_2 and phenol or catechol to the active center.

Of particular interest is the observation of a scarcely reported process, namely, aerobic Cu^{II}-centered hydroxylation and oxidation of phenol in the absence of H_2O_2.[23] The production of *o*-quinone from phenol catalyzed by $CuA\beta_{1-20}$ follows pre-equilibrium kinetics, yielding $k_{cat} = 3.90 \times 10^{-3}\ s^{-1}$ and $K'_{app} = 1.23$ mM (Figure 1a) that represent an 8.67×10^4-fold first-order rate acceleration with respect to aerobic autooxidation of phenol. The k_{cat} value is lower than that for catechol oxidation and indicates that the hydroxylation step here must be the rate-limiting step. Otherwise, these two reactions would have similar k_{cat} values attributed to the oxidation of a bound catechol upon hydroxylation of a bound phenol. Moreover, the use of deuterated phenol as substrate reveals a significant kinetic isotope effect (KIE); k_{cat} values of 1.12×10^{-3} and $0.0442\ s^{-1}$ are obtained in the absence and presence, respectively, of H_2O_2 (100 mM) and represent KIE values of 3.46 and 4.77, respectively. The results indicate that hydroxylation and cleavage of the *o*-C–H bond of phenol is the rate-limiting step, which is followed by a faster step to form *o*-quinone. The different KIE values for the hydroxylation of phenol in the presence and absence of H_2O_2 suggest that the rate-determining step in these two cases may be different and/or possibly involve additional pathways. The K'_{app} values are not significantly different between phenol and deuterated phenol (1.31 and 1.23 mM for the latter in the presence and absence of H_2O_2, respectively) which suggests that k_{cat} does not significantly contribute to the magnitude of K'_{app} and that the two substrates may have a similar binding mode. The mechanistic reasoning for the conversion of phenol to *o*-quinone may be attributed to the fact that the Cu^{II}/Cu^{I} redox equilibrium can be achieved upon phenol binding, followed by electron transfer to afford a Cu^{I}–phenol radical, which is thought to be stabilized through resonance structures with delocalization of the free radical at the *ortho* and *para* positions[23a] (Figure 3, step a). This intermediate is then attacked by dioxygen and followed by electron transfer to possibly form a Cu^{II} superoxide center which may be further stabilized by a dinuclear center (step b). It was proposed previously that the free radical was attached directly by triplet dioxygen[23a] which, however, is not symmetrically favorable. Coupling of the superoxide radical to the bound phenol radical at an *ortho* position is then expected to be a favorable step (step c), which is followed by transfer of electrons and an oxygen atom to afford the final quinone product (step d). Involvement of a dinuclear center for the catalysis is possible as discussed below.

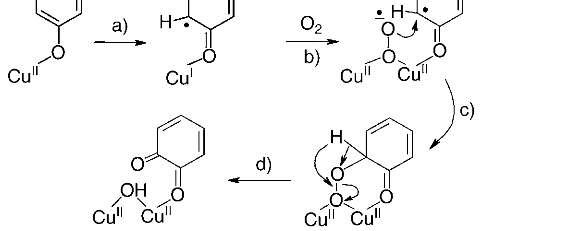

Figure 3. Proposed mechanism for aerobic hydroxylation and oxidation of phenol in the absence of H_2O_2, with the binding of phenol and reduction of the metal center as the key step (a). The binding of dioxygen and formation of superoxide (b) is proposed to be assisted by a dinuclear center. See text for details.

As the hydroxylation and oxidation of phenol is a multi-electron-transfer process, the involvement of two metal centers is suspected. The activity profile for phenol oxidation is similar to that for the Zn^{II} dilution experiment for catechol oxidation. The data can be fitted equally well to the Hill equation [Eq. (3)] to afford $\theta = 1.80$ ($r^2 = 0.98$) and a quadratic equation for single-metal binding ($r^2 = 0.98$), consistent with either a mononuclear oxidation[23] or a cooperative mechanism involving a dinuclear center,[16] or a combination of both pathways.

To monitor substrate binding, a "slow" substrate, 4,5-dichlorocatechol (DCC), which is approximately 200 times slower than catechol in terms of k_{cat}, was titrated into a solution of $CuA\beta_{1-20}$ (0.2 mM) in the presence or absence of H_2O_2 (100 mM) and monitored by UV/Vis spectroscopy at 25°C at pH 7.0 (Figure 4). Similar spectra were obtained

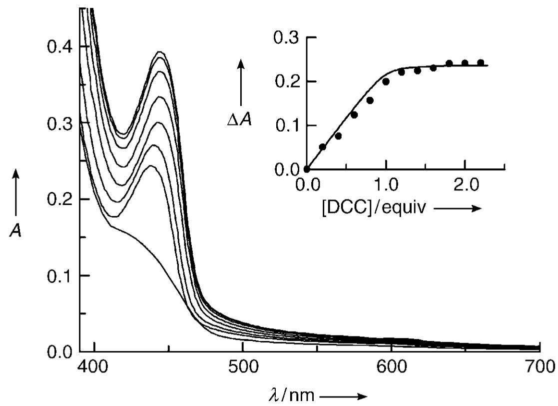

Figure 4. Titration of DCC to CuAβ (0.2 mM) in the presence of H_2O_2 (100 mM) in HEPES buffer (100 mM) at pH 7.0 monitored by UV/Vis spectroscopy. Analogous spectra were obtained in the absence of H_2O_2. The inset shows the change in absorbance at $\lambda = 437$ nm as a function of the number of equivalents of DCC added with respect to $(Cu^{II}A\beta)_2$, consistent with the formation of a 1:1 DCC–$(Cu^{II}A\beta)_2$ adduct.

through the course of the titration, indicating that H_2O_2 was not involved in the binding of DCC to $Cu^{II}A\beta$ under the experimental conditions. The absorption at 437 nm increased upon addition of DCC to $(Cu^{II}A\beta)_2$ and reached saturation at more than 1.2 equivalents of DCC, and the data were fitted to a single-substrate binding model to yield a dissociation constant of 0.24 mM. The result provides direct evidence for catechol binding to a dicopper(II) center, consistent with the observations with a chemical model[24] and the mechanism proposed above.

In conclusion, we have established the catalytic activities of $CuA\beta_{1-20}$ toward the relatively inactive species (according to their k_0 values) catechol and phenol in the presence and absence of H_2O_2 in aqueous solutions at near-physiological conditions. The reaction patterns are consistent with the mechanisms carried out by Type-3 copper centers as observed with catechol oxidase and tyrosinase and their dinuclear model systems.[8,22] So far these results are unique in metal-centered redox chemistry related to Alzheimer's disease and are expected to offer further insight into the neuropathology of this disease, as it is suspected to be linked with, besides many other factors, the oxidation of mono- and diphenol-containing neurotransmitters such as dopamine, epinephrine, norepinephrine, and serotonin.[25,26] Furthermore, the association of this highly reactive Cu–oxygen chemistry with Alzheimer's disease can better define the role of metallo-β-amyloids in the neuropathology of this disease and possibly lead to different treatment strategies toward this disease.

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[10] Phenol hydroxylation/oxidation and catechol oxidation were performed as previously reported[11] with minor changes to suit the current studies. The same molar concentrations of phenol or catechol and 3-methyl-2-benzothiazolinone hydrazone (MBTH, an indicator of *o*-quinone) were mixed in HEPES buffer (100 mM) at pH 7.00 in the presence of $CuA\beta_{1-20}$ at 5.0 μM (phenol hydroxylation/oxidation) or 0.50 μM (catechol oxidation). The red adduct of the *o*-quinone product was monitored at 500 nm ($\varepsilon = 32{,}500\,M^{-1}cm^{-1}$; see Figure 1c). The autooxidation of phenol and catechol were determined under the same conditions in the absence of $CuA\beta_{1-20}$. The 1–20 fragment of Aβ was synthesized at the Peptide Center of the University of South Florida. The identity of the peptide was confirmed with a Bruker MALDI-TOF mass spectrometer.

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[12] Rates were determined on a Varian CARY50 spectrophotometer equipped with a temperature controller. The rate law for pre-equilibrium kinetics [Eq. (1)], as followed by $Cu^{II}A\beta$, is shown in Equation (2), in which k_{cat} and K'_{app} are the first-order rate constant and apparent dissociation constant, respectively, S is the substrate, P is the product, and v_{bg} is the autooxidation rate.

$$Cu^{II}A\beta + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A\beta Cu^{II}\text{–}S \xrightarrow{k_{cat}} Cu^{II}A\beta + P \quad (1)$$

$$v = v_{bg} + \frac{k_{cat}[CuA\beta][S]}{K'_{app}+[S]} \quad (2)$$

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[17] The Hill equation can serve as a general model for cooperativity as reflected by the Hill coefficient θ, which deviates from unity in the presence of cooperativity [Eq. (3); K_x is the intrinsic dissociation constant].

$$\frac{v_0}{v_{max}} = \frac{[CuA\beta]^{\theta}}{K_x + [CuA\beta]^{\theta}} \quad (3)$$

[18] In case of two-substrate catalysis, such as phenol hydroxylation/oxidation and catechol oxidation in the presence of H_2O_2, both substrates can interact with the metal center independently. The data are fitted to the Hanes plot [Eq. (4)] to yield true values of substrate dissociation constants K'.[19]

$$\frac{[S]}{v_0} = \frac{(1+K'_{\alpha}/[H_2O_2])}{v_{max}}[S] + \frac{K'}{v_{max}}\left(1+\frac{K_i}{[H_2O_2]}\right) \quad (4)$$

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