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Heme−Cu Bound Aβ Peptides: Spectroscopic Characterization, Reactivity, and Relevance to Alzheimer's Disease

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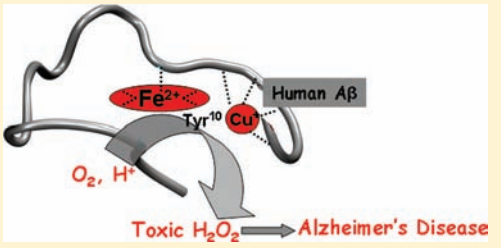
Supporting Information

ABSTRACT: Recently, it has been shown that heme binds to Aβ peptides which may play a major role in Alzheimer's disease (AD). This study illustrates that Aβ peptides can bind both Cu and heme cofactors at the same time. Both cofactors have unique spectroscopic and electrochemical features which are unaffected in the presence of the other, implying that they are electronically, chemically, and electrochemically uncoupled. These data clearly indicate that Cu cannot bind to three histidine residues simultaneously in Cu−Aβ complexes as previously proposed, since one of the histidines is involved in binding heme. The heme−Aβ and the heme−Cu−Aβ peptide complexes function as peroxidases. Interestingly, the Cu−Aβ complex also exhibits peroxidase activity, which may have significant implications in AD. Both Cu^{+}−Aβ and heme (Fe^{2+})−Aβ complexes reduce O_2 to H_2O_2 quantitatively. Only one of the two electrons that are required for the reduction of O_2 to H_2O_2 is derived from the reduced metal site, while the Tyr^{10} residue of the native Aβ peptide donates the second electron. This Tyr^{10} residue, the source of electron for the generation of partially reduced oxygen species (PROS, e.g., H_2O_2) is absent in rodents, which do not get affected by AD. When both heme and Cu are bound to the Aβ peptides, which is likely to happen physiologically, the amount of toxic PROS generated is maximum, implying that heme−Cu−Aβ complexes could potentially be most toxic for AD.



1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative pathology resulting in the most common form of dementia. According to a current estimate, large fractions of the elderly people are suffering from AD.[1] The characteristic pathological feature of AD is the large accumulation of amyloid beta (Aβ) peptides in the brain.[2−4] Aβ peptide is generated from a large transmembrane amyloid precursor protein (APP) by proteolysis.[5] Aβ is normally found in biological fluids and contains 39−42 amino acid residues.[6] Two enzymes β and γ secretases cleave APP to form Aβ.[7] The length of the Aβ peptide is related to amyloidogenesis and Aβ(1−42) forms the highest fraction of the amyloid deposition.[3] Recent studies suggest that small soluble oligomeric forms or pore like proto fibrils are more toxic compared to fibrillar forms.[8,9] The hypothesis of self-aggregation of Aβ alone is insufficient to explain the accumulation of Aβ in specific regions of the brain. Transition metals, Zn^{2+}, Cu^{2+} and Fe^{3+} (to a lesser extent), have been invoked to be responsible for this aggregation of the Aβ peptides, since they are found at much higher concentrations in the neocortex of the brain, the region that is affected by AD.[10−13] It has also been shown that Aβ aggregates from post-mortem AD affected tissues, when treated with metal chelators, produce soluble Aβ peptides, further indicating the role of these transition metals in aggregating these peptides.[14,15]

The redox active transition metals, Fe and Cu, generate toxic reactive oxygen species (ROS), causing oxidative stress (Scheme 1),[16,17] which are associated with AD and are in fact believed to precede the formation of the amyloid aggregates and hence could be the cause for the early signs of AD.[3] In the presence of the reduced transition metal ions (Fe^{2+}, Cu^{+}), there is spontaneous generation of freely permeable, neurotoxic partially reduced oxygen species (PROS), for example, $HO_2^{\bullet-}$, H_2O_2, HO·, and so forth.[18,19] Ascorbate (vitamin C), α-tocopherol (vitamin E), or glutathione can act as endogenous reducing agents.[1] Highly reactive hydroxyl radicals formed in this process generate lipid peroxidation adducts and nucleic acid adducts which are characteristics of AD pathology.[20−23] Often in the absence of external reducing agents, Aβ side chains also get oxidized, forming proteolysis resistant soluble dimers cross-linked possibly via tyrosine or histidine residues of the Aβ peptides.[24] However, the exact nature and mechanism of dimerization is poorly understood.

A large number of *in vitro* and *in vivo* studies support the direct involvement of the transition metals in AD.[25] It is well documented that all the metal binding sites are in the N-terminal hydrophilic region of the Aβ peptide.[26,27] The contribution of Zn^{2+} ions to the amyloid deposition in transgenic mice (note, normally rodents do not show AD) is strong evidence of its plaque forming properties.[28−30] Zn has been proposed to be coordinated by four to six ligands in Zn^{2+}−Aβ complexes. NMR studies indicate that three histidine residues (i.e., His^{6}, His^{13}, and His^{14}) and the carboxylate side chain of Glu^{11} are involved in Zn ligation.[31−33] Asp^{1} and water are likely to be the other ligands.[34]

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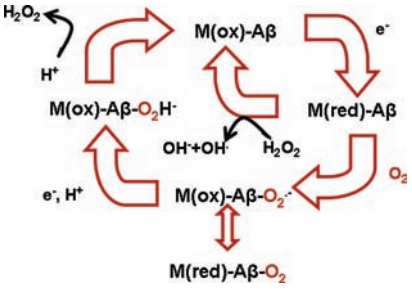
Scheme 1. A Schematic Representation of Transition Metal Induced ROS Generation[a]



[a] M = transition metals like Fe and Cu.

Tyr[10] and Arg[5] are not proposed to be associated with Zn binding.[35]

Unlike Zn^{2+}, Cu^{2+} can either accelerate or inhibit amyloidogenesis depending on the condition and type of aggregated state.[36] Though Cu^{2+} induced Aβ deposition is still questionable, it plays a significant role in the generation of ROS (Scheme 1) via a Fenton type reaction.[18,27] The coordination environment of Cu^{2+} bound Aβ has been controversial and a number of models have been suggested. Initial studies suggest that the Cu^{2+} bound Aβ peptide has a 3N1O coordination environment.[37,38] The 3N coordination[39] can either be from three histidine residues,[17] or two histidine residues and the N-terminus of the peptide.[27,40] The O binding residue can be donated by Asp[1], Asp[7], Glu[3], Glu[11] or it can be aqueous buffer derived.[41] More recent studies indicate that Cu−Aβ complexes contain two different species, component I and component II which are in equilibrium in the pH range 6−9. Different coordination environments have been proposed for these two components based on various spectroscopic data. HYSCORE data suggests the coordinating ligands for component I to be two histidine residues (His[6] and His[13]/His[14]), one N-terminus residue, and the carboxylate group of Asp[1].[42] Component II has been proposed to be comprised of three histidine residues along with the carboxylate group of Asp[1].[42] Two other models for component I have been reported having a similar Cu^{2+} coordination environment as above, but having an additional fifth ligand. One such study reports the carbonyl group of the peptide linkage of Asp[1]−Ala[2] as the fifth ligand using various EPR spectroscopic techniques like CW, ESEEM, pulse ENDOR, and pulse HYSCORE, while another group proposes the peptide backbone carbonyl of the remaining histidine group to be the fifth ligand based on ^{13}C and ^{1}H NMR.[43−45] The corresponding component II has been proposed to consist of one histidine (any of the three histidines available), the carbonyl group of the amide linkage of Ala[2]−Glu[3], a deprotonated nitrogen of an amide bond, and the N-terminus and the carboxylate group of Asp[1].[43−45] Yet another group reports binding of Cu^{2+} through the N-terminus along with His[6], His[13], and the carbonyl moiety of Asp[1] in component I and by three histidine ligands along with the carbonyl oxygen atom of the amide bond of Ala[2]−Glu[3] in component II using HYSCORE spectroscopy on isotopically labeled peptides.[46] Cu^{+}−Aβ is the reactive species that is directly involved in ROS formation.[47] As a result, determination of the coordination environment of Cu^{+}−Aβ has also been of interest. Two groups proposed a linear bis-His coordination to Cu^{+} through His[13] and His[14] using EXAFS and IR spectroscopy.[48−50] Cu^{+} coordination has also been studied by NMR spectroscopy.[51]

Recent studies invoke that heme is associated with AD. It has been shown that heme binds to the Aβ peptides forming heme−Aβ complexes, which results in a deficiency of heme required for normal biological functions.[52,53] It has further been observed that the characteristic pathological features of AD directly correlate with those observed for heme deficiency, thus, further suggesting that heme might have a direct role in AD.[54] More recently, the active site environment of such heme−Aβ complexes has been spectroscopically defined, and it has been shown that either His[13] or His[14] is the heme-coordinating residue of the Aβ peptides. These heme−Aβ complexes have also been shown to function as peroxidases, where the Arg[5] residue has been identified as the acidic residue in the distal pocket essential for peroxidase activity.[55] These heme-Aβ complexes catalytically oxidize neurotransmitters like serotonin, 3,4-dihydroxyphenylalanine, and 4-hydroxyphenylpyruvic acid by H_2O_2, which might trigger abnormal neurotransmission in AD patients.[56,57] Interestingly, both His[13] and Arg[5] residues of the Aβ peptides associated with heme binding are lacking in rodents, which do not get affected by AD, implicating the possible role of heme in AD. However, some studies also invoke that heme binding to Aβ prevents its aggregation and hence reduces the cytotoxicity caused by oxidative stress.[58]

Aβ peptides are available in both the extracellular and intracellular spaces of the AD brain. A total of 31 μg g^{-1}Aβ has been obtained in AD brain which is almost 15 times the amount when compared to non-AD brain.[1,59] This huge amount of Aβ peptide can likely bind Cu (extracellular concentration of 0.2−1.7 μM)[41,60] and heme (less than 30 nM abundance in brain)[61,62] simultaneously *in vivo*. Thus, a physiologically relevant study should focus on the combined effect of heme and Cu on Aβ. In this study, we report that human Aβ(1−40) and Aβ(1−16) peptides can bind both heme and Cu cofactors simultaneously. Absorption and EPR spectroscopy were used to characterize these active sites. In parallel, cyclic voltammetry (CV) and PROS detection assays of wild-type and Tyr[10]Gly mutant of Aβ(1−16) were used to investigate their O_2 reducing properties. Peroxidase assays were performed to complement the above results. These results have been used to develop a structure−function correlation of the active site of Aβ peptides formed after binding both heme and Cu.

2. MATERIALS AND METHODS

All reagents were of the highest grade commercially available and were used without further purification. Aβ peptides (1−40) (sequence: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val), (1−16) (sequence: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys), and Tyr[10]Gly mutant of Aβ(1−16) have been used for this study. All peptides were purchased from GL Biochem (Shanghai) Ltd. with >95% purity. Hemin, copper sulfate, and the buffers were purchased from Sigma. All the peptide stock solutions were made in 100 mM Hepes buffer, hemin solution was made in 1 M NaOH solution, and $CuSO_4$ was dissolved in nanopure water. Peptide stock solutions were 0.5 mM, $CuSO_4$ stock solution was 10 mM, and heme stock solution was 5 mM. Heme−Aβ complexes were prepared by incubating 1 equiv of both Aβ and heme solutions for ~1 h. The Fe of heme is in +3 oxidation state, unless specified in the text. Cu−Aβ complexes were prepared by incubating 1 equiv of Aβ and 0.8 equiv of $CuSO_4$ solution for ~1 h. The oxidation state of Cu in the Cu−Aβ complexes is +2, unless specified. Heme−Cu−Aβ complexes were prepared by incubating 0.8 equiv of $CuSO_4$ with 1 equiv of the

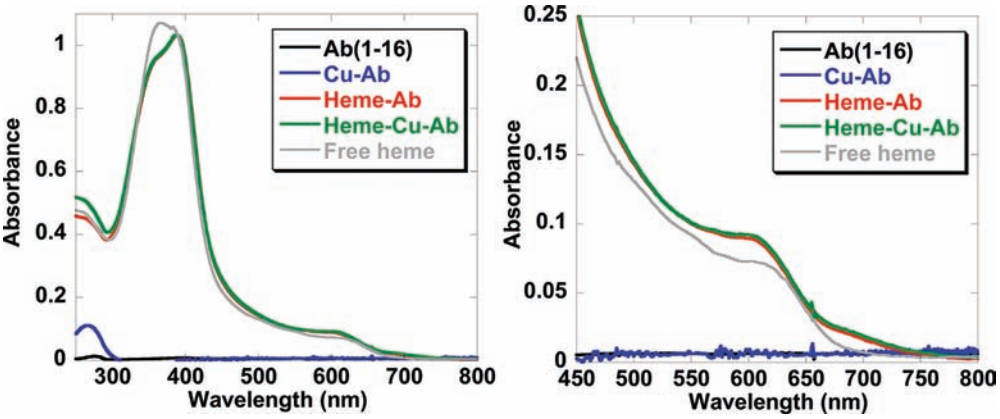


Figure 1. The absorption spectra of Aβ(1−16) in black, Cu-Aβ(1−16) in blue, heme−Aβ(1−16) in red, heme−Cu−Aβ(1−16) in green and free heme in gray. All the data were recorded at pH 7 in 100 mM Hepes buffer. Concentrations of the peptide solutions are 0.02 mM. Heme has Fe in +3 oxidation state and Cu is in +2 oxidation state.

heme−Aβ complex for ∼1 h. Heme−Cu−Aβ complexes were also prepared by incubating 1 equiv of hemin with 1 equiv of the Cu−Aβ complex for ∼1 h. In heme−Cu−Aβ complex, Fe is in +3 oxidation state and Cu is in +2 oxidation state, unless otherwise specified.

Absorption spectra were recorded by adding 40 μL of the heme−Aβ (0.5 mM), Cu−Aβ (0.5 mM), or heme−Cu−Aβ (0.5 mM) complex solution in 1 mL of buffer (100 mM Hepes at pH 7). All spectral data were obtained by an UV−vis diode array spectrophotometer (Agilent 8453).

EPR spectra were obtained by a Jeol (JES FA200) spectrophotometer. EPR samples were 0.5 mM in concentration and were run at 77 K in a liquid nitrogen finger dewar. Cu−Aβ solution was at pH 7. Heme−Aβ and heme−Cu−Aβ EPR samples at pH 7 were prepared by lowering the pH of a high pH sample (pH ∼ 11) with 0.5 M H_2SO_4.

Cyclic voltammetry was performed on a CH Instruments potentiostat (model 710D). A home-built solution cell was made where a Teflon hollow disk was tightly fit on a graphite working electrode (∼100 μL volume). A Pt wire and an Ag wire were used as counter and reference electrode, respectively. Aβ buffered solutions with 1 M KCl were prepared. The Fe^{3+}/Fe^{2+} potential for a 1 mM $K_4[Fe(CN)_6]$ solutions in 1 M KCl was determined to be 200 mV using this setup. This value is +450 mV versus NHE. Thus, the *E*-values measured against this Ag reference electrode have to be corrected by +250 mV to scale them versus NHE. Scan rates were varied between 20 and 50 mV/s to obtain the Cu^{2+}/Cu^{+} potential, and 1 V/s to obtain the heme Fe^{3+}/Fe^{2+} potential. The experiments were performed in degassed buffers to eliminate the O_2 reduction by the Aβ−cofactor complex as well as the graphite working electrode. The Cu CVs have been obtained at pH 7. Heme CVs have been obtained at a high pH (pH ∼ 10) which is ∼3 units higher than the pK_a of the heme site[55] and is thus the pure and physiologically relevant form.

3,3′,5,5′-Tetramethylbenzidine (TMB) was used as the substrate for peroxidase activity measurement.[63] Ten milligrams of TMB was dissolved in 0.5 mL of glacial AcOH and 10 mL of AcOH/NaOAc buffer (1 M, pH 4.5). The solution was diluted to 25 mL with water. This was followed by addition of 100 μL, 30 vol H_2O_2. Ten microliters of 0.05 mM protein sample was added to the above solution. Kinetic traces were obtained by monitoring the increase of the 652 nm absorption band with time.

For PROS calculation, a xylenol orange assay was performed as follows.[64] A total of 4.9 mg of Mohr's salt and 3.9 mg of xylenol orange were dissolved in 5 mL, 250 mM H_2SO_4 and stirred for 10 min. A total of 200 μL of this solution was taken in 1.8 mL of nanopure water and a calibration curve for the quantitative estimation of H_2O_2 was obtained for 0.05, 0.1, 0.5, 1, 2.5, 5, and 10 μM concentrations of H_2O_2 by recording their absorbance at 560 nm (Supporting Information Figure S1). The calibration curve was expressed as absorbance at a fixed wavelength of 560 nm versus concentration of H_2O_2 in micromolar units for a 2 mL volume. For the detection of PROS of an unknown quantity, a blank was obtained in the UV−vis spectrophotometer with 1.8 mL of nanopure water in a cuvette. A total of 200 μL of the xylenol orange solution was added to this cuvette and the absorbance was recorded. This served as the control. The cofactor−Aβ complex was reduced by ascorbic acid (for Cu−Aβ) or dithionite (for heme−Aβ and heme−Cu−Aβ) under anaerobic conditions (observed by absorption and EPR spectroscopy), followed by their reoxidation by O_2 (followed by absorption and EPR spectroscopy). A total of 200 μL of 0.025 mM reoxidized solution was added to the cuvette containing the control. Absorbance of this solution was recorded. The value of absorbance of the above solution (after subtracting the control) at 560 nm when plotted on the calibration curve yielded the corresponding H_2O_2 concentration.

3. RESULTS AND ANALYSIS

3.1. Absorption Spectroscopy. It has recently been shown that 1 equiv of heme covalently binds to Aβ(1−16) and Aβ-(1−40) peptides. His^{13} or His^{14} of the Aβ peptide is the heme-coordinating residue and Arg^5 is present at the distal site of heme.[55] The absorption spectrum of heme bound Aβ(1−16) is characterized by an intensity decrease of the Soret band and a shift of the Q-band relative to heme (Figure 1, red and gray, respectively). The absorption spectrum of 1 equiv Cu incubated Aβ(1−16) shows the 280 nm peptide band, and does not exhibit ligand field transitions associated with Cu at these concentrations (Figure 1, blue). On addition of 1 equiv of Cu to the heme bound Aβ(1−16) peptide, the absorption spectrum shows characteristic features of the heme−Aβ complex with the appearance of no additional bands associated with Cu^{2+} (Figure 1, green).

3.2. EPR Spectroscopy. The 77 K EPR spectra of the heme bound Aβ(1−16) and Aβ(1−40) peptides at pH 7 show an axial high spin (S = 5/2) iron signal with g ∼ 6.0, with partial rhombic character (Figure 2, red and purple, respectively). No additional high field, low spin signal was observed at this temperature. The EPR spectra of the Cu^{2+} loaded Aβ(1−16) and Aβ(1−40) complexes at pH 7 show characteristic type 2 Cu^{2+} signal in the high field region of the spectrum, with $A_{//} \sim 168 \times 10^{-4}$ cm^{-1}, $g_{//} \sim 2.25$, and $g_{\perp} \sim 2.038$ (Figure 3, blue and pink, respectively).[65] When Cu^{2+} was added to the preformed heme−Aβ(1−16) and heme−Aβ(1−40) complexes, they show a high spin S = 5/2 signal with *g* ∼ 6.0, associated with the heme center (Figure 2, dark and

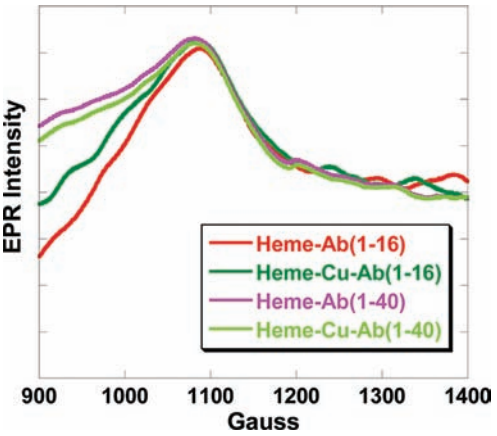


Figure 2. The 77 K EPR spectra of the low field region of heme−Aβ(1−16) in red, heme−Cu−Aβ(1−16) in dark green, heme−Aβ(1−40) in purple, and heme−Cu−Aβ(1−40) in light green. Sample concentrations are 0.5 mM in 100 mM Hepes buffer at pH 7. Heme has Fe in +3 oxidation state and Cu is in +2 oxidation state.

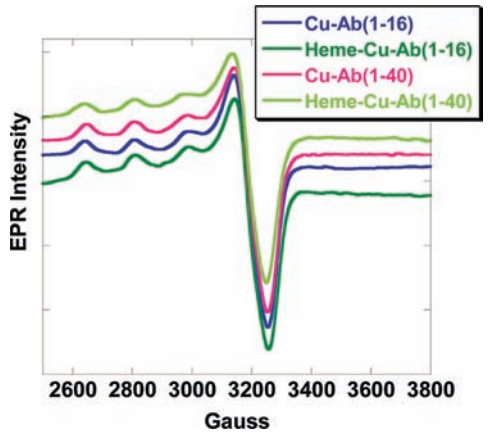


Figure 3. The 77 K EPR spectra of the high field region of Cu−Aβ(1−16) in blue, heme−Cu−Aβ(1−16) in dark green, Cu−Aβ(1−40) in pink, and heme−Cu−Aβ(1−40) in light green. Sample concentrations are 0.5 mM in 100 mM Hepes buffer at pH 7. Heme has Fe in +3 oxidation state and Cu is in +2 oxidation state.

Table 1. Reduction Potentials (V) of Cu−Aβ, Heme−Aβ, and Heme−Cu−Aβ Complexes

	Cu−Aβ	heme−Aβ	heme−Cu−Aβ
Cu center	+0.03		+0.01
Heme center		−0.43	−0.49

light green, respectively) and an additional S = 1/2 axial Cu^{2+} signal having $A_{//} \sim 168 \times 10^{-4}$ cm^{-1}, $g_{//} \sim 2.25$, and $g_{\perp} \sim 2.038$ (Figure 3, dark and light green, respectively). Thus, the high field region of the EPR spectra of the heme−Cu−Aβ(1−40) and heme−Cu−Aβ(1−16) complexes is identical to that of the EPR spectra of Cu−Aβ(1−40) and Cu−Aβ(1−16), respectively (Figure 3). Similarly, the low field region of the EPR spectra of the heme−Cu−Aβ(1−40) and heme−Cu−Aβ(1−16) complexes is identical to that of the EPR spectra of the heme−Aβ-(1−40) and heme−Aβ(1−16) complexes, respectively (Figure 2).

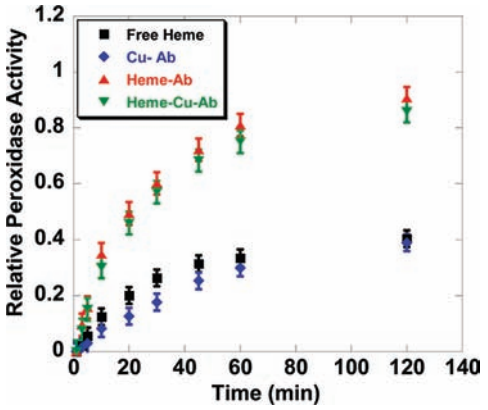


Figure 4. Kinetic traces for peroxidase activity, monitoring the increase of 652 nm absorbance intensity, for heme−Aβ (red), heme−Cu−Aβ (green), Cu−Aβ (blue), and free heme (black). Detailed information is mentioned in the Materials and Methods. Heme has Fe in +3 oxidation state and Cu is in +2 oxidation state.

Thus, the EPR data clearly illustrates that the heme−Cu−Aβ complexes contain two electronically uncoupled paramagnetic centers that remain unperturbed in the presence of each other. Identical EPR spectra are obtained when the peptides were incubated first with Cu, followed by heme, indicating the formation of the same heme−Cu−Aβ complexes.

3.3. Cyclic Voltammetry. Recently, cyclic voltammogram (CV) results of Cu−Aβ complexes have been reported.[66,67] The CV of Cu−Aβ(1−16) complex under anaerobic conditions shows a quasi-reversible process with an $E^0 \sim 0.03$ V (Figure S2A), which is very different from the CV of free Cu^{2+} in solution (Figure S3). The heme−Cu−Aβ(1−16) complex shows the Cu^{2+}/Cu^{+} redox process at ~0.01 V (Figure S2 A). In both heme−Aβ and heme−Cu−Aβ complexes, the $Fe^{3+/2+}$ CV could be clearly observed under anaerobic conditions at faster scan rates. The CV of the heme−Aβ and the heme−Cu−Aβ complexes shows a $Fe^{3+/2+}$ process at −0.43 and −0.49 V, respectively (Figure S2B). Thus, the heme−Cu−Aβ(1−16) complex exhibits the electrochemical properties of both Cu−Aβ(1−16) and heme−Aβ(1−16) complexes. The electrochemical behavior of both the individual Cu−Aβ and heme−Aβ sites is retained in the heme−Cu−Aβ complex (Table 1).

3.4. Peroxidase Activity. The heme−Aβ complexes, where the heme is coordinated by His[13] or His[14], have been demonstrated to function as peroxidases.[55,56] When the peroxidase activity was measured by the catalytic oxidation of 3, 3′, 5, 5′-tetramethylbenzidine (TMB), by H_2O_2 for the heme−Cu−Aβ(1−16) peptide complex, it shows activity similar to that of the heme−Aβ complex (Figure 4). Interestingly, the Cu bound Aβ(1−16) shows peroxidase activity similar to that of free heme. This further demonstrates that the heme and Cu have discrete binding sites and Cu addition does not affect the peroxidase activity of the heme active site.[68]

3.5. PROS Generation. The reduction of O_2 by Cu^{+}−Aβ, heme(Fe^{2+})−Aβ, and heme(Fe^{2+})−Cu^{+}−Aβ peptide complexes was investigated under homogeneous condition. When 1 equiv of reducing agent (ascorbic acid for Cu−Aβ and dithionite for heme−Aβ) was added to the Cu−Aβ and heme−Aβ peptide solutions, the resting Cu^{2+} site was completely reduced to Cu^{+} (observed by EPR spectroscopy) and the Fe^{3+} of the heme was reduced to Fe^{2+} (followed by absorption and EPR spectroscopy). When O_2 was introduced to the fully reduced Cu^{+}−Aβ(1−16) and heme(Fe^{2+})−Aβ(1−16) complexes, it reoxidized the Cu^{+}

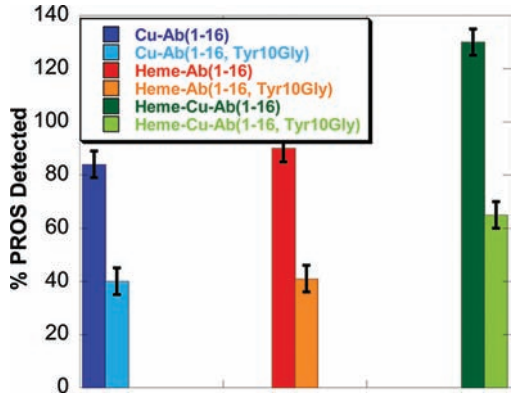
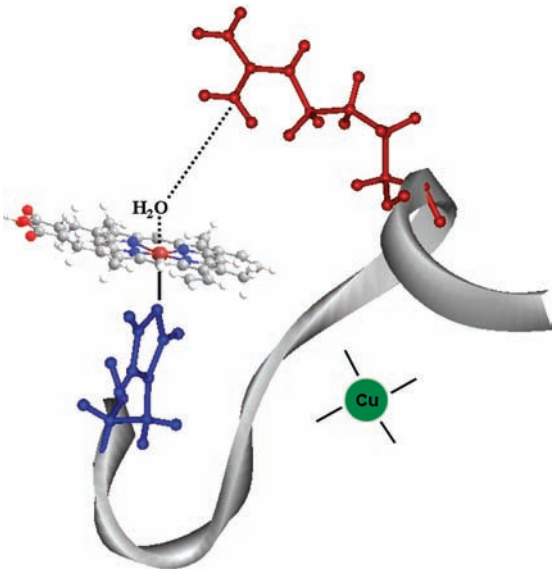


Figure 5. Percentage of PROS detected for Cu−Aβ(1−16) in dark blue, Cu−Tyr^{10}GlyAβ(1−16) in light blue, heme−Aβ(1−16) in red, heme−Tyr^{10}GlyAβ(1−16) in orange, heme−Cu−Aβ(1−16) in dark green, and heme−Cu−Tyr^{10}GlyAβ(1−16) in light green. Cu^{+} and Fe^{2+} are the reactive species that react with O_2 to form PROS.

Scheme 2. Schematic Representation of Simultaneous Heme and Cu Binding to Aβ(1−16)[a]



[a] His^{13} and Arg^{5} residues involved in heme binding are highlighted in blue and red, respectively. The Cu coordinating ligands are not specified.

site to the Cu^{2+} form and the Fe^{2+} to the Fe^{3+} form of the heme site, respectively, as detected by EPR and absorption spectroscopy. Similar reduction of the Cu^{2+} and heme(Fe^{3+}) sites followed by their reoxidation in the presence of O_2 was observed for the heme−Cu−Aβ complex. This reduction of O_2 by the reduced Cu^{+}−Aβ, heme(Fe^{2+})−Aβ, and heme(Fe^{2+})−Cu^{+}−Aβ peptides can either produce superoxide ($O_2^{\bullet-}$), H_2O_2, or it could be a 4 e^{-}/4 H^{+} process producing H_2O. Any H_2O_2 formed during this oxidation process (either due to the 2 e^{-} reduction of O_2 or by the disproportionation of $O_2^{\bullet-}$) could be detected by using a xylenol orange assay (details in Materials and Methods). The results indicate that Cu^{+}−Aβ produces ∼84 ± 5% H_2O_2, heme(Fe^{2+})−Aβ generates ∼90 ± 5% H_2O_2, and heme(Fe^{2+})−Cu^{+}−Aβ results in ∼130 ± 5% H_2O_2 (Figure 5). Thus, both the reduced heme(Fe^{2+}) and Cu^{+} sites are reoxidized by atomospheric O_2 to generate approximately equivalent amounts of H_2O_2. When both the heme(Fe^{2+}) and Cu^{+} sites are present, the amount of PROS generated is much more than the individual sites. In control experiments, H_2O_2 was added to the Cu−Aβ, heme−Aβ, and Cu−heme−Aβ complexes, incubated and then detected using the same assay protocol. Almost quantitative amounts of initial H_2O_2 added could be recovered, indicating that at these concentrations of H_2O_2, the peroxidase activity of these complexes did not compromise the PROS detection. The amounts of H_2O_2 detected for the Cu^{+}−Aβ and heme(Fe^{2+})−Aβ complexes (84−90%, Figure 5) imply that both these complexes catalyze the 2 e^{-} reduction of O_2 to H_2O_2 in aqueous buffered solutions having physiological pHs.[69,70] However, the H_2O_2 produced in the case of heme(Fe^{2+})−Cu^{+}−Aβ complex (∼130%, Figure 5) is derived from a 2 e^{-} reduction and a 1 e^{-} reduction of O_2.[71]

As mentioned above, EPR experiments indicate that the Cu^{+} and Fe^{2+} sites of Cu^{+}−Aβ and heme(Fe^{2+})−Aβ are oxidized by O_2 forming Cu^{2+} and Fe^{3+}, respectively. Thus, only 1 e^{-} is donated to O_2 by the metal center. This should reduce O_2 to $O_2^{\bullet-}$ which would, after disproportionation, show ∼50% H_2O_2 and not greater than ∼84% as observed experimentally (Figure 5). Similarly, in the case of heme(Fe^{2+})−Cu^{+}−Aβ, the presence of two redox active sites (heme−Fe and Cu) can provide two electrons for O_2 reduction, which should produce 100% H_2O_2 and not 130% H_2O_2. Therefore, the additional electron, required for the observed 2 e^{-} reduction of O_2 to H_2O_2 (for Cu^{+}−Aβ and heme(Fe^{2+})−Aβ complexes) and 3 e^{-} reduction of O_2 to H_2O_2 (for heme(Fe^{2+})−Cu^{+}−Aβ complex), must be derived from the Aβ peptide. Tyrosine (the 10th residue of Aβ(1−16) peptide) is known to be able to donate an e^{-} during O_2 activation in several enzymatic systems.[72−75] Similar H_2O_2 detection assays of the reaction of Cu^{+}−Aβ, heme(Fe^{2+})−Aβ, and heme(Fe^{2+})−Cu^{+}−Aβ complexes of the Tyr^{10}Gly mutant of Aβ(1−16) with O_2 show only 40−45% PROS for the Cu^{+}−Aβ and heme(Fe^{2+})−Aβ complexes and ∼65% PROS for the heme(Fe^{2+})−Cu^{+}−Aβ complex (Figure 5). Thus, H_2O_2 generation is decreased by ∼50% in the absence of the Tyr^{10} residue for the Cu^{+}−Aβ and heme(Fe^{2+})−Aβ complexes and ∼66% for the heme(Fe^{2+})−Cu^{+}−Aβ complex. This clearly indicates that the Tyr residue is donating an e^{-} during the oxidation of Cu^{+}−Aβ, heme(Fe^{2+})−Aβ, and heme(Fe^{2+})−Cu^{+}−Aβ complexes by molecular O_2. Note that oxidation of Tyr by one electron should produce a TyrO· (phenoxyl) radical species which has a characteristic EPR spectrum. We do not observe this signal in our data. This implies that the TyrO· radical is possibly dimerizing in the reaction time scale. This process may play a fundamental role in AD pathology and investigations into its mechanism are in progress.[24,76,77]

4. DISCUSSION

Several research groups have reported Cu binding to Aβ peptides.[17,37−39,41−46,48,49] Although there are still some ambiguities regarding the identitiy of the amino acids responsible for binding Cu, it has been well established that Cu bound Aβ may play a vital role in AD. Similarly, heme bound Aβ has also been suggested to be associated with AD. Recently, it has been shown that His^{13} and Arg^{5}, two of the three amino acids absent in rodents which do not get affected by AD, form the heme binding site.[55] Physiologically, both heme and Cu are available in the

Scheme 3. Amino Acid Sequence of Aβ(1−16) Peptides in Human (Top) and Rats, Mice, and Chinese Hamsters (Bottom), Highlighting the Residues That Differ between the Two Peptide Sequences

brain in such concentrations that it is likely that both of these metal cofactors can bind Aβ peptides. Our EPR data indicate that the Cu binding to free Aβ peptide and Cu binding to heme bound Aβ peptide are spectroscopically identical (Figure 3). Likewise, the EPR signal from heme when complexed with free Aβ and Cu bound Aβ are the same (Figure 2). The absorption spectra of the heme−Aβ complex and heme−Cu−Aβ complex are similar, indicating that heme remains unperturbed in the presence of Cu (Figure 1). CV of the Cu−Aβ, heme−Aβ, and heme−Cu−Aβ shows only small perturbation of the $Cu^{2+/+}$ and the heme $Fe^{3+/2+}$ couple. Thus, our spectroscopic and electrochemical data indicate that first, both heme and Cu can bind Aβ at the same time producing very little effect on the other site, and second, the sites are magnetically uncoupled (Scheme 2).

Our results indicate that the Cu sites in the Cu−Aβ and heme−Cu−Aβ complexes are identical in nature (Figures 2, 3, Table 1). Recent spectroscopic studies of site specific mutants have identified either His^{13} or His^{14} to be the heme-binding residue.[55] These data also exhibit dramatic changes in the spectra in the absence of His^{13} or His^{14} indicating that His^{13} or His^{14} is occupied in binding heme. Thus, only His^{6} and His^{13}/His^{14} are available to coordinate to Cu in heme−Cu−Aβ. While some studies have suggested the involvement of all three histidine residues (His^{6}, His^{13}, and His^{14}) in binding Cu^{2+},[17,41,78,79] others have refuted this.[27,40,42−45] The present study clearly demonstrates that Cu−Aβ cannot bind to all three histidine ligands simultaneously.

Heme−Aβ complexes show significant enhancement of peroxidase activity relative to free heme (Figure 4). Our results indicate that Cu−Aβ also has some peroxidase activity, a fact previously unknown. This may have profound implications in AD. The Cu bound heme−Aβ peptides retain the peroxidase activity of heme−Aβ.

CV data indicate that both the Cu and the heme sites can be reduced by physiological reducing agents. While the heme site ($E°$ = −0.42 V, i.e., −0.17 V vs NHE) can be reduced by glutathione (E^0 = −0.25 V vs NHE)[80−84] or NADH ($E°$ = −0.45 V vs NHE),[85] the Cu site ($E°$ = 0.03−0.01 V, i.e., 0.28−0.26 V vs NHE) can be reduced by the above reducing agents as well as by ascorbic acid.

The reduction of O_2 by Aβ (free and metal mediated) has been actively followed.[1,16,17,41] Some studies report that Aβ-(1−40) in aqueous solution gets oxidized to Aβ radical producing neurotoxic ROS based on mass spectrometry and EPR spin trapping experiments.[86] However, this possibility of spontaneous generation of free radicals derived from the peptide was later ruled out by control EPR experiments.[87] More recent studies propose metal ion (e.g., Fe^{3+}, Cu^{2+}) mediated generation of H_2O_2 by Aβ peptides.[88] Aβ(1−42) in the presence of catalytic amounts of transition metals (Fe^{3+} and Cu^{2+}) reduces the metal center and subsequently generates equivalent amounts of H_2O_2.[89] The amount of H_2O_2 produced follows a 2 e^- transfer pathway and is dependent on the oxygen availability.[89] In the presence of excess endogenous reducing agents (e.g., cholesterol, catecholamine, vitamin C), Cu−Aβ(1−42) generates catalytic H_2O_2 following a 2 e^- reduction of O_2.[90] It has been proposed that the 2 e^-'s are derived from the Aβ peptide which reduces 2 equiv of Cu^{2+} bound to Aβ(1−42) to Cu^+, which subsequently transfers the electron to O_2. Alternatively, two molecules of Cu−Aβ can each donate an electron to O_2 to form H_2O_2. Met^{35} has also been suggested to be responsible for providing reducing equivalents. In this study, oxidation of reduced Cu^+−Aβ, heme-(Fe^{2+})−Aβ, and heme(Fe^{2+})−Cu^+−Aβ (in the absence of any excess reductant) by molecular O_2 indicates that the amounts of H_2O_2 generated by these species are ∼84%, ∼90%, and ∼130%, respectively (Figure 5). Thus, both heme(Fe^{2+})−Aβ and Cu^+−Aβ complexes reduce O_2 by 2 e^- to H_2O_2 in aqueous buffered solutions at physiologic pHs. One electron for this process is derived from the reduced metal cofactor (i.e., Cu or heme) while the other electron is derived from oxidation of the Tyr^{10} residue. This is clearly indicated by 40−45% PROS generation upon reoxidation of the reduced Tyr^{10}Gly Aβ(1−16) mutant complexes of Cu^+ and heme(Fe^{2+}), which is half of what is produced in the wild-type, indicating a 1 e^- reduction of O_2 to O_2^- (i.e., Cu^+ and Fe^{2+} both donate 1 e^-).[91] Since heme(Fe^{2+})−Cu^+−Aβ contains three electrons (one each from Cu, Fe, and Tyr^{10}), the amount of H_2O_2 generated is ∼130% (expected is 150%), which decreases to ∼65% in the case of heme(Fe^{2+})−Cu^+−Aβ (Tyr^{10}Gly) complex which now has two reduced sites. Thus, our 2 e^- reduction of O_2 to H_2O_2 is in agreement with previous findings.[89,90] However, in contrast to the past proposed models (which invoke two Cu^+ centers present in Aβ or two molecules of Cu^+−Aβ or a Cu^+−Aβ and Met^{35} as the source of electrons for O_2 reduction), we demonstrate that one reducing equivalent for O_2 reduction is derived from the reduced metal cofactor (i.e., heme(Fe^{2+}) or Cu^+) and the other from the Tyr^{10} residue. More importantly, when both heme and Cu are bound to the Aβ peptide, the toxic PROS formation is maximum (Figure 5), implying that the presence of both these cofactors could potentially have an impact in the pathology of AD.

Rodent Aβ peptide sequence varies from the human counterpart at the positions 5, 10, and 13 (Scheme 3). So far, His^{13} has been invoked in binding Cu. Arg^5 has been proposed to impose steric and conformational constrains on the Cu bound Aβ peptide.[45,92] Our previous results showed that Arg^5 and His^{13} are involved in heme binding.[55] The current results indicate that Tyr^{10} provides an electron during the 2 e^- reduction of O_2 to H_2O_2 by either Cu^+ or heme(Fe^{2+}) bound Aβ peptides. H_2O_2 is a freely diffusing molecule that, if not quenched by catalases, can induce significant oxidative stress in a biological system. Thus, all three residues, Arg^5, Tyr^{10}, and His^{13}, unique to human Aβ peptides, play key roles in the pathology of AD.

5. CONCLUSION

In summary, the Aβ peptides can bind to both heme and Cu cofactors simultaneously. These cofactors have unique spectroscopic features that remain unperturbed in the presence of each other. These data do not support the three histidine binding site model for Cu−Aβ complexes as previously proposed. The heme−Aβ peptide complexes show peroxidase activity. Interestingly, the Cu−Aβ complexes also show peroxidase activity, which can have significant implications in AD. Both Cu^+−Aβ and heme(Fe^{2+})−Aβ complexes reduce O_2 by 2 e^- to produce H_2O_2. One electron required for this O_2 reduction is derived from the metal cofactor and the other from the Tyr^{10} residue.

The electrochemical data suggests that both the Cu and heme−Aβ complexes can be reduced under physiological conditions and they are prone to generate oxidative stress. When both cofactors are bound to the Aβ peptide, which is a likely physiological situation, maximum amounts of toxic PROS are formed.

■ ASSOCIATED CONTENT

Supporting Information. Complete refs 14, 24, and 89; calibration curve for H_2O_2 detection; CV plots of Cu−Aβ, heme−Aβ, heme−Cu-Aβ and free Cu^{2+}. This material is available free of charge via the Internet at http://pubs.acs.org.

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