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Perspective

The Role of Heme and Copper in Alzheimer's Disease and Type 2 Diabetes Mellitus

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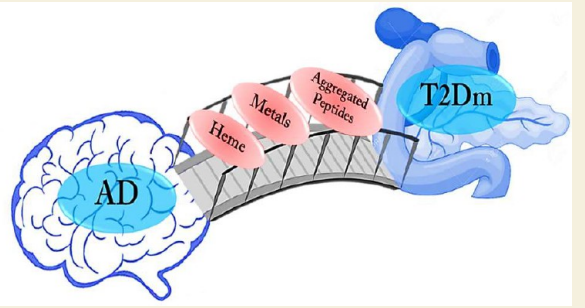
ABSTRACT: Beyond the well-explored proposition of protein aggregation or amyloidosis as the central event in amyloidogenic diseases like Alzheimer's Disease (AD), and Type 2 Diabetes Mellitus (T2Dm); there are alternative hypotheses, now becoming increasingly evident, which suggest that the small biomolecules like redox noninnocent metals (Fe, Cu, Zn, etc.) and cofactors (Heme) have a definite influence in the onset and extent of such degenerative maladies. Dyshomeostasis of these components remains as one of the common features in both AD and T2Dm etiology. Recent advances in this course reveal that the metal/cofactor-peptide interactions and covalent binding can alarmingly enhance and modify the toxic reactivities, oxidize vital biomolecules, significantly contribute to the oxidative stress leading to cell apoptosis, and may precede the amyloid fibrils formation by altering their native folds. This perspective highlights this aspect of amyloidogenic pathology which revolves around the impact of the metals and cofactors in the pathogenic courses of AD and T2Dm including the active site environments, altered reactivities, and the probable mechanisms involving some highly reactive intermediates as well. It also discusses some *in vitro* metal chelation or heme sequestration strategies which might serve as a possible remedy. These findings might open up a new paradigm in our conventional understanding of amyloidogenic diseases. Moreover, the interaction of the active sites with small molecules elucidates potential biochemical reactivities that can inspire designing of drug candidates for such pathologies.



KEYWORDS: *Alzheimer's disease, Type 2 Diabetes, heme, copper, spectroscopy, kinetics, intermediates*

1. INTRODUCTION

Amyloidosis is a vital hallmark in several degenerative maladies and so far more than 30 human proteins have been identified which potentially lead to these diseased conditions, collectively termed as "amyloidogenic diseases" or "amyloidoses".[1−6] All such diseases show the unique pathological feature of protein/peptide depositions in a form of cross β-sheet fibrils that are localized across definite organs or are accumulated systematically throughout the body (Scheme 1).[7−10] Amyloidosis can evolve either from a normal native protein arrangement, i.e., sporadic or from a protein having mutagenesis which is familial or hereditary.[1,4,11] There can also be some infectious forms of amyloidosis like prion protein aggregation that can cause the transmissible spongiform encephalopathies.[12−14] A few common amyloidegenic diseases are Alzheimer's disease (AD), type 2 diabetes mellitus (T2Dm), dialysis related amyloidosis (DRA), frontotemporal dementia (FTD), Huntington's disease (HD), multiple system atrophy (MSA), familial amyloid polyneuropathy (FAP), and transthyretin (TTR) amyloidoses, etc.; among which presently AD and T2Dm have become endemic all over the world.[1,15−19] To date, researchers have tried to formulate theories in order to address the cause and effect of these abnormal protein aggregation and associated malfunctions in the disease etiology, although these are inadequate as yet.[20,21]

The silver tsunami of 21st century, AD, is a neurodegenerative disease, characterized by a gradual loss of neurons in the hippocampus and cortex region, resulting in the shrinkage of brain.[22,23] It is also a common form of dementia with cognitive decline and behavioral function loss including memory, thinking abilities, etc. This proteinopathy symptomatically consists two types of misfolded protein deposits inside the brain; one is the neurofibrillary tangles (NFTs) having hyperphosphorylated Tau protein and the other one is the cellular plaques with aggregated Amyloid β (Aβ) peptides.[24,25] Aβ is a peptide product of the improper cleavage of a larger trans-membrane protein, amyloid precursor protein

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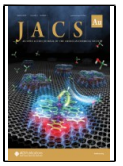

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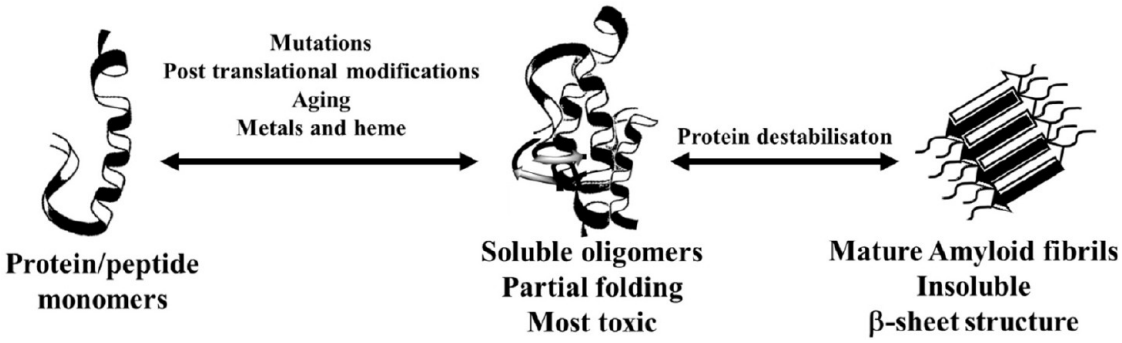
Scheme 1[a]



[a]Native peptide or protein monomers having a random-coil structure, aggregates to form oligomers and protofibrils with partial folding and finally to insoluble mature fibrillar form. The soluble oligomers are considered as the most toxic form of amyloids.

(APP).[26,27] In 1991, the proposal of J. Hardy and D. Allsop considered Aβ protein deposition as the primary influence to drive the 'amyloid cascade hypothesis' in AD.[28] This hypothesis is strongly endorsed by extensive genetic, pharmacological, pathological, and biochemical evidence, and therefore, the main scientific focus lingers on and around these particular proteins, making the earlier therapeutic approaches target primarily on the production pathway or the aggregation mechanism of these peptides.[29−31] Numerous clinical trials with small molecules and vaccines (β-secretase/BACE1 inhibitors, tramiprosate, colostrinin, scyllo-inositol, aducanumab (Aduhelm) etc.) have been steered to aim the AD related Aβ peptide formation, aggregation, or its clearance, which accounts for the maximum number of clinical tests (22.3% until 2019), although they remain largely unsuccessful in their medical trials.[21,32−34] Other known hypotheses include the Neurotransmitters hypothesis (the second most tested ∼19%), Cholinergic hypothesis, Tau propagation hypothesis, Calcium homeostasis and NMDA hypothesis, Neurovascular hypothesis, Inflammatory hypothesis, Lymphatic system hypothesis, Metal ion hypothesis, and the most recent Heme hypothesis.[34] Each of these hypotheses can explain certain aspects of the disease etiology, however, does not provide an inclusive scenario of the disease origin or associated toxicities and abnormalities observed in AD brain owing to the fact that AD is a complex multifactorial pathology, seeking multidirectional therapeutic approaches.

Along this line, the aggregation of another amyloidogenic peptide, islet amyloid polypeptide protein (IAPP) which deposits around the β-cell of pancreas is held responsible for the decreased β-cell mass, hyperamylinemia of pancreas, and the higher oxidative stress, which happen to be the central events in Type 2 Diabetes mellitus (T2Dm).[35−37] T2Dm is known as a chronic metabolic disorder, attributed to insulin resistance, decreased insulin secretion, glucose misbalance, β-cell loss, and most importantly the IAPP islet amyloidosis.[38] Recently, IAPP, in the form of oligomers is considered to be the most toxic form of amyloidogenic protein which is also membrane permeant and may contribute to β-cell apoptosis; although no *in vivo* evidence can be found in the human pancreas.[3,39,40] All such obvious reasons lead this particular amyloidogenic peptide to be targeted deliberately over the past decades to craft potential drug substances.[41−43] For instance, compounds like polyphenols and flavonoids, etc. have been tried to inhibit IAPP peptide aggregation and their cytotoxicity *in vitro*, and vaccines are also injected to raise antitoxic oligomer antibodies which are supposed to prevent IAPP oligomerization.[36,43] However, these have not resulted in stopping the β-cell apoptosis *in vivo*. Unfortunately, these trials are not very successful or are in a preclinical stage to be considered as impending therapeutics against the malfunctional protein generation, clearance, or aggregation. These point toward the reconsideration of the amyloid hypothesis, which is primarily based on particular amyloidogenic proteins and indicate inclusion of the other essential interfaces involved that positively affect the disease etiology.[21] Hence, in parallel with these protein-targeted researches, scientists have started excursions in other directions as well.

In both AD and T2Dm, multiple pathological changes are found to occur, one such change is in the metal homeostasis in the affected organs.[44−46] In the recent past, biometals like Cu, Zn, and Fe have been found to accumulate in and around the amyloid plaques in the AD brain.[47,48] Metal interference in T2Dm pathogenesis is also prominent as a contributor to the oxidative stress and proinflammatory environment in vascular, renal, and neural tissues.[49] As metal exposure of a soluble protein can perturb its native folding, it can cause proteins to form amyloid aggregates, while in some cases, metal is claimed to inhibit the fibrillization process as well.[50−56] As a matter of fact, with degenerative disorders and with aging, there is actually a significant interruption in the homeostasis of essential biometals which can also be the cause of the metal deficiency found in cellular compartments, as well as metal accumulation in microscopic proteinopathies.[57−59] Metal chelation therapy, although under controversy, has shown some positive impact in Alzheimer's disease and diabetic patients, further corroborating the suspected role of metals in such pathophysiology.[44,60−62] Fe, the most abundant transition metal in the human body, is also been implicated in this disease pathology in the form of a heme cofactor. Similar to the metal ions, heme is more recently found to have specific interactions with the amyloidogenic proteins/peptides causing cytotoxic effects in body.[63−65] There are several changes in heme metabolism, observed in the AD brain, along with its colocalization within the senile plaques.[62,66,67] The presence of heme casts a similar effect in T2Dm as well. This redox active cofactor can instigate oxidative stress by producing reactive oxygen species (ROS) and in turn accelerate the mitochondrial decay and damage other cellular components.[64,68,69] Thus, metals and heme may as well be a significant hallmark for identifying the individuals who are predisposed to these degenerative pathologies. Owing to the considerable similar-

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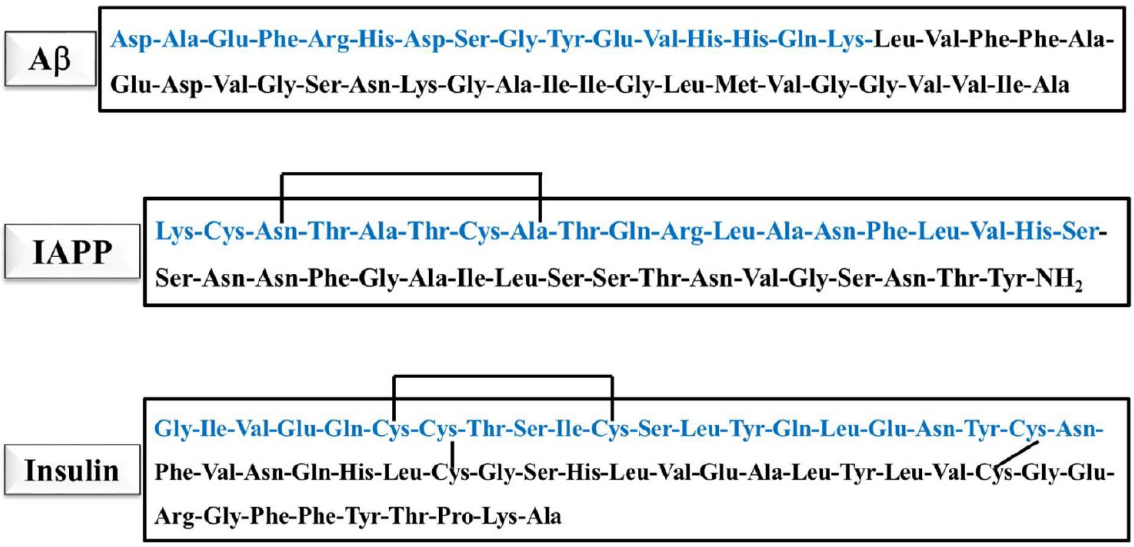
Scheme 2. Amino Acid Sequence of Human Aβ, IAPP, and Insulin[a]



[a]The blue segment is hydrophilic and the rest is hydrophobic region of human Aβ and IAPP; the blue part is chain A and rest is chain B of insulin.

ities between AD and T2Dm, researchers have been trying to find basic connections so that the remedial approach may become easy and holistic. Without undermining the previous approaches toward disease pathogenesis, another aspect involving metals and redox cofactor-like heme has been the focus here. These small molecules might serve as the missing link between AD and T2Dm as they are directly involved in coordination with amyloidogenic peptides like Aβ, IAPP, and insulin as well (Scheme 2).[70−74]

Moreover, their interactions show similar cytotoxic effects in the affected organs, making them more vulnerable than healthy individuals. This perspective deals with heme and metal involvement in the etiology of AD and T2Dm, demonstrating a brief overview of the process of their binding to peptides, the active site environments, their altered reactivities, the effects in cellular environments and on the other vital biomolecules, and the reactive oxidants that are responsible for enhanced toxicity in their respective mechanisms. Lastly, it elaborates the effect of chelators which might direct toward an imminent cure.

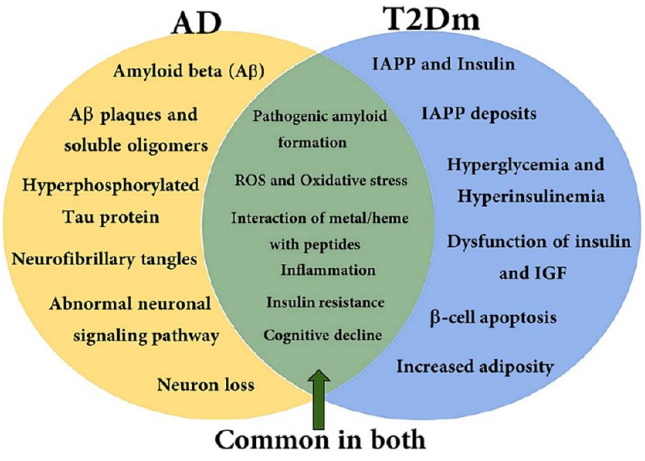
2. CONNECTIONS BETWEEN AD AND T2DM

Conventionally, AD and T2Dm are thought to be two independent disorders, however, the mounting epidemiological, observational, and basic molecular level research evidence have linked T2Dm as an increased risk of AD and vice versa.[75,76] Such similarities between AD and T2Dm have justified the proposed designation of AD as 'Type 3 Diabetes (T3Dm)'.[77,78]

2.1. Epidemiology

Prevailing characteristic features like protein misfolding, aggregate deposition, metal and heme binding, oxidative stress generation, altered glucose metabolism, hyperglycemia and insulin resistance, premature senescence and slow cognitive deterioration with aging; all these can be found in the histopathology of both these maladies (Scheme 3).[79,80] All these are considered as the outcome of proteinopathy, i.e., the abnormal conformational changes of proteins which have native physiological functions otherwise.[22,42] Common risk factors are higher cholesterol, aging degenerations, hampered metabolism, amyloid depositions, tau hyper-phosphorylation,

Scheme 3. Commonalities between AD and T2Dm



increased glycogen synthesis kinase 3 (GSK3) activity, cardiovascular problems, inflammation, interaction of genetic and nongenetic factors with apolipoprotein E4 (ApoE4), cell apoptosis, oxidative stress, mitochondrial damage and so on.[39,81,82] Mutations in the Aβ peptide which increases its propensity to fibrilize are identified in early onset of familial AD while a similar kind of mutation in IAPP has been found to enhance the risk of T2Dm.[81,83]

2.2. Aβ and IAPP

IAPP, the neuroendocrine peptide cosecreted along with insulin from pancreatic β-cells, can exert a pathological role in cognitive functions as it can cross and damage the blood brain barrier (BBB) and parallely can interact and co-deposit with Aβ-40/42 and tau peptides in the cerebrovascular system and gray matter in the aging brains, common to both AD and T2Dm.[40,76,78] It has been found that Aβ and IAPP can interact with each other and the latter promoted the former's aggregation in a seeding fashion, leading to the cross-seed oligomer formation.[40,73] IAPP dyshomeostasis aggravates the Aβ-42 toxicity via ROS generation and the interruption of insulin degrading enzyme activity which are responsible for IAPP, insulin, and Aβ degradation.[76] Such Aβ-IAPP crosstalk

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has important consequences in neuronal network function, insulin resistance, and in corresponding glucose imbalance. Eventually it contributes to the metabolic dysfunction in brain, leading to the onset and progression of diabetes-associated dementia.[84] Interestingly, Resveratrol, a phenolic compound, has been found to inhibit the pathological effects of both IAPP and Aβ, showing the above-discussed link between these two diseases.[85,86]

2.3. Aβ and Insulin

During AD, the brain suffers from neuron loss and also the ability to process glucose and to respond to insulin and insulin-like growth factor (IGF) which are necessary for synaptic plasticity and essential cognitive function, and the depletion of which may lead to dementia.[75] Impaired insulin signaling, one of the prominent symptoms in diabetes, also plays a critical role in AD pathogenesis as insulin is found to indirectly enhance the cleavage of APP by controlling the γ-secretase complex and thus can increase the Aβ level.[87] Such "brain diabetes phenotype" can result in the appearance of classical AD molecular biomarkers. Moreover, hyperinsulinemia and hyperglycemia may increase the quantity of free radicals and eventually decrease the capability of antioxidants, which may successively lead to further difficulties in the disease pathway.[75] Based on preclinical research, a few drugs or insulin sensitizers used to treat diabetes, including DPP-4 inhibitors (gliptins), GLP-1, or GLP-1R agonists, have shown to be a promising approach to fight against AD also, providing some neuronal protection in elderly patients.[88]

2.4. Metals and Heme

The essential trace elements have an optimum concentration (mostly in μg scale) to function effectively in humans and are strictly regulated throughout the system. Specifically, the biometals like Cu, Zn, and Fe are necessary to perform as cofactors for different enzymatic activities and mitochondrial and neuronal functions, etc.[89] Imbalance of any of these metals in the body may lead to pathological conditions. In both AD and T2Dm, such irregularity has been observed and, in both cases, it interferes with the respective protein aggregation and toxicity.[44,45,61,90,91] Cu and Zn are found to directly coordinate to Aβ peptide while Fe has a higher concentration in the senile plaques.[92,93] Fe overload may accelerate the neuronal Aβ production which consequently can worsen the cognitive decline.[92] Free Cu and Fe are involved in the development of T2dm pathology as well. Similar to that for AD, Cu levels are found to be altered in diabetes as well.[94,95] In fact, the Cu level becomes considerably higher compared to that of the healthy subjects, and such increased serum concentration of Cu has been linked to conditions like distressed structure of arterial walls, infections, and oxidative stress, etc.[93,96,97] The irregular metabolism of Cu along with Zn affects the function of important antioxidant enzymes like superoxide dismutase (SOD), compromising the cell lifetime and the natural defense system in the body.[51,92,98] A Cu chelating substance, tetrathiomolybdate essentially shows an advantageous role in the T2Dm pathogenesis, further validating the toxic role of Cu in this malady.[62,99] Moreover, Cu can act as a catalyst in Fe oxidation and its deposition in tissues.[98] Being redox-active, excess Fe and Cu can enhance the reactive oxygen species (ROS) generation, and the subsequent toxic hydroxyl radical formation gives rise to a pro-inflammatory environment in vascular, renal, and neural tissues and can act as a trigger for insulin resistance.[51,100]

Heme appears to be another common element in both these diseases. This common redox cofactor is known to perform several toxic effects independently as well as when bound to peptides or proteins.[63,67,101] Reports suggest that heme metabolism is changed in AD brain and is closely allied to many undesirable observations in the disease's pathology. In the AD brain a higher level of heme *b* (250%) has been found, and this excess heme content can enhance oxidative stress, hampering the normal mitochondrial activity.[102] Besides this, the altered metabolism of heme; including unregulated iron gathering, increased heme oxygenase, ferrochelatase, biliverdin reductase A, and heme degradation products can be seen in AD pathology.[64,103−105] Moreover, the monomeric form of APP, the precursor of the Aβ peptide, is found to be decreased by ∼50% in the heme deficient AD brain.[64] In different parts of the brain, colocalization of hemoglobin (a form of heme) with Aβ is observed in the senile plaques which are characteristic to neuropathology. Such coexistence also promotes Aβ oligomerization.[106] The presence of heme-rich deposits alongside the Aβ deposits is another vital evidence of heme involvement in AD.[53] The cerebral amyloid angiopathy (CAA) is a common neuropathological finding in AD which is characterized by Aβ depositions around the blood vessel wall. These depositions considerably weaken the cerebral vessel walls causing a high chance of rupture and hemorrhage.[107,108] Thus, the micro-hemorrhages and consequent heme exposure inside the brain might authenticate the possibility of the heme involvement in disease etiology. The colocalization of heme-rich deposits together with Aβ plaques are confirmed in AD by staining the affected brain.[24,109] The heme hypothesis has its confirmative evidence from the recent researches that established heme-Aβ binding *in vitro*, which might account for heme deficiency and other cytopathology observed in AD.[67,102,110−112] Thus, all these findings collectively propose heme to be another key to the Alzheimer's pathogenesis. It is notable that heme-bound Aβ can behave as peroxidase and can catalyze the oxidation of many significant biomolecules in H_2O_2 medium which can be portrayed as a reason for abnormal neurotransmission and cognitive decline.[110,113] T2Dm also shows much similar inclusion of heme in its etiology. Coincidentally, many of the symptoms and pathological markers of AD are also seen in diabetic patients such as the higher serum concentration of Fe, higher heme-Fe ingestion, etc.[65,114] Heme can also coordinate to IAPP and its cohormone insulin *in vitro* similar to Aβ peptide.[70,115,116] All these heme−peptide complexes are capable of generating ROS and subsequent oxidative stress leading to β-cell apoptosis and decreased cell mass in pancreas.[117] Analogous to the CAA condition induced by Aβ depositions, the IAPP aggregates are also demonstrated to exert hemolytic activities against red blood cells (RBCs) confirming their cytotoxicity and an increased amount of free hemoglobin in the serum, making the role of heme in T2Dm pathology more relevant.[118,119] Hence, small molecules like metal ions and heme might act as a common bridge between these two amyloidogenic diseases, and this concept needs a greater focus in global research.

3. HEME IN AD AND T2DM

3.1. Active Site Environment of Heme-Aβ

The 1:1 heme-Aβ active-site consists of a high-spin five-coordinated heme-Fe(III) center likely connected to the His13 residue in the primary binding sphere (Scheme 2). The 241

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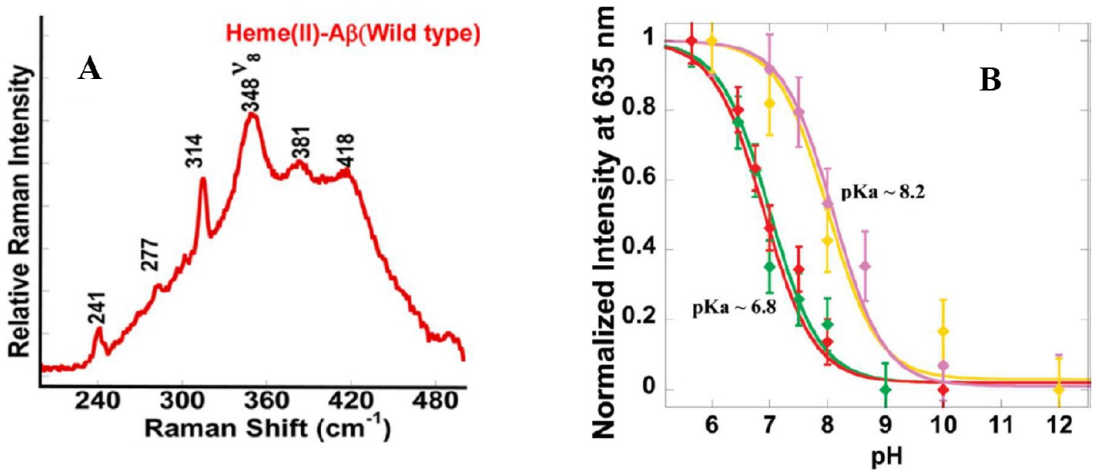
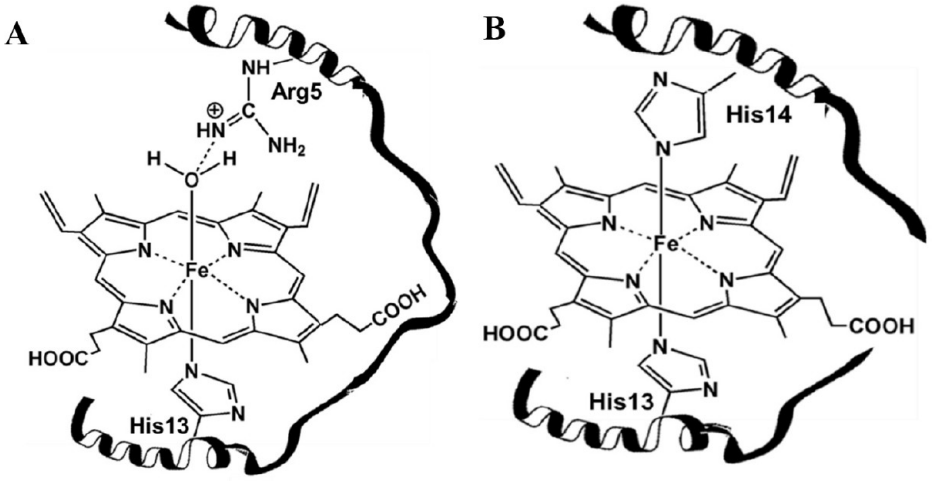


Figure 1. (A) Lower frequency rR spectrum of reduced heme-Aβ (in 100 mM, pH 7, phosphate buffer, at concentration 0.02 mM, excited at ~413.1 nm, laser power ≈15 mW on the sample). (B) pK_a plots of heme-Aβ complexes with error bars: (1−40), red; (1−16), green; (10−20), yellow; Arg5Asn mutant of (1−16) peptide, pink. Part A is adapted from ref 67. Copyright 2015 American Chemical Society. Part B is adapted from ref 112. Copyright 2011 American Chemical Society.

Scheme 4. Schematic Representation of the Active Site Environment of (A) High Spin Heme-Aβ and (B) Low Spin Heme-Aβ. Part A is Reproduced from Reference 112. Copyright 2011 American Chemical Society. Part B is Reproduced from Reference 122. Copyright 2013 American Chemical Society



cm^{-1} stretching vibration in rR spectrum confirms this His coordination in the proximal pocket (Figure 1A). pK_a perturbation reveals the trans axial sixth ligand to be a water derived residue with a $H_2O \rightleftharpoons OH^-$ equilibrium, with a pK_a of ~6.8 ± 0.3 (Figure 1B). Furthermore, for Arg mutated Aβ, the metal−peptide complex shows a relatively higher pK_a, indicating that, at physiological pH, a H-bond between the labile sixth water-derived ligand and the positively charged guanidinium side chain of Arg5 is present at a distal site that can stabilize the hydroxide form of the axial ligand relative to its protonated form.[70,112]

Importantly, this strategic arrangement of Arg5 in the second sphere makes heme-Aβ complex behave as a peroxidase.[111,117,120,121] There is also some minor amount of a six-coordinated low-spin component present along with the major high-spin component. Increasing the concentration of Aβ with respect to heme enhances the relative population of this low-spin component having bis-His ligations in the axial positions. His14 from another Aβ peptide strand acts as the second axial residue. Such coordination makes the active site of heme-Aβ similar to that of Cytochrome *b*. H-bonding by Arg5 possibly assists in the binding of the second His.[122] Scheme 4 shows the active site environments of high spin and low spin heme-Aβ complexes.

3.2. Active Site Environment of Heme-IAPP

Spectroscopic and mutagenesis studies indicate that a 1:1 heme-hIAPP complex has an analogous active site environment as heme-Aβ. This active site also comprises a high-spin Fe(III) center with an exchangeable weak field H_2O ligand having a pK_a of ~7.3 ± 0.2 (Figure 2A, 2B), confirmed by absorption, EPR, and rR spectroscopy. Here, His18 binds porphyrin-Fe(III) axially while the Arg11 moiety provides an additional second sphere interaction to support the essential active site structure of this complex (Scheme 2). Arg11 residue is proposed to form H-binding or ion pairing to the propionate side chain of heme group unlike in heme-Aβ where it is directly H-bonded to the sixth axial water-derived ligand (Scheme 5).[116] This small alteration in the second sphere of these two complexes exerts a huge dissimilarity in their function. Heme-Aβ can act as a peroxidase as its Arg5 induces a pull effect

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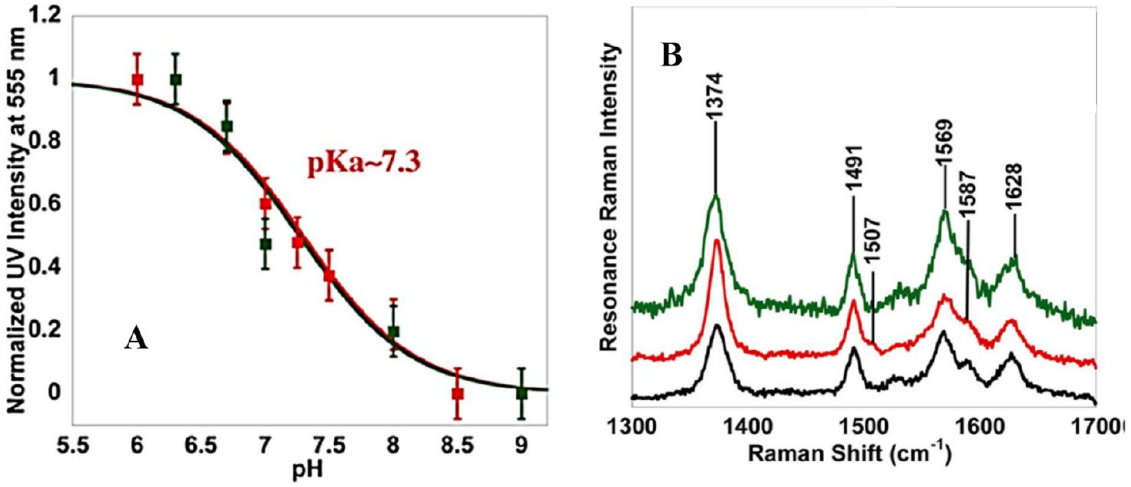
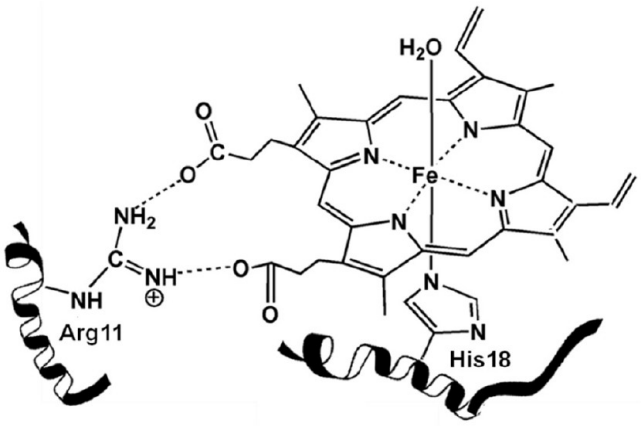


Figure 2. (A) pK_a plots of heme-IAPP complexes with error bars: (1−37) IAPP, red; (1−19) IAPP, green B). High frequency rR spectra of free heme, black, and heme-IAPP complexes: IAPP(1−37), red, and IAPP(1−19), green, at pH 7 at room temperature. Adapted from ref 116. Copyright 2013 American Chemical Society.

Scheme 5. Schematic Representation of the Active Site Environment of Heme-IAPP, Showing the Primary and Secondary Coordination Residues. Reproduced from Reference 116. Copyright 2013 American Chemical Society



which facilitates the O−O bond heterolysis, necessary for the active intermediate formation in the peroxidase pathway, while in contrast, heme-hIAPP cannot act as a peroxidase because its Arg11 moiety only provides a structural support to the active site.[117]

3.3. Active Site Environment of Heme-Insulin

In vitro experiments under physiological conditions indicate that heme binds to insulin peptide as well. The equimolar ratio of heme and insulin produces the heme-insulin complex having a mixture of components, a major high-spin mono His bound species and a low-spin bis-His coordinated minor species (Scheme 6). The associated pK_a of 1:1 heme-insulin complex is found to be 8.5 ± 0.2 which corresponds well to the H2O ↔ OH- equilibrium alike, the heme proteins such as myoglobin having a water derived ligand distal to the heme center (Figure 3A).[74]

Molecular docking studies reveal that the binding residue of insulin is His5, while Tyr26 and Phe1 promote electrostatic or hydrophobic interactions, whereas Val2 forms an H-bond to the heme center, assisting in the heme-insulin complex formation (Scheme 2).[73] In the presence of excess insulin relative to heme, the amount of low-spin bis-His component becomes higher as is indicated by the increase of the characteristic marker bands in rR spectroscopy (Figure 3B). Moreover, when the pH of the medium is increased, both the high-spin and low-spin complexes convert to the hydroxide bound form (Scheme 6).[74]

3.4. Reactivity

3.4.1. Peroxidase Activity and Substrate Oxidation of Heme-A*β*. A detrimental reactivity exhibited by heme-A*β* under physiological conditions is the peroxidase-like reactivity.[70,112,123] Natural peroxidases like horse radish peroxidase (HRP), chloroperoxidase, cytochrome *c* peroxidase, etc. can oxidize organic substrates in the presence of peroxides.[121,124,125] Recent reports reveal that a 1:1 high-spin heme-A*β* complex shows ∼3 times greater peroxidase activity as compared to free heme using H_2O_2 as the oxidant and 3,3′,5,5′-tetramethylbenzidine (TMB) as the substrate (Figure 4).[112] The low-spin bis-His coordinated heme-A*β* exhibits a higher reactivity, relative to its high-spin counterpart.[126]

All natural peroxidases contain a highly conserved Arg residue in the distal pocket which imparts a "pull effect" facilitating the O−O bond cleavage and generating the high valent Fe-oxo intermediates, which are the active oxidants (Scheme 7). It is to be noted that the rate of substrate oxidation for natural peroxidases like Myeloperoxidase (MPO) and HRP are ∼10^7 M^{-1} S^{1-} while for heme-A*β* it is ∼10^{-2} M^{-1} S^{1-}.[113,121,127] This significant second sphere influence is exhibited by Arg5 residue in A*β*. This is experimentally demonstrated by the fact that heme-A*β* ceases to exhibit its enhanced peroxidase activity in the Arg5Asn mutated heme-A*β*.[112]

In the peroxidase pathway the first intermediate is Fe(III)-OOH or Compound 0 in which Arg5 protonates the distal O and helps in cleaving the O−O bond heterolytically, forming the high valent Fe^{IV}=O porphyrin π cation radical or Compound I in the active site.[124,128] This intermediate has been trapped and characterized using its characteristic absorption feature (675 nm, Figure 5A), an axial broad EPR signal which only appears below 30 K (Figure 5B) and the oxidation state marker band at 1368 cm^{-1} and ν(Fe-O) vibration at 843 cm^{-1} in rR (Figure 5C, 5D). H/D isotope

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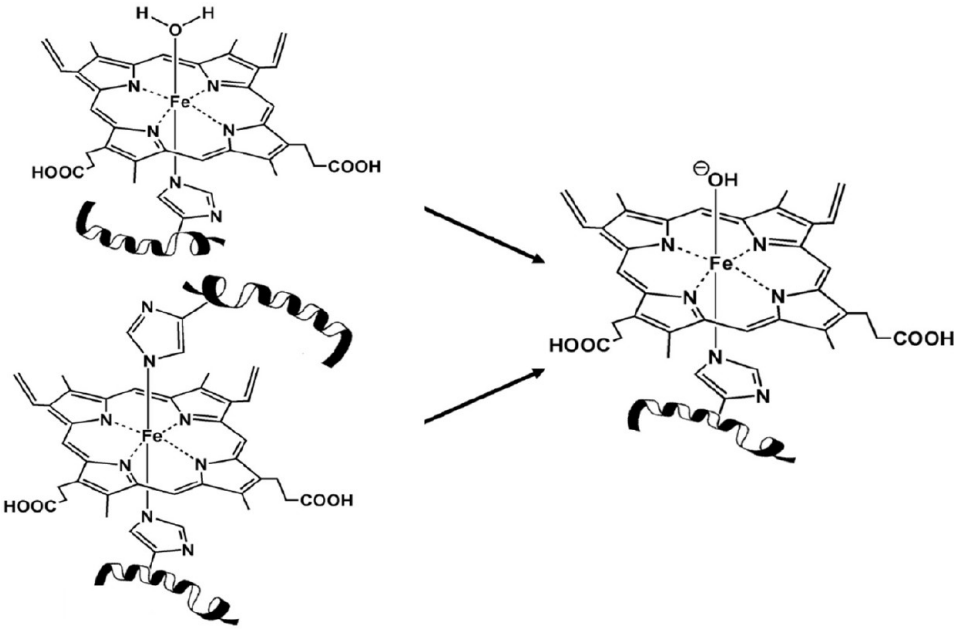
Scheme 6. Active Site Structures of High Spin and Low Spin Heme-Insulin Complexes, Both of Which Generate a Hydroxide Bound High Spin Complex at Alkaline pH. Reproduced from Reference 74. Copyright 2021 World Scientific Publishing Company



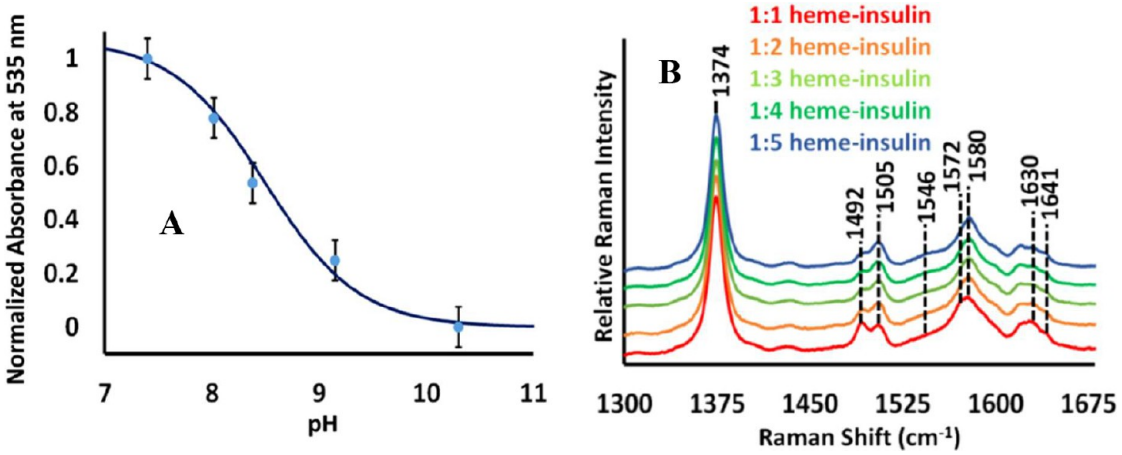


Figure 3. (A) pK_a plot of 1:1 heme-insulin complex with error bar. (B) High-frequency rR spectra (excitation 413.1 nm) of heme-insulin complexes with different heme-to-insulin ratios at pH 7.4 in 100 mM phosphate buffer. Adapted from ref 74. Copyright 2021 World Scientific Publishing Company.

experiment proves that this protonation assisted O−O bond cleavage is the rate-determining step (r.d.s.) in Compound I formation (K_H/K_D value of ∼2). The absence of any isotope effect in the decay of this intermediate is in line with the previous reports of other natural peroxidases stating that decay of Compound I occurs via outer sphere electron transfer process (Table 1).[110]

Both in the presence and in the absence of substrates, compound I decays to another high valent intermediate Fe^{IV}═O porphyrin or Compound II. The redox active Tyr10 residue can be involved in the redox pathway leading to the decay of Compound I, which is established from its ∼3 times slower decay rate for heme-Aβ (Tyr10Phe) compared to that of its native form (Table 1). Thus, Tyr10 seems to provide a natural protective role against these highly oxidizing intermediates.[110]

Owing to the peroxidase-like activity, heme-Aβ can oxidize the key physiological messenger molecules like serotonin (5-hydroxytryptamine or 5-HT), DOPA (3,4-dihydroxyphenylalanine) and other neurotransmitters in the presence of peracid/peroxide at physiological pH. Catalytic oxidation of serotonin by heme-Aβ and H_2O_2/mCPBA produces its corresponding symmetric dimer, dihydroxybitryptamine (DHT) as a major product, identified using HPLC (Scheme 8).[103,108] Following its kinetics, this oxidation reaction is found to be of first order with respect to both the catalyst and oxidant. The active oxidant in the case of the catalytic oxidation by heme-Aβ and mCPBA is found to be the transient intermediate, compound I. Heme-Aβ shows the highest catalytic activity at neutral pH due to the optimum condition where both the ease of active oxidant formation via Arg's pull effect (favorable below pH 7) and the ionization of the phenolic OH of 5-HT to generate the actual substrate (favorable above pH 7) can act in favor.[115] Quite expectedly, the absence of Arg5, hampers the rate of serotonin oxidation as

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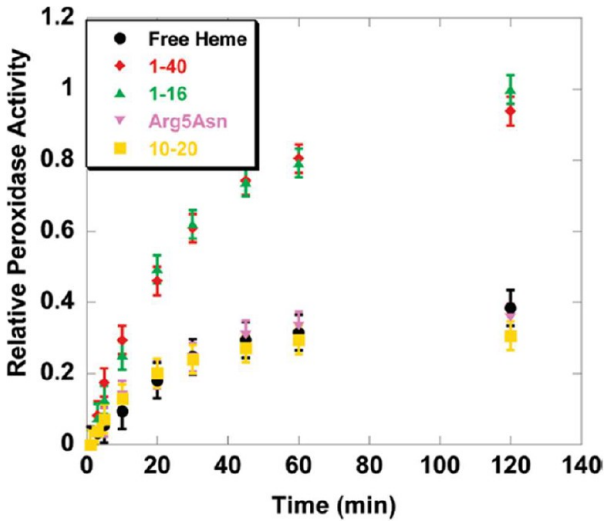
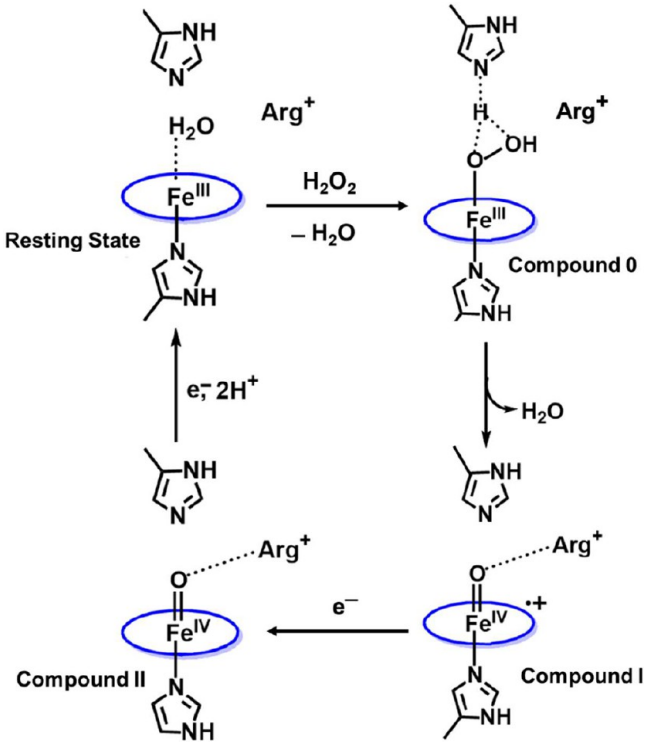


Figure 4. Kinetic traces for peroxidase activity, monitoring the increase of the 652 nm absorbance intensity, for different heme-Aβ complexes: (1−40), red; (1−16), green; (10−20), yellow; Arg5Asn mutant of (1−16), pink; and free heme, black. Adapted from ref 112. Copyright 2011 American Chemical Society.

Scheme 7. Catalytic Cycle of Peroxidase Enzymes with Axial His Ligation, Showing the Role of Arg Residue in the Distal Site. Reproduced with Permission from Reference 124. Copyright 2010 Elsevier



in its absence, formation of active oxidant becomes much slower compared to the native heme-Aβ complex, further reminiscing the second sphere impact of Arg5.[110] The noninnocent redox active Tyr10 affects this oxidation rate as well. Heme-Aβ(Tyr10Phe) shows slower substrate oxidation rate relative to the wild type heme-Aβ at physiological pH while the K_M value increases with the increase in pH of the medium, owing to the ability of proton translocation by Tyr residue that assists in compound I generation.[115] On the other hand, Try10 is thought to be a natural defense against highly reactive compound I as suggested by the slower decay of the intermediate in its absence, in the Aβ(Tyr10Phe) mutant.[110]

Notably, the altered metabolism of cholinergic, catecholaminergic, and serotonergic neurotransmitter system and their oxidations are common in AD patients.[122,129,130] A decreased level of serotonin specifically in the temporal and frontal cortex region and in the cerebrospinal fluid (CSF) are significant manifestations in the disease pathology.[34,131] Therefore, this experimental evidence of catalytic substrate oxidation in the presence of heme-Aβ might partly explain such abnormal neurotransmission in the AD brain.

3.4.2. Peroxidase Activity and Substrate Oxidation of Heme-IAPP and Heme-Insulin. As discussed earlier, the striking difference in the arrangement of Arg residue between heme-Aβ and heme-hIAPP results in a lack of enhanced peroxidase activity of the latter complex compared to that of free heme when subjected to similar catalytic oxidation of TMB substrate in the presence of H_2O_2.[116]

On the other hand, heme-insulin is capable of showing peroxidase-like activity as heme-Aβ (Figure 6). Experimental results show that insulin can amplify the usual peroxidase activity of free heme that in turn promotes production of Tyr radicals in insulin peptide chain.[73] Such radicals enhance the oxidative and nitrative stresses that are known to affect native biomolecules and important organs.[117,132,133] Tyr radicals can also end up forming the well-known di-Tyr cross-links which causes the permanent loss of physiological action of insulin by inhibiting its binding to the insulin receptor (IR) which sequentially activates the tyrosine kinase catalytic domain and cell metabolism.[73,117,134,135]

Within the β-cells, Tyr nitration may as well result in inactivation of vital proteins leading to diabetic conditions.[36] Overall, it is anticipated that such insulin dimerization will affect insulin signaling and insulin dependent glucose uptake, ultimately indicating toward the typical insulin resistance symptom in T2Dm.[73,136,137] The analogous peroxidase activity of heme-insulin similar to heme-Aβ might again demonstrate the significance of the presence of the highly conserved Arg residue in the distal pocket, which for the former complex is probably Arg22. Heme-insulin can potentially oxidize other physiologically available substrates like TMB in the presence of H_2O_2 that might lead to physiological dyshomeostasis in diabetic patients. This warrants further experimental investigations.

3.4.3. ROS Formation of Heme-Aβ. Redox active transition metals such as Fe and Cu in their reduced state can react with dioxygen to generate reactive oxygen species (ROS) via Fenton's mechanism (Scheme 9).[44,51,138] Partial reduction of O_2 produces $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, etc. which are readily diffusible through BBB and are quite neurotoxic in nature.[53,139−141] ROS can lead to lipid peroxidation and nucleic acid adducts, RNA and DNA damage and cell apoptosis, and oxidative stress induced impairment, which are common in the pathophysiology of amyloidogenic diseases.[139,142]

Xylenol orange (XO) assay detects the amount of H_2O_2 produced during the reaction of a ferrous heme-peptide complex with O_2.[143] The high-spin 1:1 heme-Aβ produces ∼90% ROS, implying a 2 e^- reduction of dioxygen. One of the electrons is donated by the heme-Fe(II) center while the second one is donated by the Tyr10 residue (Scheme 9). This

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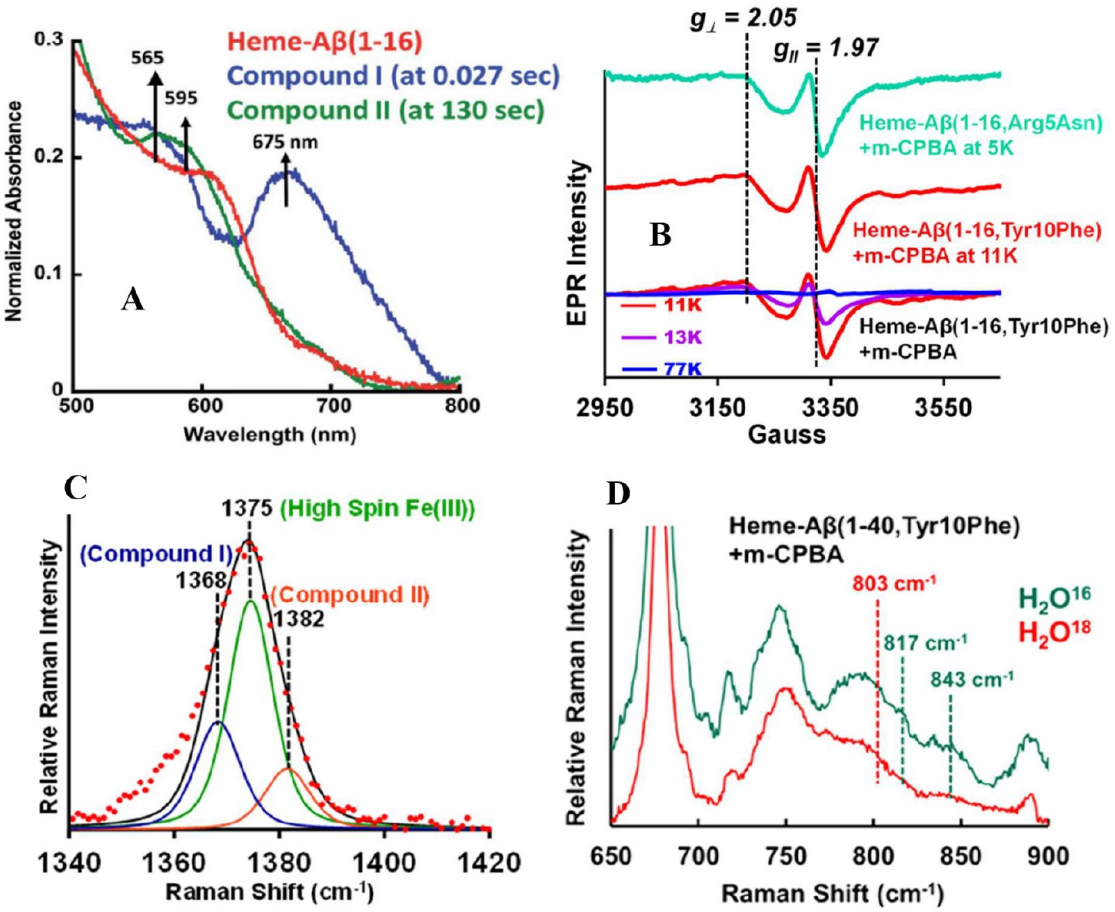

Figure 5. (A) Absorption spectrum of heme-Aβ, red; the difference spectrum at 0.027 s (compound I), blue; and the difference spectrum at 130 s (compound II), green. (B) Temperature dependent EPR spectra of heme-bound mutated Aβ with m-CPBA. (C) Experimental spectrum of heme-Aβ(Tyr16Phe) with m-CPBA, red; simulation spectrum, black; components of the rR spectrum were determined by simulating the spectra at the ν4 region. (D) Low frequency region data spectra of heme-Aβ(Tyr16Phe) with m-CPBA in H_2O^{16} medium, green, and H_2O^{18} medium, red. Data were obtained with an excitation wavelength of 413.1 nm (5 mW) at 77 K. Adapted with permission from ref 110. Copyright 2019 The Royal Society of Chemistry.

Table 1. Rate Constants of Formation and Decay of Compound I on Reaction of Heme-Aβ with m-CPBA in H_2O and in D_2O in the Presence and in the Absence of the Substrate (Serotonin) as Well as for the Reaction of the Heme-Aβ Complexes Having Site Directed Mutants with m-CPBA. Reproduced with Permission from Reference 110. Copyright 2019 The Royal Society of Chemistry

Heme-Aβ complexes	Formation rate (s^{1-})	Decay rate (s^{1-})
Heme-Aβ(1-16)	115 ± 5	1.20 ± 0.10
Heme Aβ(1 16) in D_2O	45 ± 2	1.10 ± 0.10
Heme-Aβ(1-16)+Serotonin	180 ± 5	2.05 ± 0.20
Heme Aβ(1-16,Arg5Asn)	40 ± 2	1.32 ± 0.20
Heme-Aβ(1-16,Tyr10Phe)	55 ± 3	0.40 ± 0.05

is confirmed from experimental results, which show ~50% lowering in ROS generation, by heme-Aβ when Tyr10 is replaced by Phe.[144] The tyrosyl radical (TyrO$^\bullet$) formed after one e^- transfer from Tyr residue can lead to dimer formation and peptide cross-links, an observed phenomenon in AD pathology.[145] For low-spin heme-Aβ, <40% H_2O_2 is produced during the reaction of the ferrous heme-peptide complex with O_2, suggesting a one-electron reduction of O_2 to its superoxide form which eventually disproportionates to H_2O_2 (Figure 7).[122] The e^- is donated by the heme-Fe(II) site. Here, Tyr10 is unable to donate the necessary second e^- possibly due to the lack of its favorable orientation in the bis-His coordinated active site.[122]

3.4.4. ROS Formation of Heme-IAPP and Heme-Insulin. Heme-IAPP complexes can also produce a significant amount of ROS, observed in *in vitro* experiments. Approximately 40 ± 5% H_2O_2 has been detected, indicating a similar one electron reduction of O_2 like low-spin heme-Aβ via the heme-Fe(II) center (Figure 8).[116] This can potentially exert cytotoxic effects, a phenomenon common in T2Dm etiology.

In the pancreas, alongside the heme-IAPP complex, both the mono-His and bis-His bound heme-Fe(II)-insulin complexes also form ROS, although in a negligible amount of 8−12% (Figure 8).[74]

3.4.5. Reaction Intermediate in ROS Formation of Heme-Aβ and Heme-IAPP. The formation of an Fe−O_2 intermediate is the first step in the ROS generation by the Fe(II) center (Scheme 10).[146,147] In the course of ROS formation, such oxygen-bound intermediates have been identified for heme-Aβ and heme-IAPP complexes in non-aqueous medium.[148] Using absorption, EPR, and rR spectroscopy, the Fe−O_2 intermediates of heme-Aβ and heme-IAPP are identified as six coordinated, diamagnetic, low-spin heme species which are stable only at very low temperatures (−80

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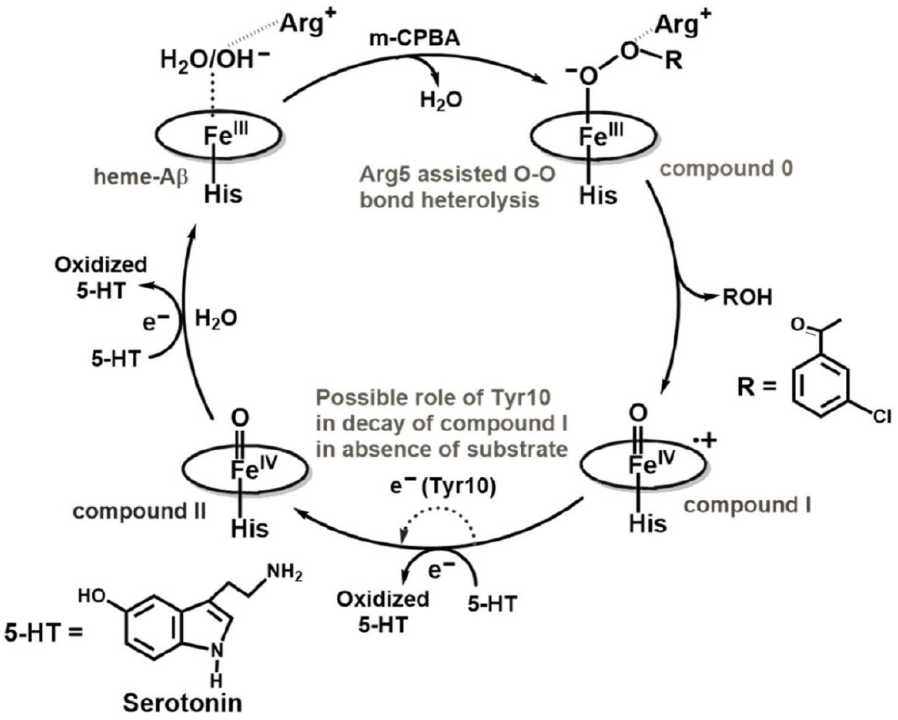
Scheme 8. Schematic Representation of the Possible Mechanism of Peroxidase Activity of Heme-Aβ and Serotonin (5-HT) Oxidation Catalyzed by It during the Reaction with m-CPBA



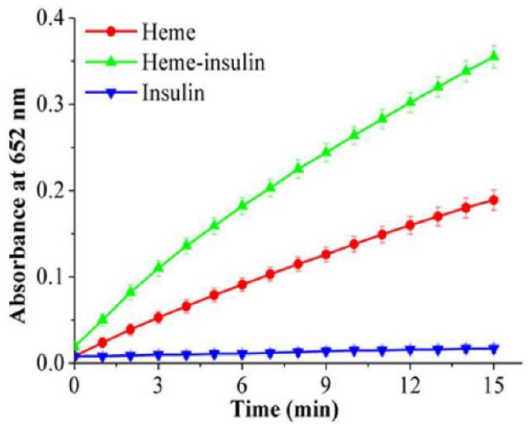
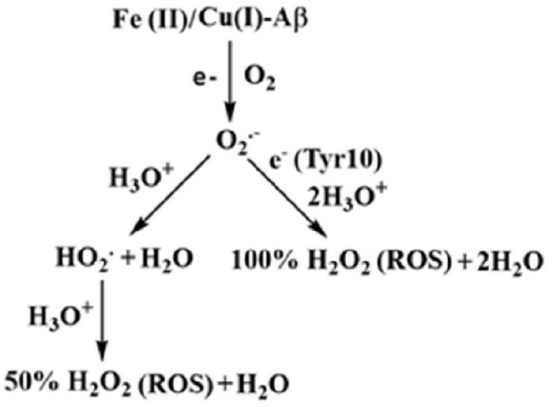


Figure 6. Kinetic traces for peroxidase activity, monitoring the increase of 652 nm absorbance intensity against time, for heme-insulin, green; free heme, red; and insulin, blue. Adapted with permission from ref 73. Copyright 2017 Elsevier.

°C). The Fe−O stretches at 575 and 577 cm^{-1} show the required isotope shift, confirming the Fe−O_2 complex formation in heme-Aβ and heme-IAPP, respectively (Figure 9A, 9B). ROS formation is suggestive of an inner sphere pathway via this Fe−O_2 complex, characteristic features of which are also in line with the other natural Fe-oxy adducts of heme proteins which have strong proximal coordination.[148]

3.4.6. Cytochrome C peroxidase Activity of Heme-Aβ. Recent experimental data demonstrates that ferrocytochrome c (Cyt c(II)) can get catalytically and stoichiometrically oxidized by heme-Fe(III)-Aβ complex in the presence of peroxides, resembling the function of naturally occurring cytochrome c peroxidase (CCP) enzyme, although at a much slower rate than that of CCP (Scheme 11).[149,150] This oxidation rate is dependent on the concentration of Cyt C(II), H_2O_2, and

Scheme 9. Schematic Representation of O_2 Reduction by the One- (50% H_2O_2) and Two- (100% H_2O_2) Electron Pathways



heme-Aβ, the chain length of Aβ peptide, ionic strength, and the pH of the medium.[151] Along with the electron transfer, Cyt c plays a vital role in the cell apoptosis mechanism, and its redox state has a significant effect on the pro-apoptotic signaling pathway. It has been observed that Cyt(III) c can induce apoptosis while Cyt(II) c cannot. Thus, the CCP-like activity of heme-Aβ complex, i.e., the oxidation of Cyt(II) c in the cytosol generating Cyt(III) c can lead to abnormal apoptosis in the AD brain.[152]

4. COPPER IN AD AND T2DM

4.1. Active Site Environment of Cu-Aβ

Owing to a high affinity for metals, the interaction of amyloidogenic Aβ peptides with metal ions, especially Cu, has been extensively investigated for a long time.[44,47] Based on the nature of the aggregated states of peptide, it has been

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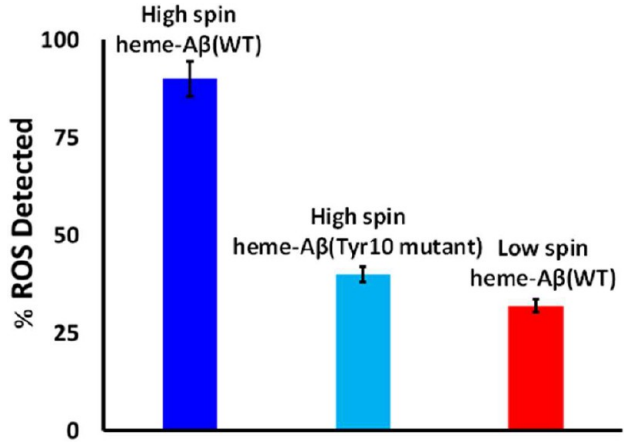


Figure 7. Percentage ROS detected for wild-type heme-Aβ high spin, blue; low spin, red; and the high spin Tyr10 mutant, cyan. Reproduced from ref 112. Copyright 2011 American Chemical Society, and ref 122, copyright 2015 American Chemical Society.

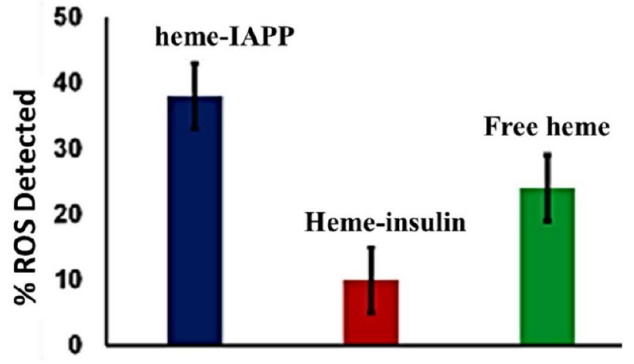


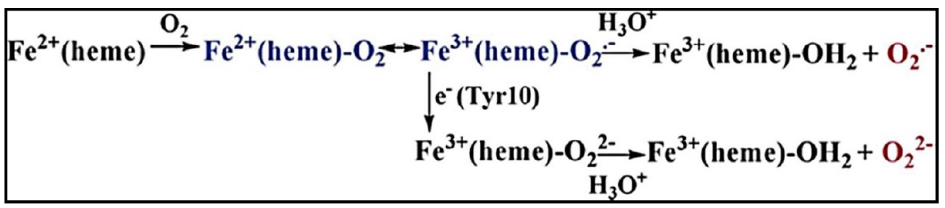
Figure 8. Percentage ROS detected for native heme-IAPP, blue; free heme, green; and heme-insulin, red.[74,116] Reproduced from ref 74. Copyright 2021 World Scientific Publishing Company, and ref 116, copyright 2013 American Chemical Society.

suggested that Cu can exert either inhibition or assistance in the Aβ fibrillation process.[50,117,153,154] Till the date, several research groups have proposed a variety of representative models for Cu(II)-Aβ active site structures employing techniques like EPR, CD, NMR, X-ray absorption spectroscopy, etc.[155−158] However, lack of any proper crystal structure makes it difficult to converge to a universally accepted model of this metal−peptide complex.

The hydrophilic region of Aβ peptide comprising the first 16 residues contains the Cu binding residues like Asp, Glu, His, and Tyr (Scheme 2). An active site of 3N1O coordination having possible N-donor ligands like three His residues or the N-terminus with two His residues and a deprotonated amide have been suggested for Cu-Aβ. The probable O-donors are Asp1, Glu3, Asp7, Glu11, aqueous buffer-derived molecule, or a carbonyl from amide linkage.[157,159,160] However, it remains a challenge to assign the distinct ligating environment for this complex, mainly because of its multiple forms in different pH values, making it further complicated. Investigation of these pH dependent components identified two components, component I and component II, which remain in equilibrium in the pH range of 5−9 (Figure 10).[72,161−163] A combination of spectroscopic studies, pH variation, exogenous ligand binding along with ligand field analysis, provides a possible geometry and electronic structure of the said components of Cu-Aβ. At physiological pH, two sets of distinct hyperfine EPR features coming from a mixture of two components can be observed, of which, component I is more dominant than component II. With an increase in pH of the medium, component II becomes more prominent, indicating that at higher pH (∼pH 9), component II is the major species while at relatively lower pH (6.5−7), component I predominates. The corresponding spectroscopic features of these two components are provided in Table 2.[72] Moreover, these two components are in pH equilibrium with a pK_a of ∼8.1, corresponding to a $H_2O \leftrightarrow OH^-$ equilibrium and which falls within the range of reported pK_a values of H_2O bound Cu complexes.[164,165] From the ligand field analysis of EPR parameters (g∥ and A∥ values), a β^2 value is calculated which is the percentage Cu 3d character in the singly occupied molecular orbital, to estimate the covalency of Cu in these two species. Component I possesses a β^2 value of 0.68 (i.e., ∼68% spin density over Cu center), and that for component II is 0.61 (i.e., ∼61% spin density over Cu), which indicates that the high pH component of Cu(II)-Aβ is more covalent owing to a lower β^2 value (Table 2). The high energy ligand field transitions in the absorption and CD spectra suggest a favorable square pyramidal penta-coordination for both the components.[72]

The titration of Cu(II)-Aβ with monodentate azide ligand reveals that azide binding is biphasic which essentially implies that one equivalent of Cu-Aβ can bind two equivalents of azide with the binding constants of 0.01 and 0.001 involving the dx^2-y^2 orbital of Cu-Aβ (Figure 11A, 11B). Hence, there must be two exchangeable ligands present in the equatorial plane to accommodate the two exogenous ligands like azide. Interestingly, this ligand exchange phenomena can only be seen in lower pH form, i.e., component I while the active site ligands in component II cannot be displaced, indicating a stronger ligand binding environment in the latter.[72] Combining such ligand exchange event with the Cu-Aβ pK_a, it may be confirmed that one of the exchangeable ligands in component I is H_2O derived which converts to its stronger nonexchangeable OH^-

Scheme 10. Schematic Representation of O_2 Reduction by Reduced Heme-Fe(II)-peptides and the Intermediates Formed during This Reaction Are Marked in Blue. Reproduced with Permission from Reference 146. Copyright 2013 The Royal Society of Chemistry



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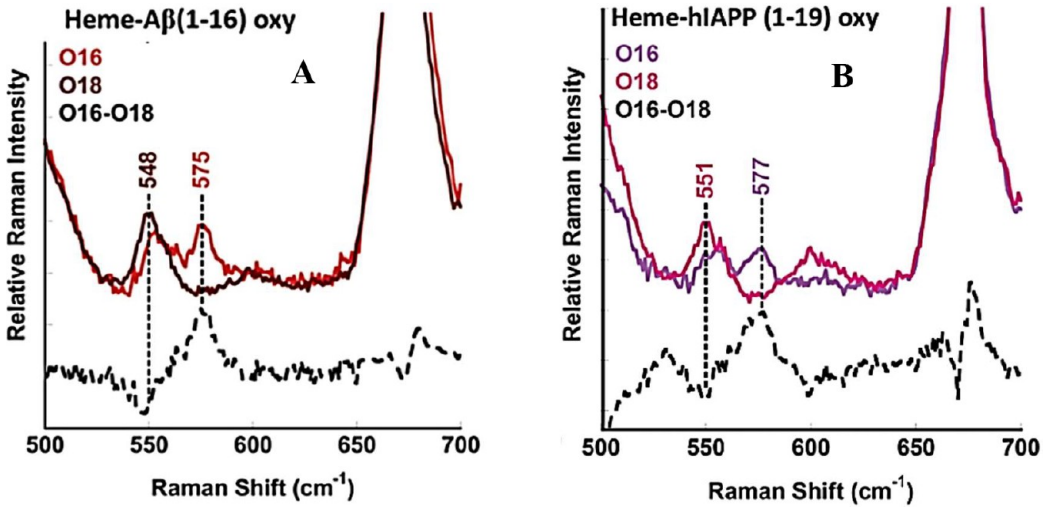
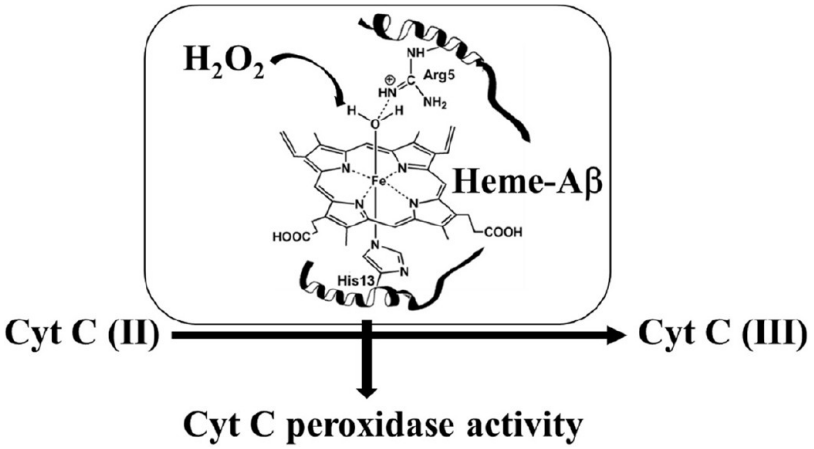


Figure 9. Low frequency resonance Raman spectra of the oxy complex of (A) heme-Aβ with O^{16}, red, and O^{18}, brown. (B) heme−IAPP with O^{16}, purple, and O^{18}, pink. Adapted with permission from ref 148. Copyright 2016 The Royal Society of Chemistry.

Scheme 11. CCP-like Activity of Heme-Aβ, Oxidizing Cyt C (substrate) in the Presence of H_2O_2[151]



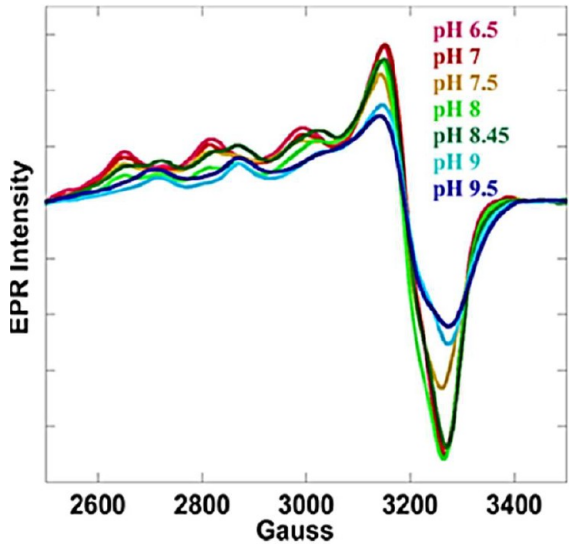


Figure 10. EPR data of the Cu-Aβ complex at different pH values (pH 6.5 in MES, pH 7, 7.5, and 8 in HEPES, and pH 8.45, 9, and 9.5 in CHES). Adapted from ref 72. Copyright 2013 American Chemical Society.

Table 2. EPR Analysis of Component I and Component II of Cu-Aβ Complex. Adapted from Reference 72. Copyright 2013 American Chemical Society

Cu-Aβ	A‖ (G)	g‖	g⊥	β^2
Component I	170	2.239	2.046	0.68
Component II	159	2.204	2.042	0.61

form in component II at the alkaline pH. The other ligand may be a carbonyl oxygen of the peptide backbone. Adjacent to this carbonyl, a -NH residue can directly form the H-bond to the hydroxide at alkaline pH, making the carbonyl stronger, hence undisplaceable by azide (Scheme 12).[72]

4.2. Active Site Environment of Cu-IAPP

IAPP, as one of the major components in T2Dm epidemiology, is found to interact with Cu, and this interaction is currently under investigation by researchers worldwide.[71,160,166,167] A few studies indicate that Cu delays the IAPP aggregation process while others claim that Cu-IAPP complex favors oligomer formation instead of the fibrillar form, making it more toxic toward pancreatic β-cells.[168−170] There are not many reports found on the active site environment of the Cu(II)-IAPP complex. Some literature evidence demonstrate that the 1−19 segment of IAPP peptide essentially

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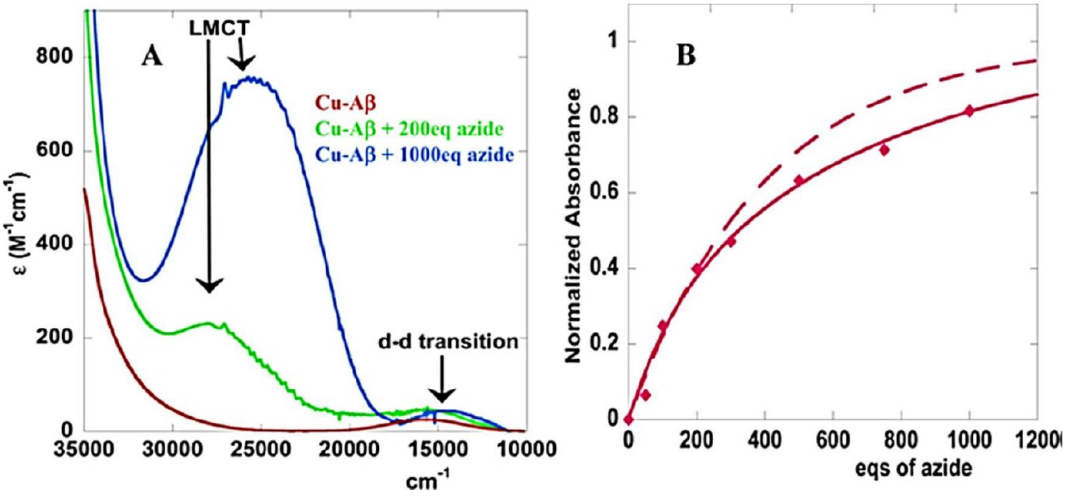
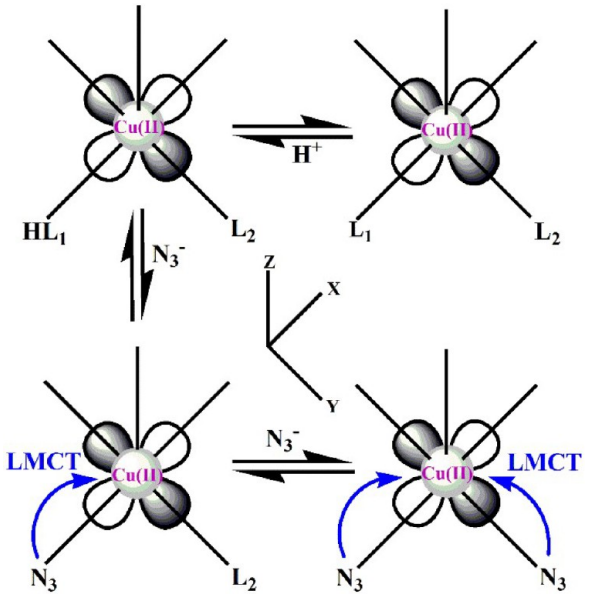


Figure 11. (A) Absorption spectra of addition of azide to Cu-Aβ; 200 equiv, green and 1000 equiv, blue; compared with Cu-Aβ without azide, red, at pH 7. The LMCT and d → d transitions have been indicated. (B) Biphasic (solid line) and monophasic (dashed line) kinetic fits of changes in normalized absorbance at 540 nm as a function of the different concentrations of azide. Adapted from ref 72. Copyright 2013 American Chemical Society.

Scheme 12. Schematic Representation of Displaceable Ligands Bound to Cu-Aβ. Reproduced from Reference 72. Copyright 2013 American Chemical Society



coordinates to metal via the His18 residue at physiological pH.[166,167] Along with the His-imidazole, three other amides from the peptide chain also contribute in the primary coordination sphere. Cu-IAPP is reported to be a square planar complex with the 4N ligating mode at pH 6.0 and above.[167] Another report highlights the Cu(II) binding to a truncated hIAPP(18−22), stating a similar role of His18 moiety while the other residues are two deprotonate amides from Ser19 and Ser20 and one O-donor from backbone carbonyl or −OH group of Ser20. This particular 3N1O fashion of the Cu-IAPP complex is found to compete for the required conformational changes to form β-sheet configurations, thus can explain the inhibitory effect of Cu in IAPP aggregation.[166] Spectroscopy and mutagenesis experiments on a 1:1 Cu(II)-IAPP complex at pH 8 show an axial EPR signal with g∥ = 2.17 and A∥ = 195 G (Table 3, Figure 12A), implying a D4h tetragonal geometry with a weak axial ligand.[71]

Table 3. EPR Parameters of the pH-Dependent Components of the Cu-IAPP Complex and Their Coordination Mode. Reproduced from Reference 71. Copyright 2017 American Chemical Society

Cu-IAPP	pH	A∥ (G)	g∥	g⊥	β^2	Coordination mode
Component I	8	195	2.17	2.03	0.635	4N
Component II	6	162	2.20	2.04	0.592	3N1O

pH perturbation studies reveal the presence of at least four distinctive species of Cu-IAPP, named as component I, component II, component III, and component IV in the 4−8 pH range of buffer. At pH 8, Component I remains as a pure species while at pH 6, both component I and component II coexist, making them physiologically and pathologically relevant. Lowering the pH of the medium to pH 5 and pH 4, component III and component IV are observed, respectively. At pH 6−8, the charge transfer band at 317 nm (31546 cm^{-1}) in the CD spectra indicates a deprotonated amide ligation to Cu, validating the presence of an anionic amide in the active site sphere of Cu(II) in components I and II (Figure 12B).[71] Furthermore, the mutagenesis experiments have aided in determining the other coordinating residues in the primary active site sphere of Cu-IAPP. It has been found that an N terminal -NH_2 group is one of the coordinating ligands in component I and II. Also, His 18 is a ligating residue in component II contrary to component I. Overall, the active site of component II is predicted as a five-coordinated square pyramidal geometry with the coordinating ligands anticipated to be an amidate (N^-), an N-terminal amine (NH_2), and a His (N) as N-donor groups, and the additional two coordinations are possibly an amide carbonyl and/or water (O). The cyclic voltammogram (CV) data points out component I as a more electron-rich species than component II. The possible binding ligands for component I are proposed to be amidate (N^-), N-terminal amine (NH_2), carbonyl (O), and an amidate (N^-) or hydroxide (HO^-) forming a square planar geometry as a result

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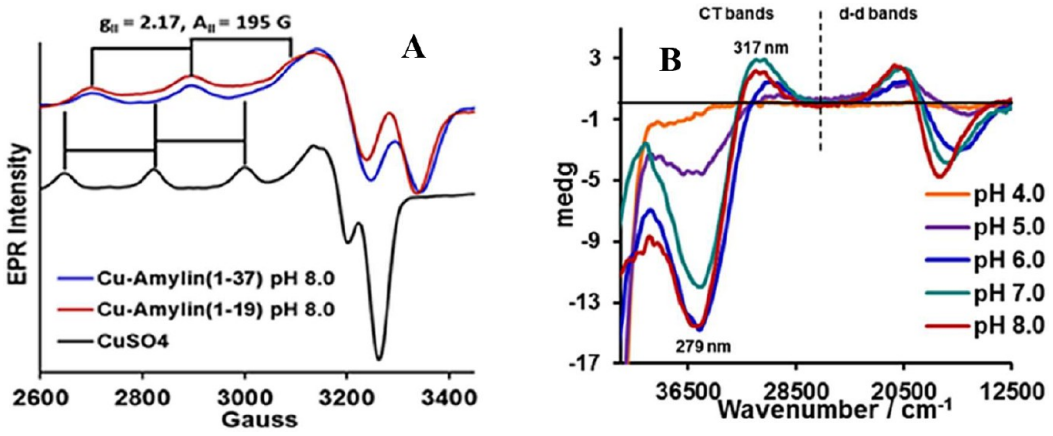
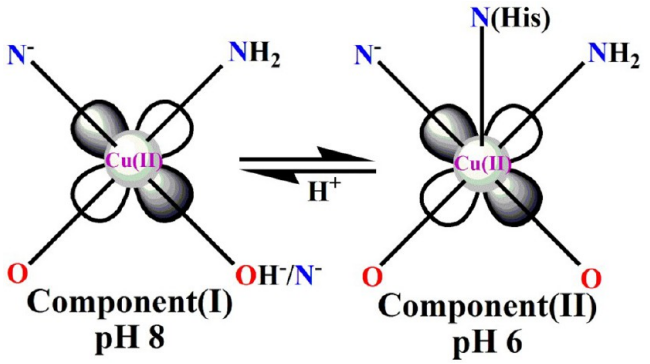


Figure 12. (A) EPR spectra of $CuSO_4$, Cu-IAPP complexes with 0.8 equiv of Cu(II) at pH 8.0 in 10 mM Mes buffer. (B) CD spectra of Cu-IAPP complex at different pH values. Adapted from ref 71. Copyright 2017 American Chemical Society.

of the loss of proton from component II.[71] The physiologically relevant forms of Cu-IAPP are shown in Scheme 13.

Scheme 13. Schematic Representation of the Physiologically Relevant Components of Cu-IAPP. Reproduced from Reference 71. Copyright 2017 American Chemical Society



4.3. Active Site Environment of Cu-Insulin

The coordination of the essential nutritional metals like Zn(II), Cu(II), and Mn(II) etc. to insulin peptide has significant physiological importance.[171,172] So far, the Zn-insulin complexes have been studied most extensively compared to other metal-insulin complexes.[173−175] Although limited, however, the Cu bound to monomeric insulin hormone has been explored to understand its electronic environment and the influence of change in the surroundings upon the interaction of the protein with metal ion.[172,176−178]

Past studies investigate the Cu(II)-insulin active site structure using absorption and EPR spectroscopy, where it is observed that in aqueous buffer solution, at pH 5, the Cu-insulin complex is similar to other tetragonal Cu(II) complexes. The metal center is found to be coordinated to the oxygen of the carboxylato groups, the nitrogen of the α- and ε-amino groups, and to the imidazole moiety. Below pH 5, there are no contributions from any N-based ligands in the coordination site. The degree of contributions from all these ligations around the Cu center differs with varying pH of the medium. However, the binding specificity of the crystalline Cu-insulin derivatives is not clear enough. Only at a very high pH value (pH 13.5), can a distinct binding site for Cu be designated owing to a sufficient increase in the ligand field strength. This binding site possibly consists of a deprotonated peptide nitrogen where the Cu(II)-N bond becomes more covalent, as monitored by the N-coupling in the EPR spectrum. Interestingly, at physiological pH, no such strong covalent binding of Cu-insulin is observed.[178] Another study reports the Cu(II)-insulin interaction based on UV−vis absorption and fluorescence emission spectroscopy, potentio-

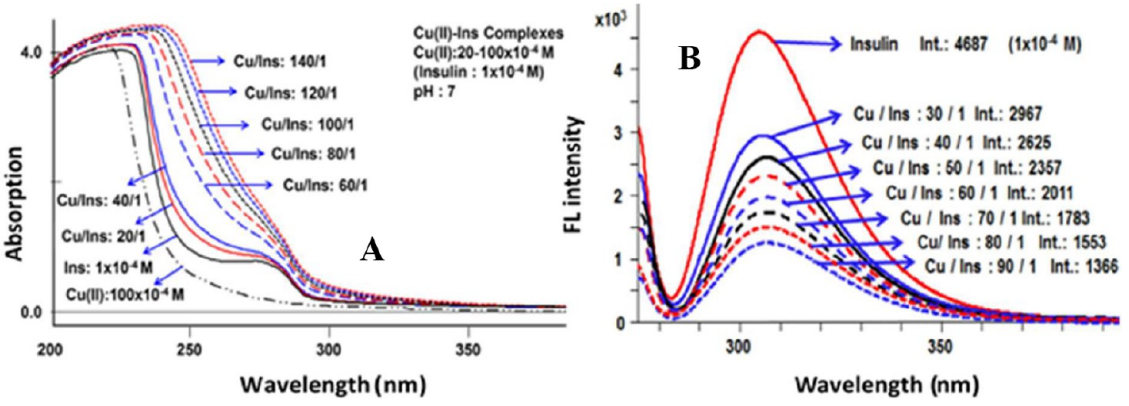


Figure 13. (A) UV−vis absorption spectra of Cu(II)-insulin solution at different molar ratios. (B) UV−vis fluorescence spectra of Cu(II)-insulin solutions at different molar ratios (λ_{Ex}, 272 nm, λ_{Em}, 306 nm); insulin, 1 × 10 $^{-4}$ M concentration. Adapted with permission from ref 176. Copyright 2022 Elsevier.

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metric acid−base titration, and liquid chromatography. It suggests that Cu binding to insulin is determined by the chelation mechanism with the formation of a M_4L-type complex and is highly stable. From the spectrophotometric titration the absorptions at 250 and 276 nm are observed, which are reminiscent of the Cu binding to peptides, specifically to the aromatic region of insulin (Figure 13A). The typical fluorescence emission of insulin at 305 nm gets quenched by almost 70% upon addition of Cu in a molar ratio of 90/1 (Cu(II)/insulin), possibly due to the metal ion coordination to the Tyr residues of insulin (Figure 13B).[176,177]

4.4. Reactivity

4.4.1. Peroxidase Activity and Substrate Oxidation of Cu-Aβ. Cu-Aβ shows peroxidase activity similar to that of free heme (heme *b*) in the presence of peroxide.[179] The reaction of Cu-Aβ with H_2O_2 at physiological pH, where component I predominates over component II, results in a species which shows ∼40% loss of spin density in the EPR spectrum (Figure 14). On increasing the concentration of Cu-Aβ, the aforesaid EPR signal loss gets enhanced. Such spin loss when combined with the absorption feature (350 and 411 nm) of this reaction mixture, indicates the formation of intermolecularly bridged Cu_2O_2 intermediates; either a side-on μ-peroxo-dicopper(II) complex or a bis (μ-oxo) dicopper(III) complex. Both these plausible intermediates are diamagnetic in nature (S = 0). However, the resultant EPR signal from Cu-Aβ and the H_2O_2 mixture does not disappear entirely, rather a species having distinct EPR parameters than the resting Cu-Aβ appears which suggests that the residual spin is not due to any unreacted Cu-Aβ but from another paramagnetic species.[180] Resonance Raman spectroscopy helps in addressing the reaction components. In the low frequency region of the rR spectrum, bands at 518 cm^{-1}, 540 cm^{-1}, and 570 cm^{-1} indicate the Cu−O vibrations of a bis(μ-oxo)copper(III) core or a Cu(II)−OOH species, while the band at 849 cm^{-1} is characteristic of the O−O vibration of a Cu(II)−OOH species (Figure 15).[181,182] The corresponding isotopic shifts of 849 and 518 cm^{-1} bands in D_2O medium further assigned these vibrations as ν(Cu−O) and ν(O−O) bands of the Cu(II)-OOH component, respectively; that is paramagnetic, contributing to the residual EPR signal (Figure 15B).[180] Moreover, the remaining two bands (540 and 570 cm^{-1}) that do not show any H/D shift, arise from a Fermi resonance and are representative of Cu−O vibrations of a diamond core bis(μ-oxo) species (Figure 15A).[182−184] Thus, rR eliminates the μ-peroxo-dicopper(II) complex as the diamagnetic intermediate in the reaction of Cu-Aβ and H_2O_2. The resultant products are shown in Scheme 14.

Similar to heme-Aβ, Cu-Aβ can also oxidize neurotransmitters like serotonin (5-HT), dopamine etc.[126,130,180,185] The catalytic oxidation of 5-HT by this catalyst has recently been investigated using spectroscopic methods like EPR, UV−vis, and rR. As discussed in the previous section, the reaction of Cu-Aβ and H_2O_2 generates a Cu(II)-OOH and a bis (μ-oxo) dicopper(III) species (Scheme 14), either of which can be the active oxidant to oxidize the substrates. Therefore, different reaction conditions have been implemented to determine the reactive oxidant in the 5-HT oxidation process. Using an excess amount of H_2O_2 (1000 equiv), the Cu(II)-OOH component is found to be produced exclusively, escaping the dimerization to form dicopper

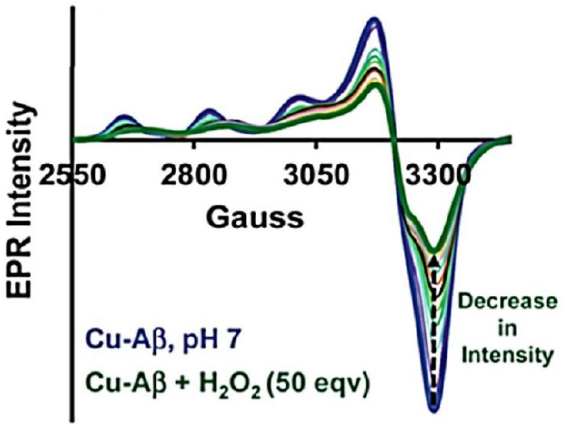

Figure 14. EPR spectra of initial Cu−Aβ, blue; Cu−Aβ with 50 equiv H_2O_2 at different times, green being the final spectrum after 120 min, in 100 mM HEPES buffer at pH 7 at 77 K. Adapted with permission from ref 180. Copyright 2021 The Royal Society of Chemistry.

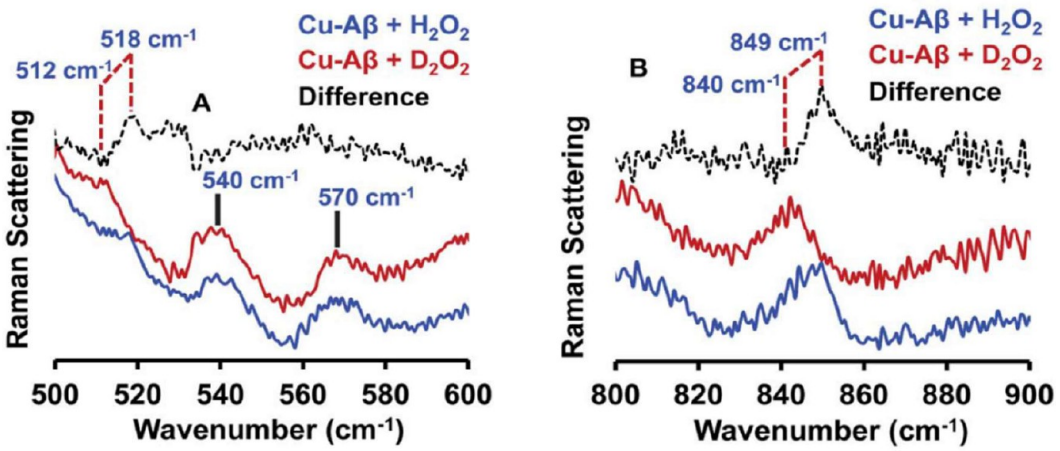

Figure 15. Resonance Raman spectra of Cu−Aβ with 50 equiv H_2O_2, blue and Cu−Aβ with 50 equiv D_2O_2 in, red; difference spectrum of H_2O_2 data from D_2O_2 data, dashed black; (A) lower energy region; (B) higher energy region. Data were obtained with an excitation wavelength of 415.4 nm (10 mW at the sample) at 77 K. Adapted with permission from ref 180. Copyright 2021 The Royal Society of Chemistry.

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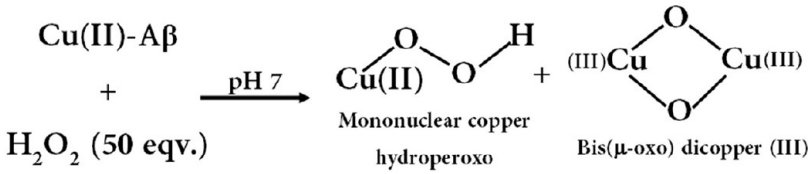
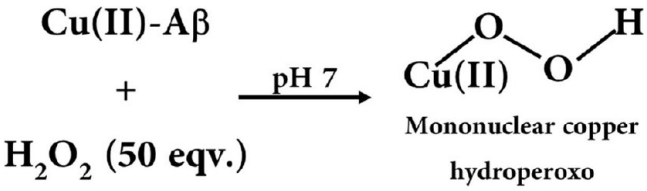
Scheme 14. Reaction of Cu−Aβ with 50 equiv H_2O_2 at pH 7. Reproduced with Permission from Reference 180. Copyright 2021 The Royal Society of Chemistry



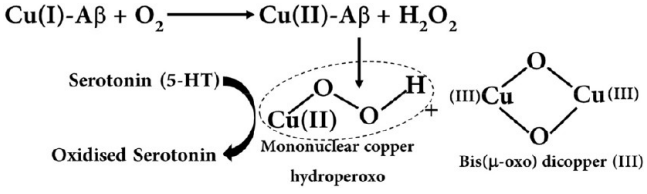
complex, and under this condition 5-HT gets promptly oxidized (Scheme 15). Furthermore, when Cu-Aβ concentration is increased, maintaining the relative ratio of Cu-Aβ:H_2O_2, the 5-HT oxidation rate becomes slower. It is to be noted here that in higher Cu-Aβ concentration, the bis (μ-oxo) dicopper(III) complex formation is enhanced. Considering these observations, it can be unambiguously concluded that the Cu(II)-OOH intermediate is the active oxidant species for 5-HT oxidation by H_2O_2 catalyzed via Cu-Aβ.[180]

Scheme 15. Reaction of Cu−Aβ with 1000 equiv H_2O_2 at pH 7. Reproduced with Permission from Reference 180. Copyright 2021 The Royal Society of Chemistry



Also, H_2O_2 is formed by the reaction of Cu(I)-Aβ with molecular O_2 via a two e^- pathway, and the generated H_2O_2 is found to be capable of 5-HT oxidation by means of the same hydroperoxide intermediate (Scheme 16).[180]

Scheme 16. Serotonin (5-HT) Oxidation Catalyzed by Cu(II)-OOH Reactive Oxidant, Produced in the Reaction of Reduced Cu-Aβ with O_2. Reproduced with Permission from Reference 180. Copyright 2021 The Royal Society of Chemistry



In this oxidation process, a known neurotoxin, tryptamine-4,5-dione (T-4,5-D) has been produced along with other products like 5-hydroxy-3-ethylamino-2-oxindole (5-HEO) and a dimer of these two, i.e., 3,3′-bis(2-aminoethyl)-5-hydroxy-[3,7′-bi-1*H*-indole]-2,4′,5′(3*H*)-trione, likely formed via aerial oxidation of T-4,5-D and 5-HEO (Scheme 17).[180,186] All these products have been characterized by absorption spectroscopy and HPLC techniques. The difference in the oxidized products of the catalytic oxidation of 5-HT by heme-Aβ and Cu-Aβ (primarily a dimer viz dihydroxybitryptamine in case of the former complex), indicates a basic difference in the mechanism and in the nature of corresponding intermediates formed in this process. Nevertheless, analogous to heme-Aβ, Cu-Aβ can play vital role in the neural toxicity and impairment in the brain signaling system as well.

4.4.2. ROS Formation of Cu-Aβ. Partially reduced oxygen species are generated in the presence of reduced Cu-Aβ. Approximately 84 ± 5% ROS has been detected using xylenol orange assay, indicating a two electron reduction of O_2 to H_2O_2 at pH 7 (Figure 16). Apart from the Cu(I) center, the redox active Tyr10 residue provides the other electron similar to what we have observed for the heme-Aβ complex. The role of Tyr is confirmed by the experimental observation where the amount of H_2O_2 generated diminished by ∼50% when the Tyr mutant is used, (Cu(I)-Aβ(Tyr10Phe)).[179]

Experimental data suggest that Cu-Aβ can also catalyze the dityrosine cross-linking via its peroxidase mechanism or during ROS formation, where generation of the tyrosyl radical is prevalent.[187,188] Such cross-linking via Cu-Aβ is similar to that of other natural peroxidases, however, less efficient. Formation of the cross-linkings can result in the formation of soluble dimers of Aβ peptide which is supposedly a key intermediate in amyloidogenesis.[189−191]

4.4.3. ROS Formation of Cu-IAPP. H_2O_2 is produced during the aggregation of IAPP peptide into its amyloid fibrillar form.[40,192] This process is found to be greatly stimulated by the presence of Cu(II) ions.[96] Owing to a suitable reduction potential, both component I (at pH 8, 206 mV vs NHE) and component II (at pH 6, 249 mV vs NHE) of the Cu-IAPP complex are easily reduced under physiological conditions by reducing agents like vitamin C, NADH, or glutathione, etc. In their reduced form Cu(I)-IAPP complexes produce ∼40% ROS at physiological pH, demonstrating a one electron reduction pathway of O_2. The other components of Cu(I)−IAPP at different pH values produce almost similar amounts of PROS (Figure 17).[71] Hence, Cu indeed can potentially play a vital role as a stimulator of oxidative stress in AD and T2Dm etiology.

5. SOME REMEDIAL APPROACHES

5.1. Heme Sequestration

In the vertebrate family, penta-coordinated globin proteins, hemoglobin (Hb), and myglobin (Mb) are well-known for their O_2 transport and storage activity and hexacoordinated cytoglobin (Cgb) and neuroglobin (Ngb) are well characterized with an essential neurological function.[193−195] The hologlobin without the heme prosthetic group is termed as apoglobin which is known to have very high affinity for heme.[196] Owing to such high heme affinity, apoglobins are proposed to sequester heme when they are administered in any heme-peptide complexes to mitigate the heme induced cytotoxicity (Scheme 18). *In vitro* experiments and spectroscopic evidence reveal that aponeuroglobin (apoNgb) can actually sequester heme from the heme(III)-Aβ complex when mixed in a 1:1 ratio, resulting in a characteristic feature of a hexacoordinated low spin heme complex that resembles

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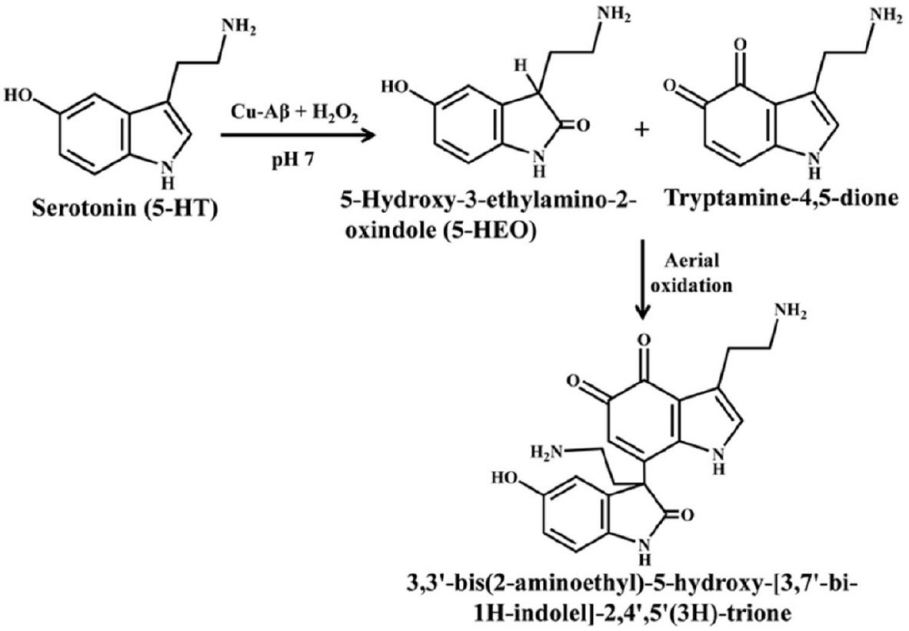
Scheme 17. Schematic Representation of Serotonin Oxidation by Cu-Aβ and H_2O_2. Reproduced with Permission from Reference 180. Copyright 2021 The Royal Society of Chemistry



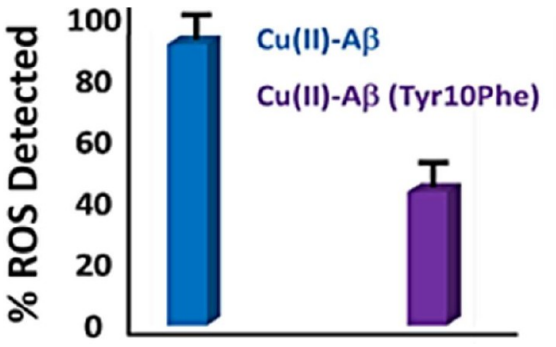


Figure 16. Percentage ROS detected for native Cu-Aβ, blue; and Cu-Aβ (Tyr10Phe), purple. Adapted from ref 179. Copyright 2011 American Chemical Society.

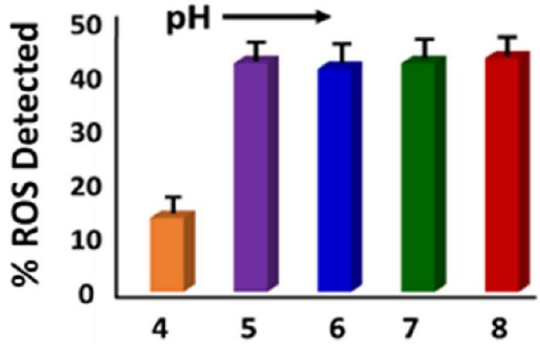


Figure 17. Percentage ROS detected for Cu-IAPP complexes at different pH values. Adapted from ref 71. Copyright 2017 American Chemical Society.

holoNgb.[197] A similar experiment using apoMb instead of apoNgb also results in the heme uptake, forming a heme complex similar to holoMb.[198] Apart from this, apoglobins can also extract heme from reduced heme(II)-Aβ complexes forming the reduced hologlobin molecules. This phenomenon not only works well for heme-Aβ, but also is applicable in heme-IAPP complexes as well.[199] Absorption, rR, and gel electrophoresis results establish that apoMb can sequester heme from a 1:1 heme-IAPP complex to constitute a six-coordinated high spin ferric active site, alike to that of Mb. In each of these cases, more than 90% heme has been transferred, confirming the high affinity of globins for heme. The transfer has been observed as a biphasic event for heme-Aβ and heme-IAPP complexes with apoMb while it is monophasic for apoNgb.[197−199] Besides heme transfer, apoglobins can also transfer heme in a ligand bound state (CO or azide bound heme-peptide systems). After heme sequestration the resultant Mb or Ngb is useful for its essential biological functions like O_2 transport, neuroprotection, and so on. Importantly, the higher amount of ROS generation by heme-Aβ and heme-IAPP complexes reduces drastically, thus diminishing the heme induced cytotoxicity and in turn providing a protection role against AD and T2Dm advancement.[197−199]

Insulin, known to control the glycemic level, can furthermore enact its defensive role by seizing heme from heme-IAPP complexes, as confirmed by spectroscopic studies (Scheme 19). The resultant heme-insulin complex, as shown earlier, can yield much less ROS (only 8−12%), hence decreases the oxidative impairments.[74]

5.2. Cu Chelator and Heme Inhibitor in Artificial Surfaces

A close interconnection of metal dyshomeostasis inside brain and the onset and progression of AD has long been established which manifests the metal chelation therapy as a feasible pharmacological option for the treatment of Alzheimer's disease. Some clinically tested chelators are desferrioxamine (DFO), used against Fe and Al overload; rasagiline, a Fe chelator; 5-chloro-7-iodo-8-hydroxyquinoline (CQ), primarily for Cu and Zn metals; hydroxyquinolne ligand (PBT2) and so on. Albeit, metal chelation therapy has shown some positive impact in Alzheimer's disease and diabetic patients; however, most of these are no longer being pursued clinically.[44,48,60−62]

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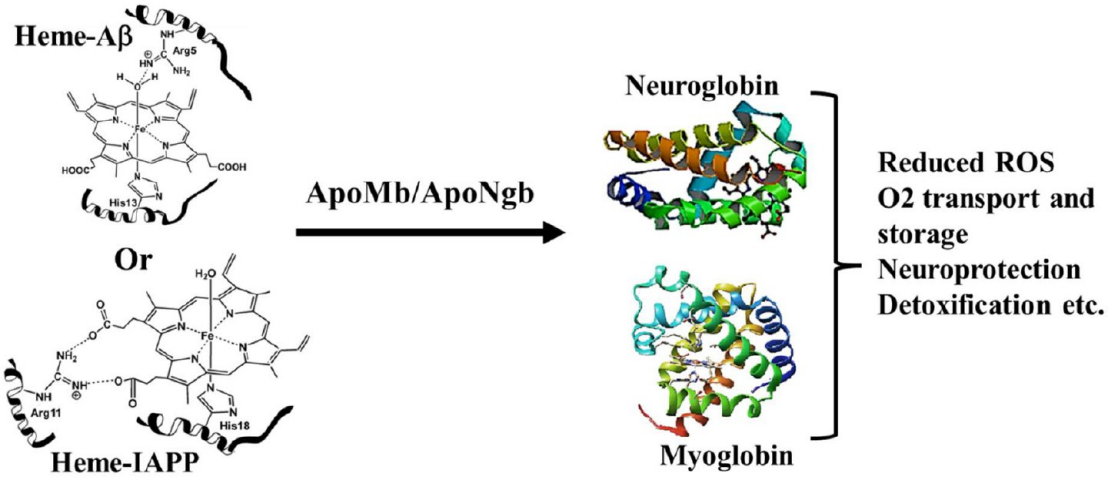
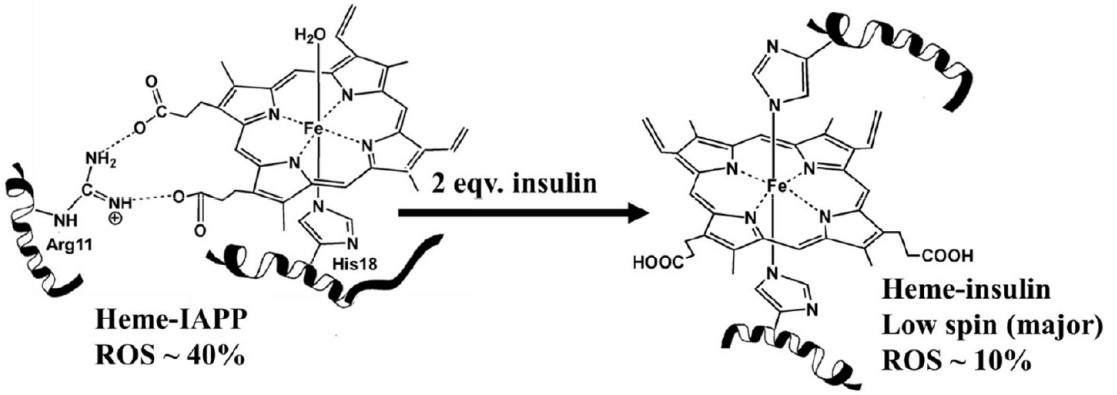
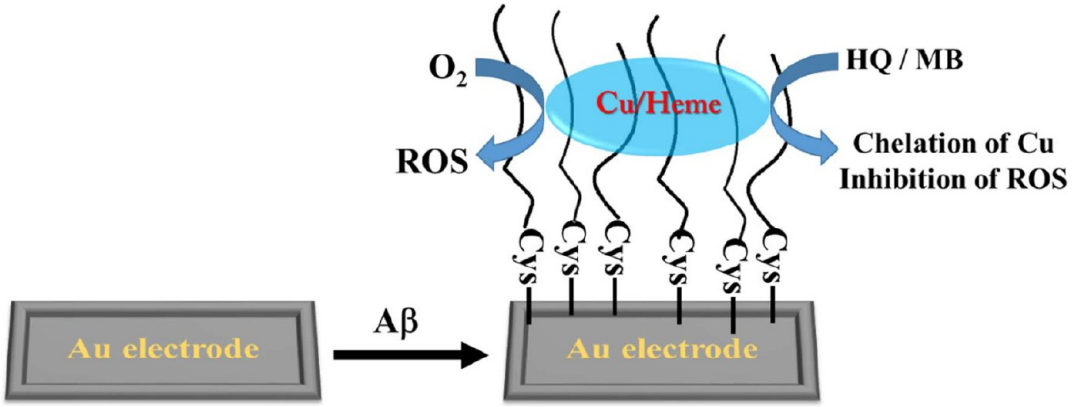
Scheme 18. Heme Sequestration by Apoglobins (ApoMb/ApoNgb) from Heme Bound Peptides



Scheme 19. Heme Sequestration by Insulin Peptide from Heme-Bound IAPP Complex. Reproduced from Reference 74. Copyright 2021 World Scientific Publishing Company



Scheme 20. Cu Chelation and ROS Inhibition (Produced by Heme) on Aβcys SAM. Reproduced from Reference 201. Copyright 2012 American Chemical Society



On a similar aspect, a platform that can probe the effect of potential drugs of AD at low concentrations has been constructed. For this, a Cys residue is attached to the truncated Aβ peptide at the C-terminus to form self-assembled monolayers (SAM) of Aβ(1−16) peptides on Au electrodes (Scheme 20). A homogeneous cluster of Aβ aggregates has been obtained when Aβcys solution is mixed with diluent 1-cysteine in a 1:9 ratio. The oligomers produced in such a system bear similar arrangements and are stable enough to assist in the investigation of their reactivity and cytotoxicity. Heme is readily attached to these peptide assemblies which serves as an artificial platform for testing biological functions and potential drug formulations.[200−202] These peptide assemblies can promptly bind both heme and Cu forming the respective SAM. With such Cu-Aβ SAM, the chelation activity of a clioquinol-like molecule, 8-hydroxyquinoline (HQ) has been observed, resulting in the removal of Cu from the SAM (Scheme 20). Larger Aβ aggregates have higher

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affinity for metals, thus making the Cu removal slower and requires a much higher concentration of HQ, compared to that from the smaller aggregates, making it more thermodynamically and kinetically efficient.[200] An effective drug in AD known to enhance the mitochondrial functions is methylene blue (MB).[203] It quenches the strong O_2 reduction current by heme-Aβ, indicating a sharp reduction in the heme induced ROS generation (Scheme 20). Thus, MB can act as an antioxidant although it takes a much greater concentration MB and extended period for the impedance of O_2 reduction for large aggregates related to isolated small aggregates.[200,201] Such inefficiency in larger aggregates or fibrils is likely due to the sterically hindered access of inhibitors to these targeted active sites. Thus, the chelation of Cu or inhibition of ROS is typically ineffective for larger aggregates, and hence considered as more toxic. The above observations require breaking of the larger fibrils to oligomers preceding any Cu and heme associated treatments.

6. CONCLUSION AND OUTLOOK

Scientists now have unanimously accepted the many common pathological links shared by AD and T2Dm. Other than the peptide aggregation, one such common aspect is the association of redox active metals like Cu, Fe, and Fe in the form of a heme prosthetic group. A recent array of investigations including the metals and heme are found to be quite significant to both AD and T2Dm pathophysiology. The observations like metal and heme dyshomeostasis, fibrillization, oxidative stress, loss of cell mass and cytotoxicity emerge to be interconnected, making these proteinopathies multifaceted. Extensive spectroscopy, mutagenesis, ESI-MS, X-ray crystallography, microscopy, and other techniques have clearly establish the covalent bindings between metal/heme to the peptides and depict their probable electronic environment of active sites together with the substantial amino acid residues in both the primary and secondary spheres.[1,204−206] Identifying the key residues in the active site of these metal-peptide complexes may help in understanding the molecular level reactivity. In most of the cases, these small biomolecules can generate or heighten the cytotoxic effects of misfolded proteins. For instance, the enhanced peroxidase activity, serotonin oxidation, ROS formation or the CCP-like activity of heme bound Aβ fit in several deleterious consequences of AD like the abnormal neurotransmission, reactive species accumulation, mitochondrial dysfunction, cell apoptosis, and so on. Similarly, in T2Dm disorder, heme-IAPP and heme-insulin show a greater amount of ROS, peroxidase activity (for heme-insulin only), and dityrosine formation which might account for some of the diabetic symptoms like insulin resistance and insulin function loss, loss of β-cell mass and β-cell dysfunction, etc. Moreover, the CAA neuropathy in AD and hemolytic activity of IAPP aggregates against RBCs can also avail free hemoglobin in affected organs making heme involvement in the pathology more relevant. The Cu analogues, viz. Cu-Aβ peptide, also can yield neurotoxic reactive oxidants that can oxidize neurotransmitters, can initialize the peptide fibrilization process via formation of dityrosine cross-links, and can form ROS while Cu-bound IAPP generates a significant amount of ROS, contributing toward the myriad of other pathological consequences. The relatively slower peroxidase activity and consequent substrate oxidation by these metal-peptide systems also match well with the late onset and gradual progression of such an "old age disease". Furthermore, *in vitro* experiments demonstrate successful removal or chelation of heme and Cu form the peptide bound state so that their induced cytotoxicity might get minimized to a certain extent. Such *in vitro* experiments using apoglobins, insulin, and MB to sequester heme or metal chelators like HQ to remove Cu may inspire approaches for *in vivo* biological systems as well.

The earlier amyloid hypothesis which considers protein aggregation as the key event leading to fatal degenerative concerns, is still pertinent, although it cannot provide a holistic scenario of such maladies. In addition, drugs targeting the amyloidogenic proteins do not show any significant symptomatic relief during their high-profile clinical trials in both the AD and T2Dm histopathology.[207] This circumstance along with the colocalization of heme/metal and the aggregated peptides in the affected organs impel scientists to the current consensus of the impact of heme and metals and their interaction to the amyloidogenic proteins in disease pathology. In fact, the cytotoxic reactivity of heme/metal bound peptides, like enhanced peroxidase activity, elevated ROS, CCP activity, protein cross-linking, neurotransmitter oxidation etc. can explain some of the critical features of AD and T2Dm pathophysiology including elevated oxidative stress, neuro-degeneration, insulin dysfunction, β-cell loss, mitochondrial dysfunction, cognitive decline, abnormal neurotransmission, and many more. Remarkably, rodent Aβ lacks the three vital amino acid residues (Arg5, Tyr10, and His13) compared to the mammalian Aβ counterpart.[70,208,209] As discussed earlier, His is the main binding residue for the small biomolecules to Aβ peptide while Arg exerts a second sphere effect in the active site, making the heme- Aβ complexes behave as peroxidase. Also, Tyr takes part in redox activity, supplying an extra electron to produce enhanced ROS, while it can in parallel induce a natural defense against the reactive ferryl oxo intermediates in mammals. The fact that rodents do not show AD thus can be attributed to the absence of these vital residues which probably make the heme binding to rodent Aβ peptide weaker and eventually exempted them from the characteristic degenerative symptoms like detrimental peroxidase activity, oxidative stress, etc.[209] Coincidentally, rats are not affected by T2Dm either.[210] The nonamyloidogenic rat-IAPP peptide comprises a different amino acid sequence compared to human IAPP, where six amino acid residues are altered, five of which are in the 20−29 region, known to be responsible for the IAPP amyloidogenicity.[211] The major alteration is the replacement of His18 residue by Arg in the nonamyloidogenic part of rat IAPP, His18 being the coordinating ligand for heme/Cu binding. This can potentially reduce the binding propensity and subsequent cellular toxicity induced by metal/heme bound IAPP. These contrasting observations of human and rodents are possibly not a mere coincidence, but rather may point out the significant impact of heme and Cu binding to the amyloidogenic peptides in the pathology of degenerative disease like AD and T2Dm. Therefore, the *in vitro* reactivities reported in this manuscript could potentially be of significance with respect to the disease pathology and await validation from *in vivo* studies.

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Author Contributions

CRediT: **Ishita Pal** conceptualization, writing-original draft, writing-review & editing; **Somdatta Ghosh Dey** conceptualization, funding acquisition, project administration, resources, supervision, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

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