In this chapter we illustrate that Aβ peptides can bind both Cu and heme cofactors at the same time. Both cofactors have unique spectroscopic and electro-chemical features which are unaffected in the presence of the other, implying that they are electronically, chemically and electrochemically uncoupled.
4.1. 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.\(^2,3,4\) Aβ peptide is generated from a large trans membrane amyloid precursor protein (APP) by proteolysis.\(^5\) Aβ is normally found in biological fluids and contains 39 to 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 a much higher concentrations in the neocortex of the brain, the region that is affected by AD.\(^10,11,12,13\) It has also been shown that Aβ aggregates from post-mortem of 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 4.1),\(^16,17\) 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), e.g. HO\(_2^−\), H\(_2\)O\(_2\), HO\(^{−}\) etc.\(^18,19\) 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.\(^20,21,22,23\) 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. It is well documented that all the metal binding sites are in the N-terminal hydrophilic region of the Aβ peptides. The contribution of Zn$^{2+}$ ions in the amyloid deposition in transgenic mice (note, normally rodents do not show AD) is a strong evidence of its plaque forming properties. 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. His6, His13 and His14) and the carboxylate side chain of Glu11 are involved in Zn ligation. Asp1 and water are likely to be the other ligands. Tyr10 and Arg5 are not proposed to be associated in Zn binding.

Unlike Zn$^{2+}$, Cu$^{2+}$ can either accelerate or inhibit amyloidogenesis depending on the condition and type of aggregated state. Though Cu$^{2+}$ induced Aβ deposition is still questionable, it plays a significant role in the generation of ROS (Scheme 4.1) via a Fenton type reaction. 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. The 3N coordination can either be from three histidine residues, or two histidine residues and the N-terminus of the peptide. The O binding residue can be donated by Asp1, Asp7, Glu3, Glu11 or be aqueous buffer derived. 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 (His6 and His13/His14), one N-terminus residue and the carboxylate group of Asp1.\textsuperscript{42} Component II has been proposed be comprised of three histidine residues along with the carboxylate group of Asp1.\textsuperscript{42} Two other models for component I have been reported having a similar Cu\textsuperscript{2+} coordination environment as above, but having an additional fifth ligand. One such study reports the carbonyl group of the peptide linkage of Asp1-Ala2 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 \textsuperscript{13}C and \textsuperscript{1}H NMR.\textsuperscript{43,44,45} 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 Ala2-Glu3, a deprotonated nitrogen of an amide bond, the N terminus and the carboxylate group of Asp1.\textsuperscript{43,44,45} Yet another group reports binding of Cu\textsuperscript{2+} through the N-terminus along with His6, His13 and the carbonyl moiety of Asp1 in component I and by three histidine ligands along with the carbonyl oxygen atom of the amide bond of Ala2-Glu3 in component II using HYSCORE spectroscopy on isotopically labeled peptides.\textsuperscript{46} Cu\textsuperscript{+}-Aβ is the reactive species that is directly involved in ROS formation.\textsuperscript{47} As a result determination of the coordination environment of Cu\textsuperscript{+}-Aβ has also been of interest. Two groups proposed a linear bis-His coordination to Cu\textsuperscript{+} through His13 and His14 using EXAFS and IR spectroscopy.\textsuperscript{48,49,50} Cu\textsuperscript{+} coordination has also been studied by NMR spectroscopy.\textsuperscript{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.\textsuperscript{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.\textsuperscript{54} More recently, the active site environment of such heme-Aβ complexes have been spectroscopically defined, and it has been shown that either His13 or His14 is the heme coordinating residue of the Aβ peptides. These heme-Aβ complexes have also been shown to function as peroxidases, where the Arg5 residue has been identified as the acidic residue in the distal pocket essential for peroxidase activity.\textsuperscript{55} These heme-Aβ complexes catalytically oxidize neurotransmitters like serotonin, 3,4-dihydroxyphenylalanine and 4-hydroxyphenylpyruvic acid by H\textsubscript{2}O\textsubscript{2}, which might trigger abnormal neurotransmission in AD patients.\textsuperscript{56,57} Interestingly, both His13 and Arg5 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.\textsuperscript{58}

Aβ peptides are available in both the extracellular and intracellular spaces of the AD brains. \SI{31}{\mu g \; g^{-1}} Aβ has been obtained in AD brain which is almost fifteen times compared to non AD brains.\textsuperscript{1,59} This huge amount of Aβ peptide can likely bind Cu (extracellular concentration of \SI{0.2–1.7}{\mu M})\textsuperscript{41,60} and heme (less than \SI{30}{nM} abundance in brain)\textsuperscript{61,62} simultaneously \textit{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\textsuperscript{10}Gly mutant of Aβ(1-16) were used to investigate their \textit{O}_2 reducing properties. Peroxidase assays were performed to compliment 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.

4.2. MATERIALS AND METHODS

4.2.1. MATERIALS

All reagents were of the highest grade commercially available and were used without further purification. Amyloid beta (Aβ) peptides (1-40) (sequence: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-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\textsuperscript{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.

4.2.2. SAMPLE PREPARATION

All the peptide stock solutions were made in \SI{100}{mM} Hepes buffer, hemin solution was made in \SI{1}{M} NaOH solution and CuSO\textsubscript{4} was dissolved in nanopure water. Peptide stock solutions were 0.5 mM, CuSO\textsubscript{4} stock solution was 10 mM and heme stock solution was 5
M. Heme-Aβ complexes were prepared by incubating 1 equivalent of Aβ with 0.8 equivalent of heme solution for ~ 1 hour. The Fe of heme is in +3 oxidation state, unless specified in the text. Cu-Aβ complexes were prepared by incubating 1 equivalent of Aβ and 0.8 equivalent of CuSO₄ solution for ~ 1 hour. The oxidation state of Cu in the Cu-Aβ complexes is +2, unless specified. Heme-Cu-Aβ complexes were prepared by incubating 0.8 equivalent of CuSO₄ with 1 equivalent of the heme-Aβ complex for ~ 1 hour. Heme-Cu-Aβ complexes were also prepared by incubating 1 equivalent of hemin with 1 equivalent of the Cu-Aβ complex for ~ 1 hour. In heme-Cu-Aβ complex, Fe is in +3 oxidation state and Cu is in +2 oxidation state, unless otherwise specified.

4.2.3. ABSORPTION SPECTROSCOPY

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 buffer (100 mM Hepes at pH 7). All spectral data were obtained by an UV-Vis diode array spectrophotometer (Agilent 8453).

4.2.4. EPR SPECTROSCOPY

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 H₂SO₄.

4.2.5. CYCLIC VOLTAMMETRY (CV)

CV were performed on a CH Instrument potentiostat (model 710D). A home built solution cell was made where a teflon hollow disc 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 Fe³⁺/Fe²⁺ potential for a 1 mM K₄[Fe(CN)₆] solutions in 1 M KCl was determined to be 200 mV using this set up. This value is +450 mV vs. NHE. Thus the E values measured against this Ag reference electrode have to be corrected by +250 mV to scale them vs. NHE. Scan rates were varied between 20 mV/s to 50 mV/s to obtain the Cu²⁺/Cu⁺ potential, and 1 V/s to obtain the heme Fe³⁺/Fe²⁺ potential. The experiments were performed in degassed buffers to eliminate the O₂ reduction by the Aβ-heme complex as well as the graphite working electrode.
4.2.6. PEROXIDASE ACTIVITY MEASUREMENT

3,3´,5,5´-tetramethylbenzidine (TMB) was used as the substrate for peroxidase activity measurement. 10 mg of TMB was dissolved in 0.5 mL glacial AcOH and 10 mL 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 volume H₂O₂. 10 µL of 0.05 mM of the protein sample was added to the above solution. Kinetic traces were obtained by monitoring the increase of the 652 nm absorption band with time.

4.2.7. PROS DETECTION ASSAY

For PROS calculation, a xylene orange assay was performed as follows. 4.9 mg of Mohr’s salt and 3.9 mg of xylene orange was dissolved in 5 mL, 250 mM H₂SO₄ and stirred for 10 minutes. 200 µL of this solution was taken in 1.8 mL of nanopure water and a calibration curve for the quantitative estimation of H₂O₂ was obtained for 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM and 10 µM concentrations of H₂O₂ by recording their absorbance at 560 nm (Figure S4.1). The calibration curve was expressed as absorbance at a fixed wavelength of 560 nm vs. concentration of H₂O₂ in µM 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 nanopure water in a cuvette. 200 µL of the xylene 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 O₂ (followed by absorption and EPR spectroscopy). 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 H₂O₂ concentration.

4.3. RESULTS AND ANALYSIS

4.3.1. ABSORPTION SPECTROSCOPY

It has recently been shown that one equivalent heme covalently binds to Aβ(1-16) and Aβ(1-40) peptides. His13 or His14 of the Aβ peptide is the heme coordinating residue and
Arg5 is present at the distal site of heme. 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 4.1, red and grey, respectively). The absorption spectrum of one equivalent Cu incubated Aβ(1-16) shows the 280 nm peptide band, and does not exhibit ligand field transitions associated with Cu at this concentration (Figure 4.1, blue). On addition of one equivalent 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 4.1, green).

![Absorption spectra](image)

**FIGURE 4.1.** The absorption spectra of Aβ(1-16), black; Cu-Aβ(1-16), blue; heme-Aβ(1-16), red; heme-Cu-Aβ(1-16), green and free heme, grey. 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.

### 4.3.2. 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 4.2, 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 4.3, blue and pink, respectively). When Cu$^{2+}$ was added to the pre-formed 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 4.2, dark and light green, respectively) and an additional $S = 1/2$ axial Cu$^{2+}$ signal having $A_{||} \approx 168 \times 10^{-4} \text{ cm}^{-1}$, $g_{||} \approx 2.25$ and $g_{\perp} \approx 2.038$ (Figure 4.3, 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 are identical to that of the EPR spectra of Cu-Aβ(1-40) and Cu-Aβ(1-16) respectively (Figure 4.3). Similarly, the low field region of the EPR spectra of the heme-Cu-Aβ(1-40) and heme-Cu-Aβ(1-16) complexes are identical to that of the EPR spectra of the heme-Aβ(1-40) and heme-Aβ(1-16) complexes respectively (Figure 4.2). Thus the EPR data clearly illustrates that the heme-Cu-Aβ complexes contain two electronically un-coupled 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.

**FIGURE 4.2.** 77 K EPR spectra of the low field region of heme-Aβ(1-16), red; heme-Cu-Aβ(1-16), dark green; heme-Aβ(1-40), purple and heme-Cu-Aβ(1-40), 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 4.3. 77 K EPR spectra of the high field region of Cu-Aβ(1-16), blue; heme-Cu-Aβ(1-16), dark green; Cu-Aβ(1-40), pink and heme-Cu-Aβ(1-40), 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.

4.3.3 CYCLIC VOLTAMMERTY

Recently cyclic voltammogram (CV) of Cu-Aβ complexes have been reported.\textsuperscript{66,67} The CV of Cu-Aβ(1-16) complex shows a quasi-reversible process with an \( E^0 \sim 0.035 \) V (vs Ag electrode) (Figure S4.2 A). The heme-Cu-Aβ(1-16) complex shows the Cu\(^{2+}/\)Cu\(^+\) redox process at \( \sim 0.012 \) V (vs Ag electrode) (Figure S4.2 A). In both heme-Aβ and heme-Cu-Aβ complexes the Fe\(^{3+}/\)Fe\(^{2+}\) CV could be clearly observed under anaerobic conditions at faster scan rates. The CV of the heme-Aβ and the heme-Cu-Aβ complexes in degassed buffer solutions show a Fe\(^{3+}/\)Fe\(^{2+}\) process at \(-0.43\) V and \(-0.49\) V, respectively (Figure S4.2 B). 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 are retained in the heme-Cu-Aβ complex (Table 4.1).
TABLE 4.1. Reduction potentials (V) of Cu-Aβ(1-16), heme-Aβ(1-16) and heme-Cu-Aβ(1-16) complexes.

<table>
<thead>
<tr>
<th></th>
<th>Cu-Aβ</th>
<th>Heme-Aβ</th>
<th>Heme-Cu-Aβ</th>
</tr>
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<tbody>
<tr>
<td>Cu center</td>
<td>+ 0.035</td>
<td></td>
<td>+0.012</td>
</tr>
<tr>
<td>Heme center</td>
<td>- 0.43</td>
<td>- 0.49</td>
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</tbody>
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4.3.4. Peroxidase Activity

The heme-Aβ complexes, where the heme is coordinated by His\textsuperscript{13} or His\textsuperscript{14}, have been demonstrated to function as peroxidases.\textsuperscript{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 4.4). 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 discreet binding sites and Cu addition does not affect the peroxidase activity of the heme active site.\textsuperscript{68}

![Figure 4.4](image-url)

**Figure 4.4.** 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 section. Heme has Fe in +3 oxidation state and Cu is in +2 oxidation state.
4.3.5. PROS GENERATION

The reduction of O$_2$ by reduced Cu$^+$-Aβ, heme(Fe$^{2+}$)-Aβ and heme(Fe$^{2+}$)-Cu$^+$-Aβ peptide complexes were investigated under homogeneous condition. When one equivalent 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 re-oxidized the Cu$^+$ 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 re-oxidation 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$_2^-$), H$_2$O$_2$ or it could be a 4 e$^-$/4 H$^+$ process producing H$_2$O. Any H$_2$O$_2$ formed during this oxidation process (either due to the 2 e$^-$ reduction of O$_2$ or by the disproportionation of O$_2^-$) could be detected by using a xylenol orange assay (details in Experimental Section). The results indicate that Cu$^+$-Aβ produces $\sim$ 84±5% H$_2$O$_2$, heme(Fe$^{2+}$)-Aβ generates $\sim$ 90±5% H$_2$O$_2$, and heme(Fe$^{2+}$)-Cu$^+$-Aβ results in $\sim$ 130±5% H$_2$O$_2$ (Figure 4.5). Thus both the reduced heme(Fe$^{2+}$) and Cu$^+$ sites are reoxidized by atmospheric O$_2$ to generate $\sim$ equivalent amounts of H$_2$O$_2$. 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$_2$O$_2$ 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$_2$O$_2$ added could be recovered, indicating that at these concentrations of H$_2$O$_2$, the peroxidase activity of these complexes did not compromise the PROS detection. The amount of H$_2$O$_2$ detected for the Cu$^+$-Aβ and heme(Fe$^{2+}$)-Aβ complexes (84-90%, Figure 4.5), imply that both these complexes catalyze the 2 e$^-$ reduction of O$_2$ to H$_2$O$_2$ in aqueous buffered solutions having physiological pHs.$^{69,70}$ However, the H$_2$O$_2$ produced in the case of heme(Fe$^{2+}$)-Cu$^+$-Aβ complex ($\sim$ 130%, Figure 4.5) is derived from a 2 e$^-$ reduction and a 1 e$^-$ reduction of O$_2$.71
Figure 4.5. % PROS detected for Cu-Aβ(1-16), dark blue; Cu-Tyr10GlyAβ(1-16), light blue; heme-Aβ(1-16), red; heme-Tyr10GlyAβ(1-16), orange; heme-Cu-Aβ(1-16), dark green and heme-Cu-Tyr10GlyAβ(1-16), light green. Cu⁺ and Fe²⁺ are the reactive species that react with O₂ to form PROS.

As mentioned above, EPR experiments indicate that the Cu⁺ and Fe²⁺ sites of Cu⁺-Aβ and heme(Fe²⁺)-Aβ are oxidized by O₂ forming Cu³⁺ and Fe³⁺ respectively. Thus only 1 e⁻ is donated to O₂ by the metal center. This should reduce O₂ to O₂⁻ which would, after disproportionation, show ~ 50% H₂O₂ and not greater than ~ 84% as observed experimentally (Figure 4.5). Similarly in the case of heme(Fe²⁺)-Cu⁺-Aβ, the presence of two redox active sites (heme-Fe and Cu) can provide two electrons for O₂ reduction, which should produce 100% H₂O₂ and not 130% H₂O₂. Therefore the additional electron, required for the observed 2 e⁻ reduction of O₂ to H₂O₂ (for Cu⁺-Aβ and heme(Fe²⁺)-Aβ complexes) and 3 e⁻ reduction of O₂ to H₂O₂ (for heme(Fe²⁺)-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 O₂ activation in several enzymatic systems.⁷²,⁷³,⁷⁴,⁷⁵ Similar H₂O₂ detection assays of the reaction of Cu⁺-Aβ, heme(Fe²⁺)-Aβ and heme(Fe²⁺)-Cu⁺-Aβ complexes of the Tyr10Gly mutant of Aβ(1-16) with O₂ show only 40-45% PROS for the Cu⁺-Aβ and heme(Fe²⁺)-Aβ complexes and ~ 65% PROS for the heme(Fe²⁺)-Cu⁺-Aβ complex (Figure 4.5). Thus, H₂O₂ generation is decreased by ~ 50% in the absence of the Tyr10 residue for the Cu⁺-Aβ and heme(Fe²⁺)-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 (phenoxy)l 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.\textsuperscript{24,76,77}

4.4. DISCUSSION

Several research groups have reported Cu binding to Aβ peptides.\textsuperscript{17,37,38,39,41,42,43,44,45,46,48,49} Although there are still some ambiguities regarding the identity of the amino acids responsible for binding Cu, it has been well established that Cu bound Aβ plays a vital role in AD. Similarly heme bound Aβ has also been invoked to be associated with AD. Recently it has been shown that His13 and Arg5, two of the three amino acids absent in rodents which do not get affected by AD, form the heme binding site.\textsuperscript{55} Physiologically both heme and Cu are available in the 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 4.3). Likewise, the EPR signal from heme when complexed with free Aβ and Cu bound Aβ are the same (Figure 4.2). 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 4.1). CV of the Cu-Aβ, heme-Aβ and heme-Cu-Aβ show only small perturbation of the Cu$^{2+}$/Cu$^+$ and the heme Fe$^{3+}$/Fe$^{2+}$ couple. Thus our spectroscopic and electrochemical data indicate that firstly, both heme and Cu can bind Aβ at the same time producing very little effect on the other site and secondly, the sites are magnetically uncoupled (Scheme 4.2).
Our results indicate that the nature of Cu sites in the Cu-Aβ and heme-Cu-Aβ complexes are identical (Figures 4.2, 4.3, Table 4.1). Recent spectroscopic studies of site specific mutants have identified either His13 or His14 to be the heme binding residue. These data also exhibit dramatic changes in the spectra in the absence of His13 or His14 indicating that His13 or His14 is occupied in binding heme. Thus, only His6 and His13/His14 are available to coordinate to Cu in heme-Cu-Aβ. While some studies have suggested the involvement of all three histidine residues (His6, His13 and His14) in binding Cu, others have refuted this. 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 4.4). 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 retains the peroxidase activity of heme-Aβ.

CV data indicates that both the Cu site and the heme sites can be reduced by physiological reducing agents. While the heme site ($E^o = -0.42$ V i.e. -0.17 V vs NHE) can be
reduced by glutathione (E⁰ = -0.250 V vs NHE)⁸⁰,⁸¹,⁸²,⁸³,⁸⁴ or NADH (E⁰ = -0.45 V vs NHE)⁸⁵, the Cu site (E⁰ = 0.035-0.01 V i.e. 0.285-0.260 V vs NHE) can be reduced by the above reducing agents as well as by ascorbic acid.

The reduction of O₂ 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. Fe³⁺, Cu²⁺) mediated generation of H₂O₂ by Aβ peptides.⁸⁸ Aβ(1-42) in the presence of catalytic amounts of transition metals (Fe³⁺ and Cu²⁺) reduces the metal center and subsequently generates equivalent amounts of H₂O₂.⁸⁹ The amount of H₂O₂ produced follows a 2 e⁻ transfer pathway and is dependant on the oxygen availability.⁸⁹ In the presence of excess endogenous reducing agents (e.g. cholesterol, catecholamines, vitamin C) Cu-Aβ(1-42) generates catalytic H₂O₂ following a 2 e⁻ reduction of O₂.⁹⁰ It has been proposed that the 2 e⁻s are derived from the Aβ peptide which reduces two equivalents of Cu²⁺ bound to Aβ(1-42) to Cu⁺, which subsequently transfers the electron to O₂. Alternatively, two molecules of Cu-Aβ can each donate an electron to O₂ to form H₂O₂. Met35 has also been invoked to be responsible for providing reducing equivalents. In this study, oxidation of reduced Cu⁺-Aβ, heme(Fe²⁺)-Aβ and heme(Fe²⁺)-Cu⁺-Aβ (in the absence of any excess reductant) by molecular O₂ indicates that the amounts of H₂O₂ generated by these species are ~84%, ~90% and ~130%, respectively (Figure 4.5). Thus both heme(Fe²⁺)-Aβ and Cu⁺-Aβ complexes reduce O₂ by 2 e⁻ to H₂O₂ 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 Tyr10 residue. This is clearly indicated by 40-45% PROS generation upon re-oxidation of the reduced Tyr10Gly Aβ(1-16) mutant complexes of Cu⁺ and heme(Fe²⁺), which is half of what is produced in the wild type, indicating a 1 e⁻ reduction of O₂ to O₂⁻ (i.e. Cu⁺ and Fe²⁺ both donate 1 e⁻).⁹¹ Since heme(Fe²⁺)-Cu⁺-Aβ contains three electrons (one each from Cu, Fe and Tyr10), the amount of H₂O₂ generated is ~ 130 % (expected is 150 %), which decreases to ~ 65 % in the case of heme(Fe²⁺)-Cu⁻-Aβ (Tyr10Gly) complex which now has two reduced sites. Thus our 2 e⁻ reduction of O₂ to H₂O₂ 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 Met35 as the source of electrons for O₂ 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 Tyr10 residue. More importantly, when both heme and Cu are bound to the Aβ peptide, the toxic PROS formation is maximum (Figure 4.5), 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 4.3). So far His13 has been invoked in binding Cu. Arg5 has been propose to impose steric and conformational constrains on the Cu bound Aβ peptide.\textsuperscript{45,92} Our previous results showed that Arg5 and His13 are involved in heme binding.\textsuperscript{55} The current results indicate that Tyr10 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, Arg5, Tyr10 and His13, unique to human Aβ peptides, play key roles in the pathology of AD.

**Scheme 4.3.** 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.

\[
\text{Asp-Ala-Glu-Phe-Arg}^5\text{-His-Asp-Ser-Gly-Tyr}^{10}\text{-Glu-Val-His}^{13}\text{-His-Gln-Lys}
\]

\[
\text{Asp-Ala-Glu-Phe-Gly}^5\text{-His-Asp-Ser-Gly-Phe}^{10}\text{-Glu-Val-Arg}^{13}\text{-His-Gln-Lys}
\]

### 4.5. SUMMARY

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 Tyr10 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.

4.6. REFERENCES

1) Rauk, A. Chemical Society Reviews, 2009, 38, 2698.
2) Glenner, G. G.; Wong, C. W. Biochemical and Biophysical Research Communication
1984, 120, 885.
10) Masters, C. L.; Tanzi, R. E. Proceedings of the National Academy of Science, 2003,
100, 11193.
12) Smith, D. J.; Cappai, R.; Barnham, K. J.; Biochimia Et Biophysica Acta, 2007, 1768,
1976.
13) Lovell, M. A.; Robertson, J. D.; Teesdale, W. J.; Campbell, J. L.; Markesbery, W. R.;
Journal of Neurological Sciences, 1998, 158, 47.


42) Drew, S. C; Noble, C, J; Masters, C, L; Hanson, G, R; Barnham, K, J. *Journal of American Chemical Society* 2009, 131, 1195.


44) Hureau, C; Coppel, Y; Dorlet, P; Solari, P, L; Sayen, S; Guillon, E; Sabater, L.; Faller, P. *Angewandte. Chemie Internatinal Edition* 2009, 48, 9522.


Shearer, J.; Callan, P. E.; Tran, T.; Szalai, V. A. *Chemical Communication*. 2010, 46, 9137.


Atamna, H.; Boyle, K. *Proceedings of the National Academy of Science*, 2006, 103, 3381.


Sassa, S., 2004. Why heme needs to be degraded to iron, biliverdin IXalpha, and carbon monoxide? Antioxid Redox Signal 6, 819.
Peroxidase activity reflects the rate of 3,3′,5,5′-tetramethylbenzidine (TMB) oxidation. Because the rate of heme-\( \alpha \beta \) catalyzed TMB oxidation is faster than the rate of Cu-\( \alpha \beta \) catalyzed oxidation, the reaction likely proceeds through a faster kinetic pathway (i.e. through the heme-\( \alpha \beta \) site), when both heme and Cu are simultaneously present in the peptide. Hence heme-Cu-\( \alpha \beta \) peroxidase activity is not a summation of the heme-\( \alpha \beta \) and Cu-\( \alpha \beta \) peroxidase activities.

1 e\(^-\) reduction of \( \text{O}_2 \) produces superoxide, \( \text{O}_2^- \), which after disproportionation should yield \( \sim 50 \% \text{H}_2\text{O}_2 \)

Theoretically, 2 e\(^-\) reduction of \( \text{O}_2 \) should produce 100 % \( \text{H}_2\text{O}_2 \) and on the other hand, 1 e\(^-\) reduction of \( \text{O}_2 \) should produce 100 % \( \text{O}_2^- \), which should then disproportionate to generate 50 % \( \text{H}_2\text{O}_2 \). Thus, effectively, 150 % \( \text{H}_2\text{O}_2 \) should be produced when one \( \text{O}_2 \) is reduced by 2 e\(^-\) and another by 1 e\(^-\).

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91) Note that the lack of the characteristic EPR signal of a TyrO species may indicate dimerization of the Aβ peptides. This is a characteristic pathology of AD where the involvement of the Tyr$^{10}$ has been invoked but not established. Further work detailing the mechanism of the process is currently under investigation.

Figure S4.1. Calibration curve for H$_2$O$_2$ detection by the xylenol orange assay protocol, (details in the Materials and Methods section). Absorbance at 560 nm vs. different concentrations of H$_2$O$_2$ (µM) have been plotted to prepare the calibration curve.
Figure S4.2. Anaerobic CV of A) Cu-Aβ(1-16), blue and heme-Cu-Aβ(1-16), green at 20 mV/s, and B) heme-Aβ(1-16), red and heme-Cu-Aβ(1-16), green at 1 V/s.