Nitric oxide (NO) is one of the most important signaling molecules present in the human body and plays complex roles in many biological processes. In this chapter we discuss that NO can bind to heme and Cu bound Aβ complexes. Interestingly, in a physiologically relevant mixed valent Cu$^+$-Fe$^{3+}$(heme)-Aβ complex, NO can bind to the heme center, and drive the Tyr10 residue mediated electron transfer from the Cu to the heme center. This results in the formation of an oxidized Cu$^{2+}$ site and Fe$^{2+}$(heme)-Aβ-NO complex. The resultant Fe$^{2+}$(heme)-NO complex dissociates from the Aβ peptides. Thus, NO also provides a mechanism for removal of heme from Aβ peptides.
6.1. INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disorder causing senile dementia.\(^1\) This terminal disease is closely associated with massive loss of neurons and synaptic breakdown in the brain. The most common biomarker of AD is the accumulation of neurotoxic amyloid beta (Aβ) peptides in the brain.\(^2,3\) Transition metals like Cu and Fe bind to Aβ peptides\(^4-6\) and in their reduced forms react with O\(_2\) generating toxic partially reduced oxygen species (PROS)\(^7,8\) leading to oxidative stress and oligomerization of Aβ peptides. Recently it has been shown that heme binds to Aβ peptides.\(^9-12\) This complexation diminishes the bioavailability of regulatory heme, leading to deficiency of heme required for normal biological processes.\(^13\) Heme deficiency gives rise to specific symptoms, which are incidentally key pathological features of AD,\(^13\) implicating a direct role of heme in this disease. The heme-Aβ complexes behave as peroxidases and can oxidize neurotransmitters like serotonin and 3,4-dihydroxyphenylalanine (DOPA) in presence of H\(_2\)O\(_2\), which might account for the abnormal neurotransmission observed in AD patients.\(^14,15\) The active site of this complex has been characterized by spectroscopic techniques. His13 and His14 of Aβ peptide have been identified as the heme coordinating residues.\(^12\) Arg5 residue present at the distal pocket H-bonds with the exchangeable water derived ligand present at the distal position and donates a proton driving the O-O bond heterolysis, making the heme-Aβ complexes function as peroxidases.\(^12\)

The Aβ peptides have recently been shown to simultaneously bind both heme and Cu. The heme and Cu sites exhibit unique spectroscopic and electrochemical features that remain unperturbed in the presence of each other.\(^16,17\) The PROS generated by the Cu\(^+\)-heme(Fe\(^{2+}\))-Aβ complexes is maximum relative to heme(Fe\(^{2+}\))-Aβ and Cu\(^+\)-Aβ complexes making them most toxic for AD.\(^16\)

Nitric oxide (NO) is one of the most important signaling molecules present in the human body and plays complex roles in many biological processes.\(^18\) It is synthesized form \(L\)-arginine by the enzyme nitric oxide synthase (NOS).\(^19,20\) Recently, NO has been associated with AD since there is a decreased level of NO in patients suffering from AD.\(^21\) Soluble guanylyl cyclase (sGC) enzyme binds NO and relays NO signal.\(^22,23\) This activated sGC elevates intracellular levels of a second messenger molecule, cyclic guanosine monophosphate (cGMP).\(^22\) This is a key signal transduction system in central nervous
system (CNS) that maintains plasticity in the hippocampus and cerebral cortex of the brain.\textsuperscript{24,25} This immensely important process for formation of new memory is hampered in AD brain possibly due to a decrease in NO concentration. Thus, decreased levels of NO observed in AD brains may contribute to memory impairment and neuronal cell death. Presently it has been accepted that aggregated Aβ peptides inhibit the NO signaling pathway and suppress the protective effects of endogenous NO in the brain.\textsuperscript{26} It has also been observed that the NO produced by neuronal NOS (nNOS) and endothelial NOS (eNOS) plays a protective role against Aβ induced neuronal cell death, cerebrovascular dysfunction and cerebral amyloid angiopathy.\textsuperscript{27} Clinical trial of NO donors and cGMP analogues as therapeutics have been found to suppress cell death, prevents inflammatory responses in brain cells and reverses learning and memory impairment through protein kinase G (PKG) activation.\textsuperscript{28} NO produced by inducible NOS (iNOS) causes neurotoxicity forming reactive nitrogen species. Elevated iNOS level and decreased nNOS level in aged rats also show similar dual character as observed in human AD.\textsuperscript{29} Thus, it appears that normal and sufficient bioavailability of NO is essential for inhibiting the risks of AD.

Here we show that NO can bind to heme and Cu bound Aβ complexes. The reduction potentials of the heme and Cu sites in the Aβ bound complexes are -0.17 V and 0.28-0.26 V vs NHE, respectively. Thus, reducing agents like vitamin C (E\textsuperscript{o} = -0.066 V vs NHE)\textsuperscript{30} can selectively reduce the Cu site of the Cu\textsuperscript{2+}-heme(Fe\textsuperscript{3+})-Aβ complex.\textsuperscript{11,16} Interestingly, in a physiologically relevant mixed valent Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ complex, NO can bind to the heme center, and drives the Tyr10 residue mediated electron transfer from the Cu to the heme center. This results in the formation of an oxidized Cu\textsuperscript{2+} site and heme(Fe\textsuperscript{2+})-Aβ-NO complex. The oxidized Cu site generated in the process is less toxic since it is less prone towards generation of oxidative stress. The resultant heme(Fe\textsuperscript{2+})-NO complex dissociates from the Aβ peptides. Thus, NO also provides a mechanism for removal of heme from Aβ peptides.

6.2. MATERIALS AND METHODS

6.2.1. MATERIALS

All reagents were of the highest grade commercially available and were used without further purification. Amyloid beta peptide, Aβ(1-16) (Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys) and its Try10Gly mutant were purchased from GL
Biochem (Shanghai) Ltd. with >95% purity. Hemin, copper sulfate and the HEPES buffer were purchased from Sigma. Sodium nitrite, sodium dithionite, ascorbic acid and sulfuric acid were purchased from Merck. D$_2$O was purchased from Cambridge Isotope Laboratory.

6.2.2. SAMPLE PREPARATION

Aβ stock solution of 0.5 mM strength was prepared by dissolving in pH 7 Hepes buffer. 5 mM hemin solution was prepared in 1 M NaOH solution. Copper sulfate solution was made of 10 mM strength in nanopure water. Ascorbic acid and sodium dithionite solutions of 20 mM strength were made by dissolving them in degassed buffer under anaerobic condition. heme(Fe$^{3+}$)-Aβ complex was prepared by incubation of 1 equivalent of Aβ with 0.8 equivalent of heme ~ 6 hours and Cu$^{2+}$-Aβ complex was prepared by incubating 0.8 equivalent of CuSO$_4$ with 1 equivalent of Aβ for ~ 1 hour. For Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ samples 0.8 equivalent of heme was incubated with 1 equivalent of Aβ for ~ 6 hours followed by incubation with 0.8 equivalent of CuSO$_4$ for ~ 1 hour. The control sample was prepared by adding heme(Fe$^{3+}$) to Cu$^{2+}$-Aβ complex (heme(Fe$^{3+}$) added to Cu$^{2+}$-Aβ and Cu$^{2+}$ added to heme(Fe$^{3+}$)-Aβ produces the same Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ complex), allowing no incubation time for the heme cofactor to bind with Aβ. All the EPR samples were either prepared at or calibrated to pH 7 by addition of dilute H$_2$SO$_4$. To obtain Cu$^{+}$-heme(Fe$^{3+}$)-Aβ, the oxidized Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ was reduced with ascorbic acid under anaerobic condition at pH 7 in a glove box. Reducing the Cu$^{2+}$ center in Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ does not affect the heme(Fe$^{3+}$) center as evident from absorption and EPR data (Figure S6.1). Scrubbed NO gas (generated by adding saturated NaNO$_2$ solution to 20 M H$_2$SO$_4$ and purified by passing the generated gas through thoroughly degassed 4 M KOH and water) saturated buffer solutions (1.94 mM) were prepared by purging NO through anaerobic buffer for about 10 minutes.

For NO complexes, one equivalent NO saturated buffer solution (strength 1.94 mM) was added to Cu$^{+}$-heme(Fe$^{3+}$)-Aβ (100 μL, 0.5 mM) in an EPR tube, in the glove box. For kinetics study, this sample was taken out of glove box within 1 minute and frozen in liquid nitrogen to obtain the initial spectrum. For subsequent data points, the same sample was thawed in the glove box and incubated; following which it was frozen again in liquid nitrogen. Similar protocol was followed to prepare the deuterated sample. One equivalent of the NO saturated solution was added to the Cu$^{+}$-heme(Fe$^{3+}$)-Aβ complex in a cuvette under anaerobic condition for obtaining the absorption data at various reaction times.
6.2.3, EPR SPECTROSCOPY

EPR samples were 0.5 mM in concentration and 100 µL in volume and were run at 77 K in a liquid nitrogen finger dewar. EPR spectra were obtained by a Jeol (JES FA200) spectrophotometer with the following parameters for the high energy region: modulation width 20 gauss; amplitude 50; time const. 300 msec; power 10 mW; frequency 9.27 GHz; and low energy region: modulation width 20 gauss; amplitude 50; time const. 30 msec; power 1 mW; frequency 9.27 GHz.

6.2.4, ABSORPTION SPECTROSCOPY

Absorption spectral data were obtained by a UV-Vis diode array spectrophotometer (Agilent 8453).

6.2.5, RESONANCE RAMAN (RR) SPECTROSCOPY

RR spectroscopy data were obtained using a Trivista 555 spectrograph (Princeton Instruments) and using 413.1 nm excitation from a Kr+ laser (Coherent, Sabre Innova SBRC-DBW-K).

6.3. RESULTS AND ANALYSIS

6.3.1, ABSORPTION SPECTROSCOPY

Cu²⁺-heme(Fe³⁺)-Aβ complex of Aβ(1-16) was prepared by incubating 0.8 equivalent of Cu²⁺ with an equivalent of heme(Fe³⁺)-Aβ peptide complex. When the mixed valent Cu⁺-heme(Fe³⁺)-Aβ complex was exposed to one equivalent of NO, it generated an absorption spectrum with the Soret band at ~ 384 nm, within a couple of seconds (Figure 6.1, light green). Gradually, with time, the Soret band red shifted to ~ 394 nm and bands at 555 nm and 585 nm appeared in the Q band region of the spectrum (Figure 6.1, purple). When one equivalent of NO was added to heme(Fe³⁺)-Aβ complex (no Cu), it formed a species having a Soret band at ~384 nm (Figure 6.1, orange), similar to the spectrum generated on instantaneous NO exposure to the Cu⁺-heme(Fe³⁺)-Aβ complex (Figure 6.1, light green). The absorption spectrum on addition of NO to the reduced heme(Fe²⁺)-Aβ complex (no Cu), generated a spectrum having Soret band at 394 nm and Q bands at 555 nm and 585 nm (Figure 6.1, dark green), very similar to that produced on prolonged NO exposure to the
mixed valent Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ complex (Figure 6.1, purple). Thus, the species formed on instantaneous exposure of NO to Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ is likely a heme(Fe\textsuperscript{3+})-NO species which then converts to a -heme(Fe\textsuperscript{2+})-NO species.

FIGURE 6.1. Absorption spectra of Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ complex with one equivalent NO after 10 secs (light green), 60 secs (purple) overlaid with heme(Fe\textsuperscript{3+})-Aβ-NO, orange and heme(Fe\textsuperscript{2+})-Aβ –NO, dark green.

6.3.2. EPR SPECTROSCOPY

The reaction of NO with the mixed valent Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ complex was monitored by EPR spectroscopy as well. The starting Fe\textsuperscript{3+} center showed a high spin $S = 5/2$ axial signal in the EPR spectrum, while the reduced Cu center was EPR silent (Figure 6.2A, red). On exposing this to one equivalent of NO, the high spin axial EPR signal diminished with the concomitant increase in EPR signal intensity in the high field, low spin region of the spectrum (Figure 6.2A, purple). An intense signal observed in the high field $g = 2$ region of the EPR spectrum indicated formation of an $S=1/2$ species (Figure 6.2B, purple). This signal is similar to that of the one electron reduced heme(Fe\textsuperscript{2+})-Aβ-NO complex (Figure 6.2B, green). Additionally, the presence of hyperfine features of an oxidized Cu species was observed with $A_{H} \sim 170 \times 10^{-4}$ cm\textsuperscript{-1}, $g_{H} \sim 2.239$ and $g_{\perp} \sim 2.046$ (Figure 6.2A, purple). These parameters are identical to the EPR parameters of the Cu\textsuperscript{2+} site of the Cu\textsuperscript{2+}-heme(Fe\textsuperscript{3+})-Aβ complex (Figure 6.2B, cyan), indicating that the Cu\textsuperscript{+} site of the Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ complex gets oxidized by one electron to Cu\textsuperscript{2+} on NO exposure as well.
Figure 6.2. EPR spectra of Cu⁺-heme(Fe³⁺)-Aβ, red; Cu⁺-heme(Fe³⁺)-Aβ + NO, purple; Cu²⁺-heme(Fe³⁺)-Aβ, cyan and heme(Fe³⁺)-Aβ + NO, green in the (A) low field and (B) high field regions.

Reaction of NO with Cu⁺-heme(Fe³⁺)-Aβ complex of a Tyr10Gly mutant was evaluated. Interestingly, for this mutant complex, the high spin $S = 5/2$ heme(Fe⁺³) signal decreased on addition of NO, implicating formation of the same heme(Fe⁺³)-NO species (Figure 6.3 A, S6.2) as the wild type peptide. However, no low spin signal appeared (Figure 6.3 B).

Figure 6.3. EPR spectra of Cu⁺-heme(Fe³⁺)-Aβ of Tyr10 mutant at pH 7, red; Cu⁺-heme(Fe³⁺)-Aβ of Tyr10 mutant + NO, final, blue and heme(Fe²⁺)-Aβ of wild type + NO, final green in the (A) low field region and (B) high field region. The red spectrum is obscured by the blue spectrum in Figure 6.3 B.
6.3.3. RESONANCE RAMAN SPECTROSCOPY (rR)

rR spectroscopy has been used to determine the coordination number, spin state and oxidation state of heme Fe.\textsuperscript{32} Excitation into the Soret band of heme results in an enhancement of vibrations associated with heme and the high frequency region of the spectrum contains characteristic marker bands that are sensitive to the oxidation and spin state and coordination of heme Fe.\textsuperscript{32-34} The oxidation state of the heme can be determined from the π-electron density marker, ν\textsubscript{4} band, while the spin and coordination state can be determined from the markers, ν\textsubscript{3}, ν\textsubscript{2}, and ν\textsubscript{10} bands, which are sensitive to the core size of the heme macrocycle.\textsuperscript{35-37} The ν\textsubscript{4}, ν\textsubscript{3}, and ν\textsubscript{2} and ν\textsubscript{10} bands for the prolonged NO exposed Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ complex were observed at 1377, 1511, 1595 and 1652 cm\textsuperscript{-1} respectively in the resonance Raman spectrum (Figure 6.4, purple) of the complex. The heme(Fe\textsuperscript{2+})-Aβ-NO (no Cu) complex of the wild type also has vibrations identical to that of the mixed valent NO complex (Figure 6.4, dark green). The resonance Raman spectrum of Fe\textsuperscript{2+}-NO complex of free heme (no Aβ, Figure 6.4, black) shows the vibrations at energies identical to those of Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ -NO and heme(Fe\textsuperscript{2+})-Aβ-NO complexes. Since Fe\textsuperscript{2+}-NO complex of free heme is five coordinate in nature, the data demonstrate that the above two complexes are also five coordinate species. Note that the above experimentally obtained frequencies are also typical of a five coordinate Fe\textsuperscript{2+}-NO species,\textsuperscript{38,39} further implying that the nitrosyl complexes formed are five coordinate in nature. The N-O stretch could not be identified because of overlapping heme ring modes.

![Graph](image)

**FIGURE 6.4.** High frequency region of rR spectra of Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ + NO, purple; heme(Fe\textsuperscript{2+})-Aβ + NO, dark green and free heme(Fe\textsuperscript{2+}) + NO, black. The data were obtained with an excitation wavelength of 413.1 nm (15 mW) at 77 K. (* indicate plasma lines from the laser.)
6.3.4. KINETICS

The kinetics of NO reaction of the Cu$^+$-heme(Fe$^{3+}$)-Aβ complex of wild type Aβ peptide was followed using EPR spectroscopy. The data shows that the rate of decrease of the high spin Fe$^{3+}$ signal is ~ 3.5 times faster than the rate of formation of the heme(Fe$^{2+}$)-NO signal and the rate of oxidation of the Cu site, the later two processes having same rates (Figure 6.5). This implies that binding of NO to the heme(Fe$^{3+}$) site forming an EPR silent heme(Fe$^{3+}$)-NO species (Figure S6.2) is relatively fast. The rate of electron transfer from the reduced Cu site to the heme(Fe$^{3+}$)-NO site forming the oxidized Cu site and heme(Fe$^{2+}$)-NO species is the rate determining step (rds).

The kinetics followed in deuterated buffer showed significant decrease in both the rates i.e. NO binding to the heme(Fe$^{3+}$) site as well as rate of electron transfer (Figure 6.5). The primary isotope effect for the first step i.e. NO binding to the heme site is ~ 18±5 and is derived from a proton coupled displacement of hydroxide by NO.$^{40}$ The primary isotope effect of the second step involving electron transfer from the reduced Cu to the ferric heme nitrosyl species was ~ 25±5.

**FIGURE 6.5.** Kinetic traces of loss of high spin EPR signal intensity in aqueous, red; and deuterated buffer, orange; increase in intensity of Cu hyperfine features in aqueous buffer, cyan; and increase in intensity of heme(Fe$^{2+}$)-NO signal intensity in aqueous, dark green and deuterated buffer, light green; along with simulations of the rates in dotted lines.
6.4. DISCUSSION

The reduction potentials of the Cu and heme sites of Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ complexes are 0.28-0.26 V and -0.17 V vs NHE,$^{11,16}$ respectively. Thus, the Cu site can selectively be reduced by abundant physiological reducing agents like vitamin C ($E'' = -0.066$ V vs NHE)$^{30}$, forming Cu$^+$-heme(Fe$^{3+}$)-Aβ, the physiologically relevant form. Aβ is known to bind reduced Cu,$^{41-43}$ and oxidized heme.$^{9-12}$ EPR and absorption data indicate that the Cu and heme sites of Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ and Cu$^+$-heme(Fe$^{3+}$)-Aβ complexes remain unperturbed in the presence of each other (Figure S6.1, S6.3).$^{16}$

From the absorption data we deduce that NO binds to the heme(Fe$^{3+}$) site of Cu$^+$-heme(Fe$^{3+}$)-Aβ forming a heme(Fe$^{3+}$)-NO species (Figure 6.1, light green). This is then reduced to form a heme(Fe$^{2+}$)-NO complex (Figure 6.1, purple) on prolong exposure.$^{44}$ The EPR results indicate simultaneous formation of a Cu$^{2+}$ site, identical to that of Cu$^{2+}$-Aβ and a heme(Fe$^{2+}$)-NO species on NO exposure to the Cu$^+$-heme(Fe$^{3+}$)-Aβ complex (Figure 6.2, purple). The resonance Raman data confirm that the final product of NO reaction with the mixed valent Cu$^+$-heme(Fe$^{3+}$)-Aβ complex (Figure 6.4, purple) is a ferrous nitrosyl species, which is five coordinate in nature (Figure 6.4, black).$^{38,39}$ This implies that reduced Cu bound Aβ directly transfers an electron to the heme(Fe$^{3+}$)-NO species reducing it to the heme(Fe$^{2+}$)-NO form. The heme then dissociates from the Aβ peptide on NO binding to generate the final five coordinate heme(Fe$^{2+}$)-NO species. Note that the interaction of NO with Cu$^+$ is known to produce Cu$^+$-nitrosyl species.$^{45}$ However, the binding constant of NO with Cu$^+$ is much less than heme.$^{46}$ Since one equivalent of NO is added in this study, the heme(Fe$^{2+}$)-NO adduct formation will be thermodynamically more favourable. However, the possibility of formation of an intermediate Cu$^+$-NO adduct prior to the formation of heme(Fe$^{3+}$)-NO cannot be eliminated.

A control sample was prepared where heme was added to Cu bound Aβ peptide but no incubation time was allowed for heme binding. This complex thus has Cu bound to Aβ, but not heme i.e. the heme is free in solution. Such a variation was chosen to evaluate the possibility of an inter molecular oxidation of the reduced Cu site by a five coordinate heme(Fe$^{3+}$)-NO species. When one equivalent of NO was added to the free heme(Fe$^{3+}$)-Cu$^+$-Aβ peptide complex, it generated an absorption spectrum corresponding to a free heme(Fe$^{3+}$)-NO complex (Figure 6.6A). However, no subsequent formation of a heme(Fe$^{2+}$)-NO signal was observed. The same reaction was also monitored by EPR spectroscopy. Similar to the Cu$^+$-heme(Fe$^{3+}$)-Aβ complex, the high spin $S = 5/2$ axial EPR signal of Fe$^{3+}$ was decreased on NO addition (Figure 6.6B), however with no development of any low spin signal (Figure 6.6C). No Cu$^{2+}$ hyperfine features were observed either. Thus, in free
heme(Fe$^{2+}$)-Cu$^{+}$-Aβ complex, where free heme can potentially bind NO, no electron transfer from the Cu$^+$ to the free heme(Fe$^{3+}$) -NO complex occurred. Thus, heme(Fe$^{3+}$)-NO needs to be bound to the Aβ peptide for electron transfer from the reduced Cu site to the ferric nitrosyl site to occur i.e. the electron transfer is intra molecular. Further, this also implies that formation of a five coordinate heme(Fe$^{2+}$)-NO species, due to heme dissociation from the Aβ peptide, occurs only after the electron transfer step, since no electron transfer is observed in this control sample containing free heme(Fe$^{3+}$) -Cu(I)-Aβ, where free heme (analogous to dissociated heme from Aβ) is five coordinate in nature. Thus the six coordinate heme(Fe$^{3+}$) -NO acts as an oxidizing agent for Cu.

**Figure 6.6.** Absorption spectra of free heme(Fe$^{3+}$)+ NO, orange and free heme(Fe$^{3+}$)-Cu$^{+}$-Aβ + NO, purple. (A) EPR spectra of free heme(Fe$^{3+}$)-Cu$^{+}$-Aβ, red; free heme(Fe$^{3+}$)-Cu$^{+}$-Aβ + NO final, purple and Cu$^{2+}$-heme(Fe$^{3+}$)-Aβ-NO, cyan in (B) low field and (C) high field regions.
Tyr10 is one of the amino acid residues absent in rodents that do not get affected by AD. The reaction of NO with Cu\(^+\)-heme(Fe\(^{3+}\))-A\(\beta\) complex of the Tyr10Gly mutant indicated formation of a heme(Fe\(^{3+}\))-NO complex, however, there was no subsequent formation of the reduced heme(Fe\(^{2+}\))-NO complex, nor did the Cu site get oxidized (Figure 6.3). It has been shown that Tyr10 does not bind either heme or Cu. Thus, Tyr10 likely mediates the electron transfer from the reduced Cu to the NO bound heme(Fe\(^{3+}\)) site. Tyrosine is well known to mediate long range electron transfer in biology. Note that given the close proximity of His13 (proposed to be involved in heme binding) and His14 (proposed to coordinate to Cu), and the fact that histidine mediate electron transfer pathways are well known in metal active sites, a His-His electron transfer pathway between the Cu and heme sites may be invoked. However, current results, which show that a Tyr10 mutant abolishes electron transfer from the Cu to the heme site and past studies (which show two electron oxidation of Cu\(^+\) and Tyr10 or heme(Fe\(^{3+}\))and Tyr10 by molecular O\(_2\)) are more consistent with a Tyr10 mediated electron transfer pathway.

The reaction of NO with the Cu\(^+\)-heme(Fe\(^{3+}\)) complex of the wild type A\(\beta\) peptide, as determined from spectroscopy and kinetics, is summarized in Scheme 1. NO binds to the Fe\(^{3+}\) center of the heme-A\(\beta\) peptide (A\(\beta\)- heme(Fe\(^{3+}\))-OH + NO + H\(^+\) \rightarrow A\(\beta\)- heme(Fe\(^{3+}\))-NO + H\(_2\)O) with a primary isotope effect of ~ 18±5. Subsequently the reduced Cu\(^+\) site acts as a reducing agent reducing the A\(\beta\) bound ferric-NO species to the A\(\beta\) bound ferrous-NO form. The rate of electron transfer from the reduced Cu site to the heme(Fe\(^{3+}\))-NO site forming the oxidized Cu site and heme(Fe\(^{2+}\))-NO species is the rds. This electron transfer is mediated by the Tyr10 residue. This step has a KIE of ~ 25±5 which could be derived from a proton coupled electron transfer (PCET) step involved in the oxidation of the Cu site, i.e. Cu\(^+\)(H\(_2\)O) \rightarrow Cu\(^{2+}\)(OH\(^-\)) + H\(^+\) +e\(^-\). PCET processes are known to have large deuterium kinetic isotope effects. Since the absence of the tyrosine residue abolishes the electron transfer from the Cu to the heme site, we propose that it is involved in the electron transfer pathway. In spite of trapping the A\(\beta\) bound heme(Fe\(^{3+}\))-NO species in EPR, its resonance Raman data could not be obtained due to its well established photolability. The heme(Fe\(^{2+}\))-NO complex formed then dissociates from the A\(\beta\) peptide forming a five coordinate species. Note that no six coordinate heme(Fe\(^{2+}\))-NO species was observed in the time dependent EPR experiments suggesting that the rate of dissociation of the heme(Fe\(^{2+}\))-NO species from the A\(\beta\) peptide (final product is a five coordinate heme(Fe\(^{3+}\))-NO species) is possibly much faster than the rate of electron transfer from Cu to heme site. The dissociation of the Fe(heme)-His(peptide
backbone) bond on NO binding is well known in several enzyme active sites.\textsuperscript{56-58} While in a protein matrix the heme stays bound near the active site due to secondary interactions (e.g. hydrogen bonding with the propionate group), in a small peptide like Aβ, it diffuses into the solution.

**SCHEME 6.1.** Schematic representation of reaction of Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ with NO. NO binds to heme(Fe\textsuperscript{3+})-Aβ in the first step. In the second step, electron is transferred from the Cu\textsuperscript{+} to the heme(Fe\textsuperscript{3+})-NO species. This is the rds and involves Tyr10. The next step is fast and involves dissociation of heme(Fe\textsuperscript{2+})-NO from the Aβ peptide. The ligands of the heme and Cu sites have not been included.

**6.5. SUMMARY**

Heme and Cu binding to Aβ peptides has been invoked to have detrimental effects in AD.\textsuperscript{16,17} Heme deficiency leads to abnormal iron homeostasis, increase in bilirubin and heme oxygenase concentration, decay of iron regulatory proteins, dysfunction in mitochondrial complex IV and oxidative stress.\textsuperscript{10,13,14} Cu bound Aβ peptides will have Cu in the reduced form under physiological conditions. Cu\textsuperscript{+} is prone to produce toxic PROS in the body generating oxidative stress.\textsuperscript{7} When heme and Cu are simultaneously bound to Aβ peptides, maximum PROS are produced.\textsuperscript{16} NO binds the heme site of the physiologically relevant Cu\textsuperscript{+}-heme(Fe\textsuperscript{3+})-Aβ peptide complex. The two otherwise electronically uncoupled paramagnetic
centers undergo electron transfer upon NO binding. This electron transfer from the Cu to the heme site is mediated by a tyrosine residue. In the process, Cu$^{2+}$ gets generated from Cu$^+$ which is much less toxic for AD relative to the reduced form and will not produce oxidative stress in the body. Moreover, once the ferric(heme) nitrosyl species gets reduced by the Cu site, the ferrous nitrosyl species formed dissociates from the Aβ peptide. Thus, NO helps in releasing heme from the Aβ peptides and can ameliorate the effects of heme binding to Aβ associated with AD. Hence NO might play a significant role in reducing the risks arising from redox-active heme and Cu bound Aβ peptides associated with AD. In fact NO has also been proposed to be involved in defending the heme-Cu active site of cytochrome c oxidase, the terminal enzyme in the respiratory chain, against antagonists like cyanide and carbon monoxide.$^{59,60}$ Interestingly, the physiological NO levels are decreased in patients affected with AD, which might imply that NO cannot implement its protective role in the body against heme and Cu. Maintaining an optimal NO level in the body might promote a significant reduction of risks associated with AD. Thus, not surprisingly NO releasing agents are already in clinical trials for AD.$^{28}$

### 6.6. REFERENCES


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Note that the pH of the experiment was ~ 7.4, and the pKa of the water derived ligand bound to the Fe3+(heme) site is 6.8, hence the hydroxide bound species was dominating at this pH.


The reorganization energy (λ) of type 2 Cu sites tends to be high. The λ involved in the oxidation of the Cu site possibly contributes to the slow electron transfer rate.


6.7. SUPPORTING INFORMATION

Figure S6.1. Absorption (A) and EPR (B) data of Cu²⁺-heme(Fe³⁺)-Aβ, orange and Cu⁺-heme(Fe³⁺)-Aβ, blue in low field region.
FIGURE S6.2. EPR spectra of heme(Fe$^{3+}$)-Aβ, red; heme(Fe$^{3+}$)-Aβ + NO final, purple and heme(Fe$^{2+}$)-Aβ + NO, green; in (A) low field and (B) high field regions.
FIGURE S6.3. A) The absorption spectra of Aβ(1-16), black; Cu^{2+}-Aβ, blue; heme(Fe^{3+})-Aβ, red; Cu^{2+}-heme(Fe^{3+})-Aβ, green and free heme, grey; B) 77 K EPR spectra of the low field region of heme(Fe^{3+})-Aβ, red, Cu^{2+}-heme(Fe^{3+})-Aβ, dark green and C) 77 K EPR spectra of the high field region of Cu^{2+}-heme(Fe^{3+})-Aβ, dark green and Cu^{2+}-Aβ, blue.