Structural and functional characterization of β -sheet heme binding peptides : de novo designed and naturally occurring

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**Structural and Functional Characterization of β -sheet
heme binding peptides: *de novo* designed and naturally
occurring**

By

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Abbreviations

Cyt c	Cytochrome C
ETC	Electron Transport Chain
PMF	Proton motive force
TMB	3,3',5,5'-tetramethylbenzidine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
HRP	Horseradish peroxidase
NO	Nitric oxide
PDB	Protein Data Bank
MP	Microperoxidases
TIM	Triosephosphate isomerase
DPC	Dodecylphosphocholine
DSS	4, 4-dimethyl-4-silapentane-1-sulfonic acid
HPLC	High Performance Liquid Chromatography
TFA	Trifluoroacetic acid
CD	Circular Dichroism
UV	Ultra violet
NMR	Nuclear Magnetic Resonance
MHz	Mega hertz
TOCSY	Total Correlation Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
DOSY	Diffusion Ordered Spectroscopy
PRE	Paramagnetic Relaxation Enhancement
MTSL	(<i>S</i> -(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl

	methanesulfonylthioate)
16-DSA	16-doxyl stearic acid
MPs	Membrane proteins
EM	Electron Microscopy
IMP	Integral Membrane Protein
PMP	Peripheral Membrane Protein
TM	Trans membrane
TDFQ	Time-resolved Trp fluorescence quenching
BAM	β -barrel assembly machinery
β-Ala	β -Alanine
δ-Ava	δ -aminovaleric acid
ϵ-Aca	ϵ -aminocaproic acid
ζ-Aha	ζ -aminoheptanoic acid
η-Aoa	η -aminooctanoic acid
NOE	Nuclear Overhauser Effect
ET	Electron transfer
AD	Alzheimer's disease
TH	Toxic Hand
OB	Oligonucleotide Binding
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic acid
CA	Carbonic anhydrase
Mb	Myoglobin
Hb	Hemoglobin
APP	Amyloid Precursor Protein

ROS	Reactive Oxygen Species
WHO	World Health Organization
FAD	Familial Alzheimer's Disease
sAD	Sporadic Alzheimer's Disease
NFT	neuro-fibrillary tangles
SP	senile plaques
AICD	APP intracellular C-terminus domain
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
SDS	Sodium dodecyl sulfate
TFE	2,2,2-Trifluoroethanol
TFA	2,2,2-Trifluoroacetic acid
LB	Luria bertani
TB	Terrific Broth
IPTG	Isopropyl β -D-1-thiogalactopyranoside
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TfT	Thioflavin T

Abstract

Heme proteins are a specialized class of metalloproteins that play a vital role in complex biological reactions, including photosynthesis and respiration. These proteins achieve their diverse structures and functions by utilizing a large number of amino acids (~more than 100 residues). Engineering of minimalistic peptides or miniproteins that can recapitulate the features of such proteins has gained significant attention in the recent years, primarily due to their applications in creating new biocatalysts and developing model systems for a better understanding of protein structure and functional attributes. Several efforts have been made towards the design of helical heme-peptides in water and membrane environment. However, fewer studies have focussed on the design of functional β -sheet peptides due to their poorly understood folding principles and a tendency for self-aggregation. In this study, we have engineered and characterized a series of β -sheet peptides in water and membrane-like environment.

First, a series of mono-heme four stranded and di-heme six stranded β -sheet peptides were designed for heme binding in a membrane-like environment. The four and six stranded peptides adopted an anti-parallel β -sheet structure and could accommodate one or two hemes respectively. The linker residues between β -strand-II/III were optimized by utilizing a β -turn (^{D} Pro-Gly) and conformationally flexible β - and ω -amino acids in the four stranded peptides to modify their heme binding pocket. Designed peptides contain increasing number of methylene groups from $n=1$ to 7 on the flexible linker residue to study its effect on heme binding and catalysis. An increase in binding affinity of designed peptides was observed with an increase in the length of ω -amino acids. The six stranded β -sheet peptide was observed to bind two

hemes cooperatively. Additionally, these peptides also serve as peroxidases and participate in electron transfer with cytochrome c in a membrane environment.

The four stranded hydrophobic peptides were further modified to improve its solubility in aqueous solutions. The heme binding pocket in these water-soluble peptides was altered by changing linker residues between β -strand II/III (^DPro-Gly turn, ω -aminoacids, Gly-Gly-Gly residues), axial coordination of heme (His/Ser coordination), and by creating a shorter binding pocket (Val9Val11). These peptides assumed a well-defined four-stranded β -sheet topology. The optimized peptides were observed to have picomolar binding affinities that are comparable to natural heme-proteins. Additionally, the peptide/heme complex exhibited high stability towards thermal- and chemical-induced denaturation.

Finally, the non-native heme interaction with self-aggregating A β 40 peptide was characterized in a membrane-like environment. A β 40 has recently been reported to bind heme in an aqueous environment. In Alzheimer's disease (AD), A β -heme complex has been found to cause a regulatory heme deficiency, initiating oxidative stress by forming a peroxidase and abrogating A β aggregation effectively. For understanding the coordination and binding of heme to A β in a membrane-bound environment, experiments were also carried out with a non-amyloidogenic segment of the peptide (A β -16). The unstructured A β -16 peptide was found to have a lower affinity compared to A β -40 peptide in both solution and membrane environment. Monomeric A β -40 has been shown to be unstructured in solution, but a bis-histidine heme coordination was observed for the α -helical A β peptide in DPC micelles. These results highlight the importance of the C-terminus of A β for maintaining heme coordination and structure of the peptide. Interestingly, heme binds to A β in a 2

peptide:1 heme stoichiometry and efficiently inhibits its aggregation within the membrane. It also behaves as a weak peroxidase and thereby contributes to a lesser oxidative stress on cells. These findings suggest a neuroprotective role of the A β /heme complex in a membrane mimetic environment.

CHAPTER 1

1. General Introduction

1.1 Heme Proteins: Overview and Biological Significance

Proteins are versatile biomolecules with an ability to catalyze complex chemical reactions in nature. The spatial arrangement of amino acids within a protein determines its fold, which in turn, influences its function. However, several proteins require the presence of an additional metal cofactor for facilitating its function. Almost half of the biological processes occurring in our body are catalyzed by metalloproteins. Out of these, heme (iron protoporphyrin XI) containing metalloproteins are the most common and abundant class present. Heme is a tetrapyrrole porphyrin macromolecule with an iron atom present at its centre. These cofactor-bound proteins carry out the essential task of electron transfer, di-oxygen binding and storage, breakdown of hydrogen peroxide as well as the oxidation of several organic substrates and metabolites. Though all heme-proteins contain a common iron cofactor, the proteins' function is dictated by a number of factors including its structure, coordination geometry of the porphyrin ring, nature of axial ligands coordinating heme iron and hydrophobic and polar residues surrounding heme binding pocket [1].

1.1.1 Globins: Myoglobin and hemoglobin are heme-proteins responsible for oxygen storage and transport, respectively. Myoglobin is a monomeric protein with a penta-coordinated heme iron, where the axial ligand coordinated to iron is a histidine residue and the sixth or the distal site is freely available to bind an oxygen molecule [2].

Figure 1: Structure of Myoglobin PDB ID: 3GK

On the other hand, hemoglobin is a tetrameric protein, where each monomer unit consists of a penta-coordinated heme iron capable of binding one oxygen molecule (four oxygen molecules in total). The oxygen binding capability of these proteins is attributed to the presence of polar and hydrophobic amino acids and steric interactions surrounding the binding cavity. A key stabilizing element for this binding interaction is hydrogen bond formation between the oxygen molecule and the distal histidine [2].

Figure 2: Structure of hemoglobin PDB ID: 1GZX

1.1.2 Cytochromes: Cytochromes are a group of heme-proteins that participate in

electron transfer and energy conservation processes by the production of ATP and oxidation of various metabolites [3]. Cytochromes are classified on the basis of the type of heme or spectral features (position of their β spectral band in reduced state), as cytochromes a (605 nm), b (~565 nm), and c (550 nm). These protein complexes switch between reduced and oxidized states to carry out their functions.

Cytochrome C (Cyt C), a component of the electron transport chain (ETC), is a highly-conserved membrane-associated protein [3]. These proteins found in the mitochondrial inner membrane are attached to heme by the formation of a covalent bond between- cysteines and the vinyl group of heme c. Cytochromes have a characteristic CXXCH heme binding motif (X denotes any amino acid) and the axial heme ligands: histidine and methionine [4] maintain a low spin-state of the Fe atom in both oxidized and reduced form. Several conserved aromatic residues are present in the binding pocket to protect heme from solvent exposure [3]. Cyt C facilitates the transfer of one electron between protein complexes III and IV in the ETC. It is released from the mitochondria into the cytosol to induce cell apoptosis [5, 6]. Recent studies have proven the antioxidant function of this protein in the mitochondria, where it scavenges superoxide and hydrogen peroxide[7].

Figure 3: Structure of Cytochrome C PDB ID: 1HRC

The bc1 protein complex (complex III) contains cytochrome b, an integral membrane protein that binds to two heme b molecules non-covalently [8]. The protein shows a bis-histidine coordination and plays a key role in facilitating electron transfer across the membrane by binding quinone and converting energy into proton motive force (PMF) [8].

Figure 4: Structure of cytochrome bc1 complex PDB ID: 1L0L. B) The hemes in the complex are highlighted

Cytochrome C Oxidase is responsible for the last step of respiration i.e. the reduction of dioxygen to water. It contains a bimetallic heme/Cu catalytic site, as well as certain additional redox cofactors [2]. The heme a in Cytochrome C Oxidase is penta-coordinated to one histidine, while three histidines coordinate copper. One of histidine residue coordinating copper is covalently attached to an adjacent tyrosine residue and this unique coordination which is common to several oxidase proteins is responsible for the formation of a tyrosine free radical during the reduction reaction [9]. Cytochrome C Oxidase's ability to reduce O_2 to water depends on the oxidation state of the two cofactors present, i.e., the bimetallic site must be in a Fe(II)-Cu(I) state to bind oxygen.

Figure 5: Structure of Cytochrome C oxidase PDB ID: 1OCC

1.1.3 Peroxidases and catalases: Peroxidases and catalases are heme-enzymes that catalyse the breakdown of their substrate, hydrogen peroxide, by forming several intermediate oxidation states of their heme iron. The breakdown of hydrogen peroxide by peroxidases results in the formation of water and oxidation of organic substrates like 3,3',5,5'-tetramethylbenzidine (TMB), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Catalase, on the other hand, catalyzes the breakdown of hydrogen peroxide to yield water and oxygen molecule. Horseradish peroxidase (HRP), one of the most characterized peroxidases contains a penta-coordinated heme b coordinated to a proximal histidine residue. The enzyme is monomeric and has a predominant helical structure. It performs a two-electron oxidation-reduction reaction via an irreversible ping-pong kinetics mechanism [10]. The catalysis reaction is initiated when peroxide binds to the high spin state of heme iron (III). This is followed by heterolytic cleavage of the O-O bond of hydrogen peroxide substrate. An intermediate state-Compound I is formed with the assistance of distal histidine and arginine residues which act as proton acceptors, and stabilize the intermediate and leaving hydroxide ion. Compound I contains a high-valent heme

Iron (IV) oxidation state and is highly unstable. Therefore, a more stable Compound II intermediate is formed by one-electron reduction and subsequent oxidation of the substrate. Compound II returns to its resting stage / initial conformation by undergoing another electron reduction and substrate oxidation.

Figure 6: Structure of Horseradish Peroxidase PDB ID: 1W4W

Figure 7: Peroxidase reaction cycle adapted from Berglund *et al*, 2002

The X-ray crystal structure of beef liver catalase reveals a tetrameric dumb-bell structure where each heme is penta-coordinated to a proximal tyrosine residue. The catalytic site also contains a distal histidine residue that participates in hydrogen bonding to stabilize reaction products, water and oxygen [11]. The catalytic

intermediate steps i.e., formation of compound I and compound II, are similar to the peroxidase reaction cycle.

Figure 8: Structure of catalase PDB ID: 1BLC. A) Beef Liver catalase structure with four heme molecules B) Heme (yellow) iron is coordinated to a proximal tyrosine residue .

Heme groups are commonly found in helical structures like the globins, cytochromes, and peroxidases and they account for almost 77% of the total structures. The tubular diameter of helices is comparable to the width of porphyrins and hence, they form favourable binding pockets for heme [11]. Heme groups are found to intercalate at the interfaces of these coiled-coiled structures. All β -sheet motifs (10%) are not uncommon in heme proteins and are found in proteins such as HasR-a heme transporter membrane protein from *Serratia marcescens* [12], nitrophorin-a nitric oxide transporter protein from *Rhodnius prolixus* [13] and Hemopexin[14]-a high-affinity heme scavenging glycoprotein. Mixed α/β heme motifs (13%) are also found in proteins such as Fix LH-an oxygen sensor [15].

1.2 All- β sheet heme-proteins

1.2.1 HasR: HasR is an outer-membrane heme receptor protein present in Gram-negative bacteria *Serratia marcescens* [16]. This β -barrel protein consists of 22 antiparallel strands with heme coordinated to two histidine residues inside the barrel. HasA, a high-affinity hemophore in *Serratia marcescens* interacts and translocates its heme group to HasR by forming a ternary complex.

Figure 9: Structure of HasR PDB ID: 3CSL. A) Structure of HasR B) Heme is coordinated to two histidines within the barrel.

1.2.2 Nitrophorin: Nitrophorins are a class of nitric-oxide transporter proteins found in blood sucking insects like *Rhodnius prolixus* [17]. They have an eight-stranded β -barrel fold similar to lipocalin proteins [18]. Heme b coordinates via a proximal histidine residue to one end of the β -barrel. The proteins bind nitric oxide (NO) in their ferric heme oxidation state and facilitate the transportation and release of NO gas into the bloodstream/tissue of the host organism. A free-ligand site available at heme (in the absence of NO) makes it possible for the protein to bind histamine, an event responsible for causing reduced inflammation in the host.

Figure 10: Structure of Nitrophorin PDB ID: 1ERX

1.2.3 Hemopexin: Hemopexin has the highest recorded affinity for heme in nature. Free heme is toxic and causes oxidative stress, inflammation and hemolysis [19]. Hemopexin scavenges free heme from the surroundings, thereby maintaining iron homeostasis and protecting cells from toxicity. The protein consists of two domains of four-stranded β -propeller structure connected by a short linker portion (of 20 residues). Heme binding pocket is situated in the linker region between the propeller domains and two histidines coordinate the heme iron (a His213 residue located in the linker region and His266 from the C-terminal domain loop region)[14].

Figure 11: Structure of Hemopexin PDB ID: 1QHU

1.3 Protein Engineering: a brief overview

Protein engineering is an emerging area in science and it involves the optimization, modification and creation of novel protein structures and functions. In particular, designing functional proteins is very challenging, as subtle modifications in the structure can majorly influence its catalytic properties. Researchers have approached this by utilizing high-throughput directed evolution techniques and computationally-designed models for re-designing / de novo design of proteins. Most design attempts have targeted protein scaffolds (with available PDB structures) as a backbone for re-engineering non-native functions [20-22].

Recent advances in the field have led to the creation of artificial peptides/proteins that bear no resemblance to their native counterparts also known as “*de novo* designing”. Short peptide models can also help delineate the minimal features required for protein structure/activity. These simpler systems are much easier to modify and incorporate a catalytic / functional binding site.

Heme-proteins are a class of abundant and diverse proteins that form an essential part of several biological mechanisms, as discussed above. In-order to gain an insight on the structure-activity relationship of these complex and important proteins, several groups have developed miniature heme-protein mimics by rational design approaches. The protein designs range from simple water-soluble single, multi-heme complexes to membrane soluble heme models[23].

One of the preliminary works on peptide-based heme systems was the characterization of microperoxidases. Microperoxidases are Cyt-C derived peptide-heme fragments that are obtained on proteolytic digestion with pepsin/trypsin[24]. The peptides (8-11 residues long) contain a covalently linked heme, bound to a

proximal histidine residue and a free distal site fully exposed to the solvent. The distal site on heme is freely available to bind to other exogenous ligands such as hydrogen peroxide, oxygen, nitric oxide etc. Microperoxidases (MP-8, MP-11) have comparable peroxidase activity to native peroxidases like HRP [24]. The parent protein Cyt C shows a weaker peroxidase activity due to its His-Met coordination and a shielded heme molecule.

Figure 12: Representative structure of Microperoxidase-11.

The residues Val11-Glu21 of horse heart Cyt C form microperoxidase-11 with His-18 axial coordination. Microperoxidase-11 is formed as a result of pepsin digestion of Cyt C. The residues Cys14-Glu21 form microperoxidase-8, another well-characterized microperoxidase which is obtained on trypsin digestion of microperoxidase-11. Figure adapted from [24].

Inspired by nature, several groups have attempted to recreate the common helix/heme/helix motif by designing mini-heme α -helical proteins. Mimochromes are a class of covalent helix-heme-helix sandwich peptides [25]. These peptides were designed with an aim to generate a minimal sequence that can fold into a helix and coat heme completely. Mimochromel consists of two identical 9-residue peptide chains that are antiparallel to each other and lie parallel to the porphyrin plane. Histidines are present at the centre of helix for axial coordination of heme. The

peptide chains are covalently linked to heme propionates through Lys side-chains and they form a pseudo- C_2 -symmetric dimer. Mimochrome6 was modified to facilitate peroxidase activity by utilizing a mono-His axial ligation and charged residues like Arg to mimic the active site of HRP [26, 27]. The peptide template consists of two asymmetric peptide chains: one with His residue present at the centre and another ligand-free peptide to create a five coordinate, ferric heme state common to peroxidases.

Figure 13: NMR derived structures of the two stereoisomers of Co(III)-Mimochrome1. A bis-histidine axial coordination to cobalt deuterio-porphyrin gives rise to two different isomers. The porphyrins are covalently linked to Lys side-chains on the peptide chains. Figure adapted from [28].

A four-helix dimer that could incorporate four heme molecules was developed by Dutton and group [29]. The maquette was designed to mimic the heme binding pocket of redox protein cytochrome b in bc1 complex. Multi-heme complexes like the cytochromes are involved in energy conservation processes like electron transfer and photosynthesis. The dimeric structure (IIa_2) can bind to four hemes parallel to its helical axis and shows spectral and electrochemical features similar to native cytochromes.

Figure 14: Model structure of the tetraheme-protein assembly. The protein coordinates heme via bis-histidine ligation and it contains a flexible linker (Cys-Gly-Gly-Gly) for the formation of a disulphide bond. Charged and hydrophobic residues (green) are present in the interior of the helix to stabilize heme-protein interactions. Figure adapted from [30].

Several groups have utilized the four-helix maquette to understand other functions of heme proteins. One of these efforts was focussed on engineering an oxygen transport protein like neuroglobin [31]. In another design, the four-helix bundle was utilized for an electron transfer function [32]. Hecht and group have utilized the electrochemical potential of these peptides towards the development of biosensors [33]. A designed four-helix heme bundle was assembled on an electrode and its peroxidase activity [34] as well as electrochemical characteristics [35] was recorded as a function of pH and ionic strength.

1.4 Methods and Strategies for Protein Engineering

1.4.1 Directed Evolution

Protein design in nature takes place by molecular evolution. Evolution is an adaptive mechanism followed by organisms that leads to their gradual alteration in-order to

cope up with different environments. These evolutionary processes are mimicked in-vitro by carrying out iterative rounds of mutagenesis, homologous recombination, screening and selection [36]. The start point involves screening protein libraries and recognizing a parent protein with a minimal activity towards the desired function. The property of the parent protein can be altered by introducing mutations. Most of the random mutations however have a deleterious effect on the activity and stability of a protein. Recombination technique has been found to be more successful in generating new functional proteins than random mutations [37, 38]. For instance, by utilizing homologous recombination, a set of 6000 proteins were generated by introducing around 70 mutations from the parent P450 protein, almost 50% were found to be well-folded, and at least 75% of the folded variants showcased catalytic activity [39]. It is observed that most activating mutations have a deleterious effect on the stability of the protein which necessitates the need for introducing stabilizing mutations before improving its activity [40].

Interest in directed evolution approach has grown significantly in the past 20 years [41-45]. The technique has been widely employed by Arnold's research group to create biocatalysts with an increased thermal stability [46, 47], non-biological functions [48] and an enhanced activity in organic solvents [49, 50]. A considerable amount of work has focussed towards evolution-based optimization of heme-proteins such as cytochrome P450 [51-53] (C-N amination, cyclopropanation activity, carbene and nitrene transfer) and cytochrome C, the first C-Si bond forming enzyme [54].

1.4.2 Computational protein design

One of the most common approaches followed for re-engineering proteins and de novo design is by using a variety of computational design algorithms [55-58].

Design efforts of catalytic proteins are concentrated towards the stabilizing the transition state intermediate, by obtaining a structural model of the active site. Algorithms have been developed for predicting the effect of these active site mutations on the enzymatic activity [59]. Sequence-based structure prediction of proteins can be made utilizing powerful algorithms [60]. Further, algorithms can also aid in designing amino acid sequences that would stabilize given protein fold [61]. The models generated from these algorithms can also account for the energy of the catalytic intermediates.

Rosetta is an ab-initio modelling program designed by Baker's research group. It utilizes Monte Carlo simulations and is based on the assumption that proteins fold into the lowest energy states as proposed by Christian Anfinsen [62]. The simulation can efficiently converge energy minima using score functions and rotamer libraries, the optimized models generated are found to be consistent with x-ray crystallography derived structures. The Rosetta platform has been designing proteins for various purposes such as creating thermostable miniaturized proteins [63-65], designing protein with non-native structures [66], creating TIM barrel catalytic fold [67], bio-catalysts for Kemp elimination reaction [68], protein cages [69, 70], Retro-Aldol Enzymes [71], Diels-Alder catalysts [72] and designing protein inhibitors that bind to a conserved region of the hemagglutinin of an influenza virus [55].

CHAPTER 2

2. Materials and Methods

2.1 Materials

Peptides were commercially synthesized from GL-Biochem (Shanghai, China) and Karebay Biochem (China). DPC was purchased from Avanti polar lipids. DPC-d₃₈, D₂O and 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were purchased from Cambridge Isotope Laboratories Inc. (Massachusetts, USA). Other chemicals including 16-DSA, Sodium dithionite, Hemin, ABTS, Cytochrome C (from equine heart), Myoglobin (from equine skeletal muscle), trypsin and pepsin were purchased from Sigma-Aldrich.

2.2 Purification of crude peptides

Crude peptides were subjected to purification by reverse phase HPLC using C₄ column (for the hydrophobic peptides) and C18 column (water-soluble peptides, microperoxidase and apomyoglobin) of 300 Å pore size, 5 µM particle size. A linear gradient 10-90% of acetonitrile/water (both solutions containing 0.1% v/v TFA) was used to elute the peptides while maintaining a constant flow rate of 2 ml/min. The major sharp peak fraction obtained was then pooled, lyophilized and verified using MALDI-TOF mass spectrometry.

2.4 Preparation of Apomyoglobin & Microperoxidase-11

Myoglobin solution (~12 mg dissolved in 10 ml water) was titrated dropwise to an acidified acetone solution (at -20 °C) [73]. The precipitate obtained after spinning down the suspension (4000 rpm, 30 mins at 4 °C) was dried by purging nitrogen gas. It was further dissolved in water containing 0.1% TFA and centrifuged at 14000 rpm

for 10 mins to remove free heme precipitate. The supernatant was subjected to purification by reverse phase HPLC with an acetonitrile linear gradient (10-90% acetonitrile at 2 ml/min flow rate) using a C₁₈ column (300 Å pore size, 5 µM particle size). The major sharp peak at 33.8 min was lyophilized, reconstituted with heme and confirmed using UV-vis spectroscopy (**Figure 15**).

Figure 15: HPLC chromatogram of apomyoglobin and its reconstitution with heme. A) HPLC purification profile of apomyoglobin with absorbance monitored at 220 nm (red) and 278 nm (green). B) UV-vis spectra of apomyoglobin (black), heme (red) and myoglobin reconstituted (blue).

Cytochrome C solution (~10 mg) and ~1 mg pepsin was dissolved in 1 ml water. The pH of the solution was adjusted to 2.0 and kept overnight at room temperature [24]. The solution was further diluted in 4 ml (10% acetonitrile, 0.1% TFA) and subjected to HPLC purification with an acetonitrile linear gradient (10-60% acetonitrile at 1ml/min flow rate) using a C₁₈ column (300 Å pore size, 5 µM particle size) (**Figure 16**). The peak containing Microperoxidase-11(44.1 min) was confirmed by mass spectrometry, lyophilized and stored in -20 °C.

Figure 16: Preparation of microperoxidase-11. A) HPLC purification of microperoxidase-11. Absorbance was monitored at 220 nm (red) and 278 nm (green). B) MALDI analysis of HPLC peak at 44 minutes shows expected molecular weight (Theoretical molecular weight - 1861.92).

2.5 Preparation of Heme

A 2.5 mM stock solution of heme (by weight) was freshly prepared in 0.1% NaOH in MilliQ water and stored under the dark. The working stock of heme was prepared by double dilution with the buffer of required pH. The concentration of heme stock was further estimated using the Pyridine Hemochrome Assay [74].

2.6 UV-vis studies (K_d determination, sodium dithionite reduction and stoichiometric analysis)

For the membrane-soluble and water-soluble peptides, the binding affinity of heme to designed peptides was characterized using a multi-well plate reader (Tecan Infinite M200 PRO) by titrating increasing concentrations of peptide (upto five equivalents) to a fixed concentration of heme prepared in 50mM sodium phosphate buffer, 2mM DPC, pH 7.2 / 5 mM Tris pH 9.0. The Soret band at 412 nm / 414 nm was monitored for complex formation for each aliquot after three-hour incubation. Wavelength scans from 350-600 nm was further recorded to monitor the shift in the absorption maxima of heme on binding to the peptides. Binding isotherms were obtained by plotting the

absorbance at 412 nm/ 414 nm versus peptide concentration. The values of $\Delta\epsilon$ and $K_{d,app}$ were derived from the ligand depletion binding model fit using Origin 9.0 for the membrane-soluble peptides.

$$\Delta A_t = \frac{\Delta\epsilon([H_t] + [P_t] + K_{d,app}) - \sqrt{([H_t] + [P_t] + K_{d,app})^2 - 4[H_t][P_t]}}{2}$$

$[H_t]$ is the total heme concentration, $[P_t]$ is the total peptide concentration, $K_{d,app}$ is the apparent dissociation constant, $\Delta\epsilon$ is the difference in extinction coefficients of bound and unbound heme, and ΔA_t is the change in absorbance for a fixed peptide concentration.

For the water-soluble peptides, heme binding was monitored in the Soret region (350-650 nm) by titrating increasing concentrations of heme (upto 5 stoichiometric equivalents) to a fixed concentration of peptides in 5 mM Tris-HCl buffer at room temperature. The K_d values were estimated by plotting absorbance at λ_{max} (414 nm) versus heme concentration using the equation for high affinity heme binding [75].

$$\Delta A = H_t \epsilon_{free} + (\epsilon_{bound} - \epsilon_{free}) \frac{([H_t] + [P_t] + K_{d,app}) - \sqrt{([H_t] + [P_t] + K_{d,app})^2 - 4[H_t][P_t]}}{2}$$

where $[H_t]$ is the total heme concentration, $[P_t]$ is the total peptide concentration, $K_{d,app}$ is the apparent dissociation constant, ϵ_{free} is the extinction coefficients of free heme, ϵ_{bound} is the extinction coefficients of bound heme, and ΔA is the change in absorbance at a given heme concentration (**Figure 17**).

Figure 17: Bound peptide 13-heme extinction coefficient determination. A) Absorption spectra of heme-peptide complex at different concentrations. B) Molar absorptivity of peptide-heme complex was determined by the slope of the linear plot monitoring Soret maxima (414 nm) peak at varying concentrations (5-40 μM) of the complex. The extinction coefficient was estimated to be $112.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Peptide-heme stoichiometry was determined using method of continuous variation or Job's plot. For the membrane-soluble peptides, 50 μM peptide and heme stocks were prepared in 2 mM DPC, 50 mM sodium phosphate buffer, pH 7.2. For the water-soluble peptides, 100 μM peptide and heme stocks were prepared in 5 mM Tris-HCl buffer, pH 9.0. Different ratios of the two stock solutions were mixed while keeping the total volume constant. The absorbance values at 412 nm / 414 nm and 356 nm were recorded for the various mole fractions of heme using a multi-well plate reader (Tecan Infinite M200 PRO). This difference in absorbance was then plotted against the mole fraction of heme. The bound and free heme extinction coefficients were determined by recording the absorption spectra at varied concentrations of peptide-heme (pre-incubated for an hour at 1:1 stoichiometry) / free heme. The λ_{max} values were then plotted against the concentration of heme to generate the extinction coefficients.

The ferrous heme spectra were obtained by addition of sodium dithionite (from a

freshly prepared 5mM stock solution) to the peptide-heme complex (20 μ M peptide, heme concentration) in 2 mM DPC 50 mM sodium phosphate buffer pH 7.2 / 5 mM Tris-HCl pH 9.0 buffer (de-aerated for 30 minutes by purging nitrogen).

For the imidazole and hydrogen peroxide binding experiments, heme-peptide complex was prepared in 5 mM Tris buffer pH 9.0 by incubating 11 μ M peptide to 10 μ M heme for 3 hours. Imidazole and hydrogen peroxide stocks of 1 M and 1 mM respectively were prepared using the identical buffer condition. Absorbance spectra of heme-peptide complex at each imidazole titration (upto 100 equivalents) and hydrogen peroxide titration (upto 10 equivalents) were monitored spectrophotometrically in the Soret region (350 nm-650 nm) at room temperature.

2.7 Competitive Binding Assays with Apomyoglobin

For the heme-transfer experiments, heme-peptide sample was prepared in 5 mM Tris-HCl buffer pH 9.0 by incubating 6 μ M peptide to 6 μ M heme for 3 hours. Apomyoglobin stock of concentration 50 μ M was prepared using the identical buffer condition. The assay was followed for an hour spectrophotometrically at room temperature after addition of apomyoglobin (final concentration 6 μ M) to the heme-peptide sample. Absorbance scans were recorded every 5 minutes from 350-650 nm in a multi-well plate reader (Tecan Infinite M200 PRO). For the competitive binding assays, equimolar concentrations of apo-peptide and apomyoglobin (6 μ M) were pre-incubated for 3 hours. Absorption spectra were monitored at the Soret region after addition of heme (final concentration 6 μ M) to the peptide + apomyoglobin sample.

2.8 Stopped-flow Kinetics Studies

A SX20 stopped flow spectrophotometer (Applied Photophysics) was used to monitor

the heme binding kinetics. Syringes containing 15 μM peptide and 15 μM heme in 5 mM Tris-HCl buffer pH 9.0 were mixed at room temperature and the absorbance values at λ_{max} were recorded versus time. The oxidation of ABTS as a measure of peroxidase activity was recorded at 700 nm versus time at room temperature. 8 μM peptide solutions containing 1 μM heme and 5 mM ABTS were mixed with 20 mM hydrogen peroxide in 5 mM Tris, pH 9.0. The absorbance values at 700 nm was converted to concentration ($\epsilon_{700\text{nm}} = 1.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) and the reaction rate was calculated by linear regression analysis. In-order to obtain the kinetic parameters for the peptide-heme complexes (V_{max} , K_m , k_{cat}/K_m), reaction rates were obtained by varying hydrogen peroxide concentrations. These reaction rates were plotted versus H_2O_2 concentration and fit to the Michaelis-Menton equation using Origin 9.0 software.

$$V = \frac{V_{\text{max}}[H_2O_2]}{K_m + [H_2O_2]}$$

V is the reaction rate ($\mu\text{M/s}$), V_{max} is the maximal velocity for the enzymatic reaction ($\mu\text{M/s}$), K_m is the Michaelis-Menton constant and $[H_2O_2]$ is the concentration of H_2O_2 used. Turnover numbers (k_{cat}) were calculated by dividing the maximal velocity V_{max} by the concentration of heme-peptide complex (0.5 μM).

For electron transfer experiment of the membrane-soluble peptides, heme-peptide samples were prepared in 2 mM DPC, 50 mM sodium phosphate buffer pH 7.2 by incubating 24 μM peptide to 24 μM heme for 3 hrs. Reduced heme-peptide was prepared by addition of sodium dithionite to the heme-peptide sample. Cytochrome c of concentration 12 μM was prepared using the identical buffer conditions. Absorbance scans were monitored from 350-600 nm in a multi-well plate reader

(Tecan Infinite M200 PRO). The kinetics of the electron transfer reaction was followed spectrophotometrically at room temperature using a stopped-flow kinetics apparatus (SX20, Applied Photophysics). The rate constant for the reaction was determined by monitoring the changes in the absorption intensity at 550 nm versus time.

2.9 Circular Dichroism and Denaturation Studies

Apo and holo peptide solutions (100 μ M) were prepared in 5 mM Tris buffer, pH 9.0. Far-UV CD studies (Chirascan-Applied Photophysics) were performed using a 0.1 mm path length cuvette with a 1 nm bandwidth and a step size of 0.5 nm at 0.5 seconds per data set. CD spectra were also recorded in the Soret region (350nm-450nm) to monitor heme environment for the heme-peptide complexes. Heme-peptide sample (10 μ M) was prepared in 5 mM Tris-HCl Buffer pH 9.0 and experiments were performed using a 1cm path length cuvette with a 1 nm bandwidth and a step size of 0.5 nm at 0.5 seconds per data set.

Thermal denaturation experiments for peptide 1 and peptide 3 (apo and holo forms- 100 μ M) were carried out from 5°C-90°C by collecting far-UV CD spectra every 5°C after 10 minutes of equilibration at each temperature using a 1mm path length cuvette (covered with a lid to prevent evaporation) at a 2nm bandwidth and a step size of 0.5 nm at 0.5 seconds per data set. The CD value (in mdeg) at positive maximum ~220-230nm (225 nm-peptide 11 apo and holo; 225 nm-peptide 13 apo, 230 nm-peptide 13 holo) was used as the point of inflection to study the thermal transition of peptides.

Fraction folded f_t for each temperature transition can be calculated using the following equation.

$$f_t = (\theta_t - \theta_u)/(\theta_f - \theta_u)$$

θ_t is the observed CD signal (mdeg) for any temperature, θ_u is the CD signal (mdeg) for the unfolded form and θ_f is the CD signal (mdeg) for the folded form. The fraction folded for each temperature transition was then plotted versus temperature. T_m values were derived using the Boltzmann equation fit for two-state transition (OriginPro 9) for the plot. For the chemical denaturation experiments, increasing concentrations of urea (0M-8M) were titrated into a 10 μ M peptide solution containing 5 mM Tris, pH 9.0. The peptide samples were equilibrated for 3 hours at room temperature at required urea concentration. The Soret region (350-650 nm) was monitored spectrophotometrically using a multi-well plate reader to observe the change in the Soret maxima on increasing concentrations of the denaturant.

2.10 NMR Spectroscopy

NMR experiments for the water-soluble peptides were performed at 278 K on a Bruker DRX 600 MHz or 700 MHz spectrometer equipped with a cryo-probe. NMR samples for the membrane-soluble peptides were prepared by dissolving lyophilized peptide powder in 125 mM deuterated DPC containing 500 μ L 90% H_2O /10 % D_2O and at pH 5.0 and recorded at 315 K. NMR samples for the water-soluble peptides were prepared by dissolving peptides (0.2-0.35 mM concentration) in 500 μ L 90% H_2O /10 % D_2O and adjusting the pH to 5.5. Homonuclear ^1H - ^1H two dimensional TOCSY (mixing time-80ms) and NOESY (mixing time-200ms) experiments were carried out for sequence-specific resonance assignments. The experiments were recorded using 64 number of scans, with spectral widths of 13 in F1 and F2 direction and using 2048 x 350 data points. NMR data were processed using TopSpin 2.1 (Bruker), and the chemical shifts were referenced directly (^1H) to the frequency of DSS. Natural abundance ^{13}C - ^1H HSQC experiments were carried out by dissolving lyophilized peptides in 100% D_2O and ^{13}C chemical shift was referenced indirectly

from the ^1H frequency of DSS [76].

To estimate diffusion coefficient of peptides, DOSYNMR experiments were carried out. 0.25 mM concentration of peptide and 0.1mM DSS was prepared in 99% D_2O solution, and were recorded on a Bruker DRX 700-MHz spectrometer equipped with a cryo-probe at 278 K. The pulse sequence was a stimulated echo bipolar gradient pulse (stebpgp1s) with the DOSY spectra acquired for each sample using a 5%-95% gradient having 16 increments (exponential array), linear ramp, diffusion gradient length (δ) 1.5 ms and big delta (Δ) 180.0 ms. Molecular weights were estimated by a further extrapolation of the Stokes-Einstein equation assuming a spherical shape where $\text{MW}_{\text{peptide}}$ and MW_{DSS} are the molecular weights of the peptide and the internal reference DSS, respectively and D_{peptide} and D_{DSS} are the diffusion coefficients of the peptides and reference compound [77].

$$\frac{\text{MW}_{\text{peptide}}}{\text{MW}_{\text{DSS}}} = \left(\frac{D_{\text{DSS}}}{D_{\text{peptide}}} \right)^3$$

PRE-experiments for the membrane soluble peptides were carried out by titrating 2mM 16-doxyl-stearic acid (16-DSA) pre-dissolved in deuterated methanol to a lyophilized peptide sample in DPC micelles. Two dimensional TOCSY spectra were carried out with and without the PRE-probe using the same experimental conditions. The intensities of the $\text{C}\alpha\text{H}/\text{NH}$ cross peak were evaluated before and after the addition of the PRE-probe and their ratio plotted for each amino acid. To facilitate MTSL (*S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) methyl methanesulfonothioate) labelling, peptide-7H6C was incubated with 10:1 MTSL:peptide concentration in 50% acetonitrile solution and incubated overnight at room temperature (>12 hours). In-order to remove excess MTSL and unlabelled peptide, the incubated sample was diluted and subjected to HPLC purification. The

major eluted peaks were analysed by mass spectrometry and the labelled peptide fraction was verified. The labelled peptide was then reconstituted in the same identical buffer conditions and two dimensional TOCSY spectra were carried out for the labelled and unlabelled peptide. The intensity of the TOCSY peaks observed for the labelled sample were used as a measure to derive distance restraints for structure calculation.

2.11 Structure Calculation:

Ensembles of 3-D structures of peptides were calculated, using CYANA 2.1 [78], from NOE-driven distance constraints and backbone dihedral angle (ϕ , ψ) constraints. Based on intensity, NOE cross-peaks observed in the 2-D NOESY spectra were categorized into strong, medium and weak and further translated to upper bound distance constraints between 2.5-5.0 Å. Backbone dihedral constraints were obtained, from PREDITOR-WISHART server based on the secondary chemical shifts of α H and NH protons. The NOE restraints and predicted dihedral angles values were used to carry out several rounds of structure calculations. Of the 100 structures generated, 20 low energy structures were selected for evaluation and analyses. PROCHECK-NMR was used to assess the stereo-chemical quality of the structure ensembles. The structures are analyzed visually by PYMOL, MOLMOL and INSIGHTII. For deriving a model peptide-heme complex, two 6Å distance constraints were assigned between the histidine 7 and 13 CH δ and CH δ while comparing distances between bis-histidine axial ligands in b-cytochromes. Of the 100 structures of the model generated, 20 low energy structures were selected for evaluation and analyses using CYANA 2.1. Heme b coordinates were inserted to the structure and the model was prepared using MOLMOL.

2.12 Resonance Raman Spectroscopy

Raman spectra of peptide-heme complexes were acquired on a LabRAM HR Raman Instrument with an argon ion laser used for excitation and an Olympus optical microscope with a 100x objective lens. The excitation wavelength used for this study was 514.5 nm. The spectrum was pre-calibrated using silicon as a reference at 520 cm⁻¹. Peptide-heme solutions of 125 μM concentration were prepared in 5 mM Tris buffer pH 9.0. The two-protein standards cytochrome C and myoglobin were also prepared using the identical buffer condition.

CHAPTER 3

3. Membrane-soluble β -sheet peptides

3.1 Chapter Overview

Membrane proteins (MPs) play an active role in numerous biological processes and serve as targets to a majority of available therapeutic drugs. However, structural information of only 2 % of MPs is available and most solved structures are of α -helical bundles. Design of novel peptides that can mimic the function of native β -sheet MPs is challenging and requires thorough knowledge of folding and function of proteins. HasR is a heme transporter β -barrel in *Serratia marcescens* with a 22 β -strand topology that accommodates heme via a bis-histidine coordination [79]. With a goal to achieve the complexities of heme binding to β -strands in membrane, four stranded and six stranded peptides were designed. These peptides like the HasR protein were designed to have a bis-histidine axial coordination and have a β -structure in a membrane-like environment.

3.2 Introduction

3.2.1 A brief overview on membrane proteins

Living cells are protected from their surrounding environment by an amphipathic layer called the cell membrane. Membrane proteins (MPs) are specialized proteins embedded within these cell membranes that facilitate the indispensable task of ion and solute transportation, signal transduction, cell metabolism and energy conservation. MPs also account for approximately a third of the proteins throughout the genome [80]. Mutations in MPs are linked with numerous diseases such as cystic fibrosis, charcot-marie tooth disease, diabetes and retinitis pigmentosa [81-84], asserting their significance as vital targets for drug development. At present, they serve as targets for more than 60% of the therapeutic drugs [85, 86]. Hence, three dimensional structures of these proteins in their native environment would lead to a deeper understanding of the structure-function relationships as well as folding principles involved in this rather abundant and important group of proteins. Despite the significant advances in structural biology techniques over the years, there have been only a few available membrane protein structures when compared to their soluble counterparts [87].

While soluble proteins are located in a hydrophilic environment, membrane proteins reside in a variable and anisotropic lipid (primarily hydrophobic in nature) environment [88]. Hence, structural characterization of these proteins is more challenging owing to their insolubility in water [80], aggregation in absence of membrane-mimetic environments (such as detergents), size constraints in techniques such as NMR and EM and achievement of sample homogeneity in the case of crystallography. This calls for a shift in our approach to gain a better insight on the governing principles of membrane protein folding mechanism.

Figure 18: Cartoon representation of integral and peripheral membrane proteins in the lipid bilayer. Integral membrane protein can either be α -helical bundle (multi-colored) or β -barrel membrane protein (blue). Extreme right is a peripheral membrane protein (pink).

Membrane proteins can be broadly classified as integral membrane proteins (IMP) or peripheral membrane proteins (PMP) on the basis of their mode of attachment to the membrane (**Figure 18**). While IMPs traverse and interact with the interior hydrophobic core of the lipid bilayer, PMPs are attached to the exterior polar head groups of the bilayer through electrostatic interactions. IMPs can be further classified as α -helical bundles and β -barrels based on their structural motifs. The dominant α -helical bundles are generally present in the cytoplasmic membrane while β -barrels are localized in the outer membranes. The factors involved in the folding and stabilization of the predominant α -helical bundles has been extensively studied, while β -barrels are sparsely reviewed with fewer than 400 structures solved [89]. As of July 2017, 3227 trans-membrane structures (2848 α -helical, 366 β -barrel) are available in the PDB (Protein Data Bank). These structures which constitute about 2% of the total deposited PDB structures. (<http://www.pdb.org>) [90].

3.2.2 Theories related to membrane protein folding

While hydrophobic interaction is the key driving force in the folding of soluble

proteins, this may not be the case for membrane proteins [91]. Unlike soluble proteins, IMPs reside in a heterogeneous bilayer environment and this would majorly influence their folding and structure. Generally, IMPs need to interact with three different environment conditions in order to insert well into a lipid bilayer, an upper hydrophilic surrounding, a polar lipid interface (around 15Å long), and an interior hydrophobic core (around 30 Å long). A combination of several other forces such as van der Waals packing, hydrogen bonding and electrostatic interactions also play a significant part [92, 93].

In 1990, a two-stage mechanism for the formation of α -helical bundles was proposed by Popot and Engelman [94, 95]. The α -helical folding event can be divided into two steps, Insertion and Folding (**Figure 19**). The insertion stage can be self-directed or mediated by the translocon complex. α -helical IMPs are characterized by long stretches of hydrophobic residues (~20 amino acids) that can traverse the entire bilayer in their helical form. Since hydrophobicity is a defining feature of α -helical bundles, partitioning of the unfolded protein in the water-bilayer interface is a key component of this stage. This is followed by formation of its secondary structure across the water-bilayer interface and its insertion into the hydrophobic core of the lipid bilayer. After insertion, the TM segments self-associate to give rise to a final tertiary and quaternary structure. Both these steps however are governed by the identities of the amino acid side chains comprising the protein sequence [96-98].

However, there are fewer theories on the folding and insertion mechanism of β -barrels. β -barrel structures are composed of 8-22 β -strands and showcase an inside-out fold where the hydrophobic residues face outward in the lipid bilayer and polar residues are localised in the interior portion of the barrels. Since the hydrogen

bonding in β -barrels is very different from the α -helical bundles, they might follow a completely different mode of folding and insertion mechanism.

Figure 19: Schematic representation of α -helical trans-membrane folding, insertion and association. A two-stage model where-in peptide folds into secondary structure followed by insertion and self-association into oligomers. Picture adapted from [99].

A recent study on OmpA folding (an 8-stranded β -barrel protein) was carried using time-resolved Trp fluorescence quenching (TDFQ) experiment [100]. In this experiment, the translocation rates of Trp across the membrane was tracked by utilizing quenchers (bromine or nitroxide spin label attached at different positions in the acyl chain of phospholipids) in the membrane. Trp mutants were made on the loops of each β -hairpin in OmpA and the membrane translocation rates were measured. It was found that the Trp present in each of the hairpins crossed the membrane at the same time. Therefore, the results suggest that β -hairpins in OmpA are translocated to the membrane in a concerted manner. Furthermore, a barrel with inter-strand hydrogen bonds needs to be formed during the translocation itself. The folding stages were also characterized using TDFQ technique: 1) an unfolded state

(U) forms an intermediate I_w in water. 2) I_w binds to the surface of the interface of the membrane to form IM_1 . 3) IM_1 proceeds to IM_2 where it forms a portion of its β -structure. 4) IM_3 , the 4- β hairpins are translocated at the centre of the membrane and tertiary structure is formed. 5) IM_3 proceeds to the native form (N), the β -barrel traverse across the entire membrane (**Figure 20**). This is strikingly different from the established α -helical folding model, where TM segments insert independently and form a tertiary structure at stage II. The BAM (β -barrel assembly machinery) chaperone complex is also known to assist in the folding and insertion of β -barrels in the outer membrane [101].

Figure 20: Model of folding and membrane insertion of OmpA. Picture adapted from Tamm *et al*, 2004.

A β -sheet/ β -barrel MP presents various challenges in its protein structure and poorly understood folding mechanisms. Therefore, an all β -sheet/ β -barrel design from first principles could provide an alternative means to understand the factors guiding membrane protein folding and stability.

3.2.3 Successful design attempts of membrane helical models

The field of protein design has been successfully employed for engineering small water-soluble protein structures [66, 102, 103]. Membrane protein design is lagging behind due to lesser established folding principles and difficulties in experimental characterization. Nevertheless, exemplary efforts have been made towards the design of small α -helical scaffolds in membrane like environments. α -helical MPs are comparatively easier to design as their TM helices form more stable intra-helical hydrogen bonding in hydrophobic nonpolar environments. Incorporating small functions such as ligand binding / catalytic activity in these scaffolds can help contribute towards an understanding of functions of complex membrane proteins such as Cytochrome P450s.

Cytochrome P450 is a heme containing membrane protein essential for metabolizing several drugs available in the market. Despite the protein's remarkable catalytic potential, there is a limited knowledge on how it functions. Synthetic heme-peptide models can serve as useful templates for understanding the complex machinery of P450 and for developing new applications. Arnold's group has successfully utilized the adaptability of the P450 active site as a scaffold for grafting new functions using directed evolution technique [104-106].

Rational design approach is utilized for: 1) Re-designing an inert protein scaffold by introducing a non-native function or 2) De novo design using first principles based on sequence guided information gained from natural proteins.

Some of the notable works in *de novo* design of α -helical scaffolds are highlighted below:

3.2.3.1 ROCKER (Transmembrane Translocation Kinetics Enhancer): Recently, Degrado's group reported a 25-residue helical peptide that self-assembles to form homo-tetramer and transport $\text{Zn}^{2+}/\text{H}^{+}$ ions across the membrane [107]. The peptide was computationally designed with two binding sites for zinc. The transportation of zinc ions across a concentration gradient was facilitated by a rocking of the structure between two conformational states such that the substrate binding pockets of the two binding sites of zinc are alternately accessible (**Figure 21**). The design was validated using X-ray crystallography (of the apo form), NMR spectroscopy (for Zn based binding titrations) and liposome flux assays (to measure the proton flux) in membrane mimicking environment.

Figure 21: Computational design model of Rocker. A scheme showing the conformational exchange between two oppositely oriented states critical for zinc transportation. Picture adapted from [107].

3.2.3.2 PRIME (Porphyrins In Membrane): PRIME, an α -helical peptide was designed by Degrado's group utilizing the sequence of a previously designed water-soluble coiled-coil di-heme-binding protein [108]. The protein constitutes an "Ala-coil" motif which enables it to fold well in a membrane-like environment. PRIME forms a homo-tetramer while it binds to iron diphenylporphrin (an unnatural cofactor) through bis-his coordination (**Figure 22**). The helices form a D₂-symmetry α -helical

bundle upon binding. UV-vis spectroscopy and analytical ultracentrifugation experiments demonstrated a 2 peptide: 1 porphyrin stoichiometry. PRIME peptide was also found to have weak affinity for heme.

Figure 22: Model structure of PRIME in the membrane (yellow). Picture adapted from [108].

3.2.3.3 ME1: Glycophorin A scaffold was re-designed by mutation of five residues to render it a heme binding function [109]. The dimeric structure of ME1 is due to the presence of a GXXXG motif in Glycophorin A and it coordinates a single heme molecule by bis-histidine coordination (**Figure 23**). The designed peptide binds heme in sub-micromolar affinity and showcases peroxidase activity (by oxidizing TMB co-substrate) in DPC micellar environment.

Figure 23: Model structure of engineered glycophorin. A dimer bound to heme in DPC micelles. Picture adapted from [109].

3.2.3.4 HETPRO (Heme-binding Transmembrane Protein): VK22, a tetrameric cationic antimicrobial peptide was used as a template for designing HETPRO [110]. Modifications were made by replacing cationic residues, introducing a GXXXG motif for inter-helical association and an Ala coil motif to improve packing interactions within helices in membrane. HETPRO adopted a dimeric structure in micelles and binds to heme in micromolar affinity via bis-histidine coordination. A 2 peptide:1 heme stoichiometry was observed for HETPRO, indicating that the bound peptide exists as an anti-parallel tetramer and it binds to 2 heme molecules (**Figure 24**).

Figure 24: Side view of tetrameric assembly HETPRO with two heme molecules. Picture adapted from [110].

3.2.4 De novo design of a β -hairpin membrane peptide

Even though β -structures are commonly found in transporters, porins, designing β -sheets is complicated due to their aggregation forming tendency. Introducing functions into this less explored structural scaffold is even more complex. The first design attempt of a functional β -sheet was carried out successfully in our laboratory. A β -hairpin (two-stranded β -sheet), the fundamental structural unit of β -barrel MPs was designed in this study [111].

Figure 25: Schematic representation of IV8 peptide with His6 residue involved in heme ligation (red). NMR solution structure of IV8 peptide modeled with DPC micelles. Picture adapted from [111].

Furthermore, the peptide termed IV8 was endowed an additional feature, accommodation of a heme prosthetic group by introduction of Histidine at the 6th position of the peptide. The peptides IV8, IV8 FA were designed and probed for heme binding, peroxidase kinetics and structural characterization in DPC micelles. The latter mutant was designed to examine the role of aromatic side-chain packing in heme binding and protein folding as observed in water-soluble models. The findings suggest that these well-folded β -hairpin peptides associate to heme b in micro-molar

affinity with a 2 peptide: 1 heme stoichiometry, while also functioning as a synthetic peroxidase. Biophysical assays performed with the Ala mutant analogue reveal that inclusion of an aromatic cross strand packing (F2-F7 edge-face packing as observed in IV8) is not a requisite for membrane protein design and does not affect the folding, stability, ligand co-ordination and activity unlike the previously reported water-soluble β -hairpin models.

3.3 Results and Discussion

3.3.1 Design of peptide 1

Figure 26: Primary structure and sequence of peptide 1.

IV-8 (Acetyl-I-F-W-D⁰P-G-H-F-V-OCH₃), a β -hairpin octapeptide was previously designed and characterized in our laboratory [111]. IV8 adopts an antiparallel β -hairpin topology in membrane and binds to heme at a lower affinity, K_d ~in high micromolar range. IV8 binds heme with 2:1 peptide/heme ratio. From this study, we realized that a high affinity heme binding would require a binding pocket within the β -sheet structure. Using IV8 sequence as the N-terminus, the peptides were extended to a four-stranded peptide (peptide 1) to optimize high-affinity heme binding. Peptide1 was designed to fold into a four stranded β -sheet structure (**Figure 26**). In-order to facilitate a heme binding within these peptides, two histidines were placed at positions 6 (strand II) and 12 (strand III) respectively. Hydrophobic amino acids with high β -sheet forming propensities (such as I1, V8, V11, M12, I17, and I18) were chosen except histidine to fold well in the membrane. The N- and C- terminus of peptides was protected by acetylation and carboxy methylation, respectively. In-order to ensure the stability of the β -sheet, a crucial parameter to be kept in mind while designing is cross-strand side chain packing, as reported in several model peptides

[112-114]. Amino acids with bulky side chains were introduced to facilitate side chain-side chain packing in the non-hydrogen bonding site of the β -sheet (F2/F7 and M12/I17). Aromatic amino acids namely tryptophan and tyrosine were inserted at position 3 (strand I) and 15 (strand IV), respectively to localize near the water-micellar interface as observed in native membrane proteins [114-116]. ^{D} Pro-Gly segment was inserted at i+1, i+2 positions respectively in-order to nucleate a type I'/II' β -turn, which is known to be vital component for stabilizing the two anti-parallel β -strands in β -sheet structures and thus providing a tight binding pocket for heme binding.

3.3.1.1 Structural Characterization using NMR spectroscopy

The four-stranded β -sheet peptide 1 was studied in DPC micelles to provide a membrane mimicking environment. The zwitter ionic characteristic of these micelles (similar to lipids found in eukaryotic membranes) and small size make these micelles an ideal condition for NMR based characterization of membrane proteins and peptides. Secondary structure of peptide 1 was verified by observing the changes in the C α H proton chemical shifts from random coil values: positive chemical shifts are observed for residues involved in β -sheet conformation, while negative chemical shifts are observed for residues involved α -helical or turn conformations (**Figure 27A**). Therefore, the H- α , C- α chemical shift index of peptides calculated using NMR spectroscopy in deuterated DPC environment could provide us with more conclusive evidence on the secondary structure of these peptides in membrane. The H- α chemical shifts are indicative of two β -hairpin structures for all peptides; four stretches of downfield shifts were observed for residues involved in β -sheet formation, upfield shifts for β -turn residues.

Figure 27: NMR characterization of peptide 1. A) α H chemical shift deviation of peptide1 B) Section of two-dimensional ^1H - ^1H NOESY spectra showing NOE connectivity between amide protons Long-range NOEs are underlined and boldfaced. C) Superimposed twenty lowest energy structures of peptide1 and D) Selected structure of peptide1 showing side chain packing within β -sheets.

The 2D NOESY spectra for peptide 1 dissolved in DPC micelles yielded significant

number of NOEs and well-dispersed spectra (7.5-9.0ppm) characteristic of a stable folded structure (**Figure 27B**). In addition to deuteration, NMR experiments were also carried out at an acidic pH condition (pH5.0) and at higher temperatures (315K), to reduce the exchange between the backbone amide hydrogen atoms of the peptide and the solvent water and hence improve the quality of the spectra. The 2D- NOESY spectra of peptides revealed sequential, medium as well as a number of long range NOEs arising from backbone-backbone, backbone-side chain and side chain-side chain cross talk. Inter-strand NOEs typical to β -sheets (strand I/ strand II, strand III/ strand IV respectively). There were several long range inter-strand NOEs observed between backbone NH protons (strand I/II -I1H/V8H, W3H/V8H, strand III/IV - V10H/I17H, H12H/I17H), side chain protons (strand I/II -F2HA/F7HA, strand III/IV - M11HA/I16HA), side chain/backbone protons (strand I/II - F2HA/V8H, strand III/IV - M11HA/I17H) that were used in structure calculations. Limited long-range NOEs were also observed between strand II and strand III (V8/V12, F7/H13, V9/G11). NOEs between strand II/strand III (V8/V12, F7/H13, V9/G11). Additionally, strong NOEs observed between W3H/H6H (H12H/Y15H), P4HA/G5H (P12HA/G13H) and G5H/H6H (G14H/Y15H) confirms the presence of a type II' turn in both these hairpins. Structure calculations utilize the intensity of NOEs as a measure of the inter-proton distances upto 5Å. Overall, NMR derived structure of peptide 1 (**Figure 27C and D**) show the presence of four stranded β -sheets / two closely placed β -hairpin structures, each consisting of three residue anti-parallel β -strands sequestered by a type II' β -turn.

3.3.1.2 Heme binding characterization of peptide 1

Binding of four stranded peptides to heme was quantified in detail using UV-vis spectroscopy. The control experiment with only heme was characterized by a broad

Soret band at $\sim 395\text{nm}$. However, on addition of peptide 1 to a fixed concentration of heme, the Soret maxima shifts to sharper dominant peak at 412nm wavelength with appearance of weak intensity α - β bands at ~ 500 - 600nm (**Figure 28C**). Reduction of the complex to its ferrous form with sodium dithionite (at its stoichiometric ratio) resulted in a red shift of the Soret peak to 426nm with more pronounced α - β bands at 530 and 560nm respectively, confirming the presence of a low spin ferric state with bis-histidine coordination for all the peptide-heme complexes (**Figure 28D**).

Figure 28: Heme binding of peptide 1. A) Heme binding isotherm of peptide 1. B) Job's Plot of peptide 1 shows peptide: heme stoichiometry 2:1. Concentration of peptide and heme was $50 \mu\text{M}$ in 2 mM DPC , 50mM sodium phosphate buffer, $\text{pH } 7.2$. C) Spectral changes of heme ($2 \mu\text{M}$) on titrating excess of peptide 1. D) Reduced spectra of peptide1-heme complex upon reduction with sodium dithionite. A Soret maximum at 412 nm is observed upon binding to all the peptides and is further red shifted to 426 nm upon reduction by sodium dithionite with low intense peaks at 530 and 560 nm indicating bis-histidine coordination

The apparent dissociation constant (K_d) for binding to heme was determined as a function of peptide concentrations, using a constant heme concentration ($2 \mu\text{M}$) and

tracing the Soret maxima at 412nm wavelength. The binding isotherm, obtained was fitted to a mutually depleting model equation and dissociation constants for each peptide was calculated (**Figures 28A, 28C, Table 2**). A weak heme binding affinity was observed for the β -turn linker peptide 1 ($K_d = 5.84\mu\text{M}$). Furthermore, the peptide binds to heme in a 2 peptide:1 heme stoichiometry. The result suggested that the binding pocket formed by the β -turn was not sufficient to accommodate heme between strand II/III. A dimerization of the peptide is required to facilitate heme binding.

3.3.2 Design optimization of peptide 2

Since the design of peptide 1 yielded a low heme-binding affinity, we created $^{\text{DP9}}$ deleted variant of peptide 1, ie; peptide 2. Peptide 2 contains a small flexible Gly linker residue between strand II/strand III. This would result in lesser packing interactions between strand II/strand III and increase the flexibility of the binding pocket. As envisioned in the design, NMR chemical shifts and NOESY spectrum clearly suggest the presence of a well folded four-stranded β -sheet (**Figure 29A and B**). Long range NOEs characteristic to β -sheet structures were observed between strand I/strand II and strand III/IV (**Figure 29B**). However, there were no long-range NOEs observed for strand II/III. NMR derived structure shows the presence of a two β -hairpin structure with a hinge like segment at G9 residue (**Figure 29C and D**). Heme binding titrations experiments suggested the design modification result in a lower affinity (estimated $K_d = 8.9\mu\text{M}$) (**Figure 30A and C**). We also observed that peptide 2 binds heme in a 2 peptide:1 heme stoichiometry (**Figure 30B**).

Figure 29: NMR characterization of peptide 2. A) αH chemical shift deviation of peptide2 B) Section of two-dimensional ^1H - ^1H NOESY spectra showing NOE connectivity between amide protons Long-range NOEs are underlined and boldfaced. C) Superimposed twenty lowest energy structures of peptide2 and D) A selected structure of peptide2 showing side chain packing within β -sheets.

Figure 30: Heme binding of peptide 2. A) Heme binding isotherm of peptide 2. B) Job's Plot of peptide 2 shows peptide:heme stoichiometry 2:1. Concentration of peptide and heme was 50 μM in 2mM DPC, 50mM sodium phosphate buffer, pH 7.2. C) Spectral changes of heme (2 μM) on titrating excess of peptide 1. D) Reduced spectra of peptide2-heme complex upon reduction with sodium dithionite. A Soret maximum at 412 nm is observed upon binding to all the peptides and is further red shifted to 426 nm upon reduction by sodium dithionite with low intense peaks at 530 and 560 nm indicating bis-histidine coordination

3.3.2.1 Design of four-stranded β -sheet peptides with ω -amino acids

Acetyl-I¹-F-W-^DP-G-**H**-F-V- β -alanine-V-M-**H**-^DP-G-Y-I-I¹⁷-OCH₃ peptide3
 Acetyl-I¹-F-W-^DP-G-**H**-F-V- δ -aminovalericacid-V-M-**H**-^DP-G-Y-I-I¹⁷-OCH₃ peptide4
 Acetyl-I¹-F-W-^DP-G-**H**-F-V- ϵ -aminocaproicacid-V-M-**H**-^DP-G-Y-I-I¹⁷-OCH₃ peptide5
 Acetyl-I¹-F-W-^DP-G-**H**-F-V- ζ -aminoheptanoicacid-V-M-**H**-^DP-G-Y-I-I¹⁷-OCH₃ peptide6
 Acetyl-I¹-F-W-^DP-G-**H**-F-V- η -aminooctanoicacid-V-M-**H**-^DP-G-Y-I-I¹⁷-OCH₃ peptide7

Figure 31: Primary structure and sequence of peptides 3-7 with residues involved in heme ligation (red). Peptide 1 consists of ^DP-G segment between β -strand 2 and 3. X denotes the linker residue between β -strand 2 and 3: β -Ala, δ -Ava, ϵ -Aca ζ -Aha and η -Aoa for peptides 3, 4, 5, 6 and 7 respectively.

Peptides 3-7 were designed by utilizing ω -amino acids between the two β -hairpins to facilitate a flexible binding pocket for heme (**Figure 31**). Furthermore, the alkyl chain-length of ω -amino acids ($H_2N-(CH_2)_n-COOH$; $n=1$:Gly, $n=2$ β -Ala, $n=4$ δ -Ava, $n=5$ ϵ -Aca, $n=6$ ζ -Aha, $n=7$ η -Aoa) was varied in peptides 2, 3, 4, 5, 6 and 7 respectively in-order to assess its effect on heme binding and catalysis.

3.3.2.2 Design of six-stranded di-heme binding β -sheet peptides

Figure 32: Primary structure and sequence of peptide 8 and its variants (peptide 9 and peptide 10) with histidine residues involved in heme ligation (red).

The di-heme binding β -sheet peptides were designed to adopt a six-stranded β -sheet structure, four histidines were placed at position 6 (strand II), 12 (strand III), 15 (strand IV) and 24 (strand V) to bind to two heme cofactors (**Figure 32**). δ -Ava residue was placed between strands II / III and strands IV/ V to provide a binding pocket to accommodate the two heme molecules. Two variants of this peptide: peptide 9 and peptide 10 were generated where histidine pairs were substituted to alanine to delineate the histidines involved in ligation and decipher the mechanism of heme ligation in the wild-type. In line with the previous design strategies, all amino acids in the third β -sheet were predominantly hydrophobic except for the four histidines. Aromatic residues (W3, Y11 and F25) are placed just below the turn to localize near the water-membrane interface. Each beta hairpin consists of ^DP-G / ^DP-^DA segments to facilitate an I'/II' β -turn nucleation.

3.3.2.3 Structures of four stranded β -sheet peptides with ω -amino acids

Figure 33 : α H chemical shift deviation of peptides 3-7. Positive deviation in α H chemical shift can be seen for residues I1, F2, W3, H6, F7, V8, M12, H13, Y16, I17, and I18 in peptides 3-7 indicating four stranded β -sheet conformation. Residues D₄-G₆ showed negative deviations for β -turn conformations.

NMR characterization of peptides 3-7 was performed in excess DPC micelles (1:250) to ensure a stable insertion of the peptides. H_{α} chemical shift signifies the presence of four β -strand regions (indicated by the positive chemical shifts) (**Figure 33**). NOESY spectra revealed no long-range NOEs between strand II/strand III, while a number of cross-strand NOEs were observed between residues in strand I/strand II, and strand III/IV respectively. Peptides 3-7 show the expected β -sheet topology with an extended conformation for the linker residue and limited packing interactions. The β -sheets in all peptides show packing interactions between residues F2/F7 and M12/I17 on the non-hydrogen bonded side of the β -sheet.

Each four-stranded peptide shows a similarity in orientation of its side chains within

the β -sheet structure. Each β -hairpin has type II' β -turn centred at $^{\text{D}}\text{P-G}$. The backbone dihedral angles (ϕ , ψ) of residues I1-W3/V10-H12 and residues H6-V8/Y15-I17 are consistent with antiparallel β -sheet conformations (**Table 1**). The side chains of residues W3, $^{\text{D}}\text{P4}$, and H6 are placed at the one face of the 1st β -hairpin. The phenyl rings of residues F2 and F7 are involved in cross-strand packing, displaying an edge-face orientation, on the other face of the hairpin. In the second β -hairpin, side chains of residues H12, $^{\text{D}}\text{P13}$ and Y15 are present on one face of the β -hairpin while M12 and I16 side chains are in proximity on the other face of the hairpin (**Figures 34 and 35**).

Figure 34: NMR based characterization of peptide 3 and peptide 7. A section of two-dimensional ^1H - ^1H NOESY spectra showing NOE connectivity between amide protons for A) peptide 3, and D) peptide 7. Long range NOEs are underlined and boldfaced. Super-imposed twenty lowest energy structures of B) peptide3, E) peptide 7 showing four stranded β -structures. A selected structure of C) peptide 3 and F) peptide 7 showing side chain packing within β -sheets.

Figure 35: NMR structures of peptide 4-6. Super-imposed twenty low energy structures and one selected structure of each peptide showing side chain packing within β -sheets. A) peptide 4, B) peptide 5 and C) peptide 6

Peptide 7 structure was refined by using MTSL as a PRE-agent to gain additional information between strands II/III. MTSL affects residues in the distance range of 20Å by enhancing their relaxation rates and causing line broadening. To facilitate MTSL labelling, A Cys6 variant of peptide 7 was used and the intensity change of 2-D TOCSY spectra was used as a measure for calculating additional constraints for

structure calculations.

Figure 36: Topology of peptide 7 in DPC micelles. A) Intensity changes monitored for peptide 7 on addition of 16-DSA. Model structure of heme-peptide 7 complex. The side chains of the two axial ligands coordinating heme are shown (purple). Residues localized at the micelle/water interface (cyan) and residues localized in the hydrophobic core of micelles (green) are highlighted.

The topology of peptide-7 in DPC micelles was analysed using 16-DSA (16-doxyl stearic acid) as a PRE-probe (**Figure 36**). 16-DSA perturbs resonances of residues present in the hydrophobic interior of the micelles, while residues localized to the water/micellar interface would be less affected. By comparing 2D-TOCSY spectra in the presence and absence of 16-DSA, intensity changes of H α resonances were analysed. We observed that residues I1, F2, V8, η -aminooctanoic acid, V10, M11, Y15, I16 and I17 were significantly perturbed by inclusion of 16-DSA, whereas residues W3, DP4, G5, H6, H12, DP13 and G14 were less affected. The presence of heme coordinating residues H6 and H12 in the interface would help in its heme coordinating function.

3.3.2.4 Structure of six stranded di-heme β -sheet peptide

Structure of six stranded β -sheet peptide 8 was characterized in detail by NMR spectroscopy. Resonance assignments of different spin systems were identified by

TOCSY followed by sequential specific assignments by overlaying NOESY spectra. The NOESY spectra revealed a large number of NOEs (sequential, medium and long range) suited for structure determination.

Figure 37: NMR based characterization of peptide 8. A) Section of two-dimensional ^1H - ^1H NOESY spectra showing NOE connectivity between amide protons for peptide 8. αH chemical shift deviation of peptide 8. Positive deviations in αH chemical shift can be seen for residues I1, F2, W3, H6, F7, A10, Y11, H12, H15, V19, A20, T24 and F25 indicating six stranded β -sheet architecture. C) Superimposed ten lowest energy structures of peptide-8. D) A selected structure of peptide-8 showing side chain packing between F2/F7, Y11/M16 and V19/F26 within β -sheets.

The $\text{H}\alpha$ chemical shift of peptide-8 depicted stretches of downfield shift values characteristic to β -sheet amino residues followed by upfield shifts for turn residues & residues placed adjacent to the linker. Some anomalies were observed like upfield shifts in M16 and H22 (**Figure 37B**). The former could be attributed to a ring current shifted $\text{H}\alpha$ due a proximal aromatic Y11 residue. Overall the chemical shift index, hints the presence of three beta-hairpins.

NOE analysis revealed many cross-strand NOEs between strand I/strand II, strand III/strand IV and strand V/ strand VI typical to β -sheets (**Figure 37A**). Long range NOEs were detected across NH backbone namely I1H/V8H, W3H/V8H,

A10H/V17H, H12H/V17H, V19H/G26H, H21H/G26H. Additionally, long-range side-chain contacts were also observed such as F2HA/F7HA, M16HA/Y11HE, M16HA/Y11HA, M16HA/Y11QB, F25HB/A20HB. Side chain-backbone cross peaks revealed long range NOEs: F2HA/V8H and Y11HA/V17H respectively. Three-dimensional structure was determined using a total of 278 NOEs (**Table 1**). The peptide 8 presents an extended conformation with three individual β -hairpin structures: each consisting of a two antiparallel β -sheet sequestered by a Π' - β turn. The orientation of side chains in each hairpin is like the four stranded peptides (**Figure 37C & D**). In the first β -hairpin, side chains of W3, ^{D} P4, H6 are located at one face of the hairpin, while F2 and F7 are situated at the opposite face of the β -hairpin and involved in edge-face stacking. In the second β -hairpin, side chains of H12, ^{D} P13, H15 are located at one face of the hairpin and Y11 and M16 side-chains are stacking on the opposite face of the hairpin. In the third β -hairpin, side chains H21, ^{D} P22, T24 are located on one face of the hairpin and A22, F25 are in proximity on the opposite face of the hairpin. While the individual β -hairpin units and their side-chain orientations could be determined to a large extent due to the presence of several long-range NOEs. The orientation of the different hairpin units could not be resolved further due to the absence of long range NOEs between strand II/strand III and strand IV/strand V.

Table 1: Summary of structural statistics of the designed peptides

	1	2	3	4	5	6	7	8
Distance constraints:								
Sequential [$ i-j = 1$]	46	38	49	61	65	62	58	84
Medium range [$1 < i-j < 4$]	12	6	12	13	19	10	16	26
Long range [$ i-j \geq 4$]	14	19	20	28	21	25	32	38
PRE-driven distance restraints							18	
Total	156	131	159	200	183	185	206	278
Dihedral-angle constraints	22	22	22	22	22	22	22	36
Deviation from mean structure (Å)								
All backbone atoms	0.30	0.37	0.20	0.45	0.35	0.44	0.14	1.32
All heavy atoms	0.57	0.65	0.42	0.84	0.81	0.93	0.50	1.49
Ramachandran plot for the mean structure (%residues)								
Most favoured region	92.3	75.0	92.3	92.3	92.3	92.3	92.3	72.6
Additionally allowed region	7.7	25.0	7.7	7.7	7.7	7.7	7.7	27.3
Generously allowed region	0	0	0	0	0	0	0	0
Disallowed region	0	0	0	0	0	0	0	0

3.3.3 Heme binding and stoichiometric analysis

3.3.3.1 Heme binding analysis for four-stranded peptides

Figure 38: Heme binding characterization of peptides with ω -aminoacids. A) Heme binding isotherms of peptides 3-7. B) Spectral changes of heme (2 μM) on titrating peptide 7. C) Reduced spectra of peptide 7-heme complex upon reduction with sodium dithionite. A Soret maximum at 412 nm is observed upon binding to all the peptides and is further red shifted to 426 nm upon reduction by sodium dithionite with low intense peaks at 530 and 560 nm indicating bis-histidine coordination. Job's Plot of D) peptide 3 E) peptide 5 F) peptide 7 shows peptide:heme stoichiometry 1:1. Concentration of peptide and heme was 50 μM in 2mM DPC, 50mM sodium phosphate buffer, pH 7.2.

Binding of four stranded peptides to heme was quantified in detail using UV-visible spectroscopy. The control experiment with only heme was characterized by a broad Soret band at ~ 395 nm. However, on addition of peptides to a fixed concentration of heme, the Soret maxima shifts to sharper dominant peak at 412 nm wavelength with appearance of weak intensity α - β bands at ~ 500 -600 nm (**Figure 38**). Reduction of the complex to its ferrous form with sodium dithionite (at its stoichiometric ratio) resulted in a red shifted Soret peak observed at 426 nm with more pronounced α - β bands at 530 and 560 nm respectively, confirming the presence of a low spin ferric

state with bis-histidine coordination for all the peptide-heme complexes.

The apparent dissociation constant (K_d) for each peptide binding to heme was determined as a function of peptide concentrations, using a constant heme concentration (2 μ M) and tracing the Soret maxima at 412 nm wavelength. The binding isotherm, obtained was fitted to a mutually depleting model equation and dissociation constants for each peptide was calculated (**Figure 38A, Table 2**).

An interesting trend was observed for the four stranded peptides with ω -amino acids (peptides 3-7) where the binding affinity increased with an increase in the length of the alkyl chain linker (**Table 2**). Peptide 3 showcased a reduced affinity to heme (K_d =2.7 μ M) while peptide 7 has the highest binding among all peptides (K_d =400 nM). These results establish that the binding pocket length is critical for mediating cofactor association. Furthermore, all these peptides bind to heme in a 1 peptide:1 heme stoichiometry as confirmed by Job's plot.

3.3.3.2 Heme binding analysis for six-stranded peptides

Heme binding with six-stranded peptides was assessed by UV-vis spectroscopy. The absorption spectra for all the peptides in the presence of the cofactor displayed a sharp peak shift at 412nm along with smaller α - β peaks at 500-600 nm indicative of bis-histidine ligation of heme (**Figure 39**). The Soret peak shift for the Ala mutants was observed only after addition of high peptide concentrations, hinting a weaker affinity while compared to wild-type peptide 8. The low resolved α - β peaks were further intensified on addition of sodium dithionite and a characteristic shifted Soret band at 428 nm further confirmed a bis-histidine binding between peptide and heme (**Figure 30**).

Figure 39: Heme binding of six stranded peptides. A) Heme binding isotherm of peptides 8, 9 and 10. B) Spectral changes of heme (1 μM) on titrating peptide 8. C) Reduced spectrum of peptide 8-heme complex upon reduction with sodium dithionite. A Soret maximum at 412 nm is observed upon binding to all the peptides and is further red shifted to 426 nm upon reduction by sodium dithionite with low intense peaks at 530 and 560 nm indicating bis-histidine coordination. D) Job's Plot of peptide 8 shows peptide:heme stoichiometry 1:2. Concentration of peptide and heme was 50 μM in 2mM DPC, 50mM sodium phosphate buffer, pH 7.2.

To compare the binding affinity of heme with the six-stranded peptides, apparent dissociation constants for each peptide was calculated by monitoring complex formation at 412 nm with increments in peptide concentrations. While peptide 8 displayed a high affinity for heme ($K_d=0.45\mu\text{M}$), the mutants showed lower affinity in the following order, peptide 10>peptide 9>peptide 8. These results conclude a cooperative heme binding, where the binding to the first site further facilitates binding to additional heme with the second site.

Table 2: Summary of heme binding affinity of the designed peptides

Peptide	K_d
	μM
1	5.8 ± 0.7
2	8.9 ± 0.9
3	2.7 ± 0.5
4	2.0 ± 0.2
5	1.6 ± 0.2
6	0.8 ± 0.2
7	0.4 ± 0.2
8	0.5 ± 0.1
9	28.1 ± 3.2
10	19.5 ± 2.1

3.3.4 Secondary structure analysis by CD spectroscopy

NMR spectroscopy could not be utilized to determine the structure of peptide-heme complexes due to the presence of a paramagnetic Fe (III) state in these complexes. A paramagnetic compound drastically alters spin coupling (through-bond and space) and hence affects the overall chemical shifts as well as induces a line broadening effect in the spectra due to a substantial increase in relaxation rates (**Figure 40**). Therefore, Far UV-CD was utilized for determining the secondary structure of the peptide-heme complexes and near-UV CD could determine the environment of heme in the presence of these peptides.

Figure 40 :¹H-NMR spectrum of peptide-cobalt porphyrin complex (1:1 stoichiometry) recorded at 298K, 50mM DPC, pH 6.5. The spectrum shows broad and attenuated amide signals with major contributions from the porphyrin ring especially at 6-6.5 and 10-11ppm range.

CD spectroscopy is a widely used technique for structure assessment of proteins and peptides by probing the distinct $n-\pi^*$ and $\pi-\pi^*$ transitions of backbone amides in the far-UV region in the presence of circularly polarized light. The far-UV spectra of peptides 1-7 in the presence/absence of heme shows a single negative minimum at around 212nm and a positive maximum at 225nm (**Figure 41**). The former is indicative of a β -sheet conformation while the latter affirms an exciton coupling between proximal aromatic residues (owing to $\pi-\pi^*$ transitions of aromatic rings). Additionally, peptide 1 showed a positive maximum at 205nm, characteristic of their three β -turn structure. CD spectra of peptide 7 in the presence of heme showed a shift in the negative minima (from 212nm in apo form to 216nm) with a more intense positive band at 230nm. These CD spectral changes indicate more β -sheet character and enhanced packing interactions for the holo peptide.

Figure 41: Far-UV CD spectra of peptide alone (black) and heme-bound (red) and near-

UV CD spectra of heme (onset) at the Soret region in peptide-heme complexes A) peptide1, B) peptide 2, C) peptide 3, D) peptide 4 E) peptide 6 and F) peptide 7. Far-UV CD spectra show β -sheet characteristic with exciton coupling due to presence of interacting aromatic amino acids. A dichroic signal in the Soret region was observed for all peptide-heme complexes indicating that heme is experiencing a chiral environment.

3.3.5 Catalysis

Peroxidase kinetics of cofactor-bound peptides was assessed by spectrophotometrically monitoring the oxidation of chromophoric substrates like ABTS. ABTS undergoes a one e^- oxidation at 700nm in the presence of peroxidases[117]. Peroxidases (E) in general utilize peroxide and oxidize certain organic compounds like TMB, ABTS etc. (AH) to colored radicals (A^*). The catalysis occurs via irreversible ping-pong kinetics, which initially involves the reaction of hydrogen peroxide (substrate) with heme (in bound form), resulting in a two-electron highly unstable reaction intermediate termed compound I (consisting a high-spin iron-IV) and water. Two subsequent single-electron transfers from substrates transform compound I to compound II, and reduce compound II further to its resting state [118].

The kinetics parameters of these synthetic peroxidases were studied in detail for ABTS as the absorbance of TMB^* formation coincides with the absorbance in Soret region (350-500nm).

Table 2 : Enzymatic parameters of peptide-heme complexes by monitoring the absorbance of ABTS* at 700nm.

Peptide	$V_{\max}$ ($\mu\text{M/s}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m ($10^4 \text{M}^{-1} \text{s}^{-1}$)
1	26.8	1.87	53.60	2.84
2	27.25	1.86	54.49	2.91
3	26.88	1.92	53.75	2.79
4	25.48	2.09	50.96	2.44
5	21.09	2.68	42.18	1.57
6	22.35	3.34	44.70	1.34
7	20.05	3.50	40.09	1.14
8	53.61	2.46	107.22	4.35
9	28.84	2.62	57.68	2.21
10	18.81	2.89	37.62	1.30
11	27.97	2.48	55.93	2.25

We observed that all peptides could function as peroxidases displaying greater activities than free heme. These peptides display moderate peroxidase activity having turnover values similar to myoglobin ($k_{\text{cat}}/K_m \cdot 10^5 \text{M}^{-1} \text{s}^{-1}$). However, their activities are much lesser than the well-studied synthetic water-soluble peroxidases. Among the four-stranded peptides, the most effective catalyst is peptide 2 while peptide 7 has the least activity. In-order to catalyze the reaction, one binding site of the bis-his heme conjugated peptides needs to be detached and to facilitate incorporation of the substrate to heme. Therefore, a peptide that associates strongly to heme, would result in a lower catalysis (due to lower affinity to the substrate i.e., hydrogen peroxide).

To probe the heme bound six-stranded peptides enzymatic potential, the peroxidase activity was determined by monitoring the oxidation of co-substrate ABTS. Peroxidase kinetics for peptide 8 and its variants was studied to reflect its activity as a consequence of heme binding. Heme and peptide alone could not function as a

peroxidase independently. Heme co-ordinates to peptide via two histidines, and in order to function as a peroxidase, the distal binding site needs to be de-attached to facilitate H_2O_2 binding to heme. Therefore, a tighter heme binding would create a competition for substrate incorporation and thus result in lower catalysis.

The initial rate velocities for these peptides showed that all peptide-heme complexes could function as peroxidases displaying greater activities than free peptide and heme (**Figure 42**). While k_{cat}/K_m values for peptide-8 variants are inversely related to their heme binding affinities such that peptide 11 is the most active, while peptide 10 is the least but this trend is not followed by peptide 8. Kinetic analyses (K_m , V_{max} values) reveal that catalysis of peptide 8 is two-fold greater than its mutants as well as four stranded peptides (**Table 3**). This could be due to the presence of two heme binding sites to accommodate the substrate H_2O_2 , which justifies its role as an efficient peroxidase (**Figure 42**).

Figure 42: Peroxidase activity of designed β -sheet peptides. A) Time course of ABTS oxidation at 700 nm for four stranded peptide-heme complexes at 4 mM H₂O₂ concentration. B) Absorbance of ABTS oxidation at 700 nm versus time for six stranded peptide-heme complexes at 4 mM H₂O₂ concentration. C) Steady-state kinetics of ABTS oxidation as a function of hydrogen peroxide concentration for peptides 2, 4, 5 and 7. D) Steady-state kinetics of ABTS oxidation as a function of hydrogen peroxide concentration for peptides 8, 9 and 10. The data was fitted to a Michaelis-Menten equation to derive catalytic parameters.

3.3.6 Electron Transfer

Figure 43 : Electron transfer activity of four-stranded and six stranded β -sheet peptides with Cytc A) Absorption spectra of heme alone (black), peptide 7-heme: oxidized (blue), reduced (orange), Cyt C: oxidized (dark cyan), reduced (red) and 2:1 equivalent mixture of reduced peptide 7-heme and oxidized Cyt C (purple). B) Absorption spectrum of a 2:1 mixture of peptide 8-heme and oxidized Cyt C (purple)/ C) Absorbance monitored at 550nm versus time for reduced peptide7-heme/Cyt C oxidized (blue) and reduce peptide-8-heme/Cyt C oxidized (red).

Important cellular functions such as photosynthesis and respiration are mediated by long-range electron transfer (ET) of heme proteins. Cytochrome C (Cyt C) is an essential part of this electron transfer chain (ETC), where it accepts electrons from the bc1 complex and transfers it to Cytochrome C Oxidase [119]. Cyt C is a highly-conserved peripheral membrane protein localized in the mitochondrial inner membrane. We have screened electron transfer activities of peptide 7 and peptide 8 with Cyt C using UV-vis spectroscopy. In **Figure 43**, the reduced peptide 7-heme

complex showed α - β bands at 530 and 560 nm respectively. The reduced Cyt C spectrum on the other hand showed α - β bands at 520 and 550 nm, respectively. The oxidized Cyt C and peptide-7-heme complex spectrum have broad and unresolved α - β bands at 500-600 nm. Interestingly, the absorption spectrum of reduced peptide-7-heme complex and oxidized Cyt C revealed an absorption spectrum corresponding to the reduced spectrum of Cyt C (with α - β bands at 520 nm and 550 nm). Furthermore, we observed similar conclusive evidence from the absorption spectra of reduced peptide 8-heme complex and oxidized Cyt C. These results suggest that the peptide-heme complexes are participating in an electron transfer with a protein present in the electron transport chain.

To monitor the rate of this electron transfer, the reaction between the peptide-heme complexes and Cyt C was monitored at 550 nm (β band of CytC reduced spectrum) using a stopped-flow apparatus. The electron transfer rate constants were determined to be 2.28s^{-1} and 1.6s^{-1} for peptide 7 and peptide 8, respectively (**Figure 43C**). Interestingly, the electron transfer rate between Cyt C and Cyt C peroxidase was estimated as 0.23 s^{-1} [120]. These results showcase an efficient electron transfer from Cyt C to the peptides in a membrane mimetic environment.

3.4 Summary

The work describes the successful design of functional multi stranded β -sheet peptides in a membrane like environment.

A contribution of a single amino acid residue in the heme binding pocket was investigated in the four-stranded membrane model peptides. The amino acid present amidst the two β -hairpins is varied from α -amino acid (Gly), ω -amino acids (β -Ala, γ -Aba, δ -Ava, ε -Aca, ζ -Aha, and η -Aoa) and a two-residue β -turn (^{D} Pro-Gly) to explore the effect of heme binding within these membrane soluble peptides as a consequence of following modifications. The NMR structures of the four-stranded peptides except peptide 1 show well-folded two- β hairpin conformations with the heme axial ligands (histidines) situated far apart. Peptide 1, on the other hand, shows two closely placed beta hairpins with proximal histidines owing to the presence of a tight β -turn residue between these two hairpin units. However, we observed a tighter binding with 1 peptide: 1 heme stoichiometry for the peptides with flexible ω -amino acid residues compared to the lower affinity and 2 peptide: 1 heme stoichiometry observed for peptides 1 and 2. The restricted β -turn in peptide 1, and small α -amino acid linker in peptide 2 could not facilitate heme inclusion within the same peptide molecule and therefore, preferentially ligates to 2 histidine residues present in 2 different peptide molecules. Additionally, all peptides bind to heme in micromolar affinity, with peptide 7 with η -Aoa linker residue demonstrating the highest affinity (0.407 μ M). Notably, the tight binding of the cofactor in II-17 η -Aoa results in a reduced peroxidase activity.

Although peptides with multiple heme binding sites have been investigated previously in membrane-soluble α -helical systems, none of the studies so far have

been carried out on β -sheets. Thus, a six-stranded β -sheet peptide was designed with two putative heme-binding sites within it. The designed peptide showcases high affinity heme binding ($0.446 \mu\text{M}$) and catalysis ($k_{\text{cat}}/k_{\text{m}}=4.5 \times 10^7$). Atomic resolution structure of peptide 8 in detergent micelles shows that the ligands (histidines) participating in heme ligation are situated far apart. However, stoichiometric analyses reveal that two hemes bind to one peptide molecule. This can only be possible if heme induces a structural rearrangement within the peptide scaffold. The modification in global fold of peptide 8 results in proximal histidine ligands that could easily accommodate two heme molecules within it. Membrane model peptides can function as peroxidases and facilitate electron-transfer with Cytochrome C across the membrane.

CHAPTER 4

4. Water soluble β -sheet peptides

4.1 Chapter overview

De novo design strategies for constructing functional peptides have been quite successful in aqueous solutions, with a majority of its efforts focussed on helical scaffolds. Design of β -sheets in aqueous solutions is complicated due to its tendency for self-assembly by forming insoluble aggregates. Recently, design of β -sheet peptides upto four antiparallel β -strands has been reported. However, achieving biological functions in these peptides like catalytic activity and ligand binding remains a challenge. Utilizing the knowledge gained from the membrane soluble peptide models, it was crucial to test the structure/functions of the hydrophobic β -sheet peptides in a different environment. Furthermore, the low affinity heme binding potential of the membrane soluble peptides could be attributed to the presence of its binding pocket buried in the micelle. We incorporated certain modifications to peptide 1 and peptide 7 to render it water-soluble to carry out heme binding experiments and structural characterization.

4.2 Introduction

4.2.1 Design of proteins in water-soluble environment

Nature has inspired scientists for several years towards designing novel proteins that can fold and encapsulate their diverse functions. Designing proteins ‘from scratch’ or *De novo* design tests our knowledge of the factors that govern structure, stability, and activity of a protein. The two-common secondary structural motifs found in nature are α -helices and β -sheets. While major contributions have been made towards design of α -helices, relatively limited attempts have been made towards designing β -sheets. The former can be attributed to the easier task of designing α -helical structures, using well-established folding principles. Further, α -helices are inherently more stable as they can independently form intra-helical hydrogen bonding within its backbone segment itself. The same rule does not hold true for a β -strand.

Independently folded β -strands could be unstable, as they need to form inter-strand hydrogen bonding. Thus, β -strands can interact with each other in a number of ways to satisfy their hydrogen bonding which often resulting in aggregation. Furthermore, their reputation for aggregation and formation of amyloid fibrils has been implicated in several neurodegenerative disorders such as AD and prion diseases [121]. However, β -sheet proteins in nature are observed to be soluble with very few cases of aggregation. These proteins follow a “negative design” feature to avoid such unwanted interactions [122]. The β -barrels exemplify this protection strategy by forming a continuous H-bonding along its barrel, to prevent any free amide group free from interacting with other β -strands. On the other hand, the β -propeller and β -sandwich proteins utilize β -bulges, prolines and charged residues on the inward side of the strands to prevent aggregation [103, 123, 124].

4.2.2 Successful designs of β -sheet structure

Designing β -sheet peptides that are monomeric and soluble in aqueous solutions is a formidable task with very few success efforts. Most of these attempts are limited to designing one or two β -hairpin units (a fundamental unit of a β -sheet consisting of two antiparallel β -strands connected by a β -turn). The constructions of a β -hairpin that are nucleated by tight two-/ three-residue β -turns have been extensively studied. Type I'/II' β -turns were created by sequences like Asn-Gly, ^{D} Pro-Gly, ^{D} Pro- ^{D} Ala segments. Balaram and Gellman's group have been successful in creating hybrid β -turns utilizing non-natural amino acids like Aib- ^{D} Ala, ^{D} Pro- β,γ,δ amino acid segments [125, 126].

4.2.2.1 Betanova: Betanova is a three-stranded 20-residue long antiparallel β -sheet designed by Serrano and group [127]. The peptide was designed by an iterative approach and was shown to be soluble and monomeric upto 2.6mM concentration. The three-dimensional structure of Betanova was determined by NMR spectroscopy and showcased the expected β -sheet topology with a right-handed twist and two-residue β -turns. This peptide also shows a cooperative thermal and chemical-induced unfolding (**Figure 44**).

4.2.2.2 Trp-zip (Tryptophan zipper): Trp-Zip peptides are 12-16 residue long β -hairpin peptides designed by Starovasnik and group [113]. The monomeric, well-folded, high thermal stability features of these peptides are due to the presence of a Trp-Trp cross-strand packing within its β -sheet (**Figure 45**).

Figure 44: NMR derived structure of Betanova. Picture adapted from [127].

Figure 45: NMR derived structure of Tryptophan zipper 1 and 2. Picture adapted from [113].

4.2.2.3 Betadoublet: Betadoublet peptide is β -sandwich peptide mimic designed by Richardson and group [128]. It contains identical units of four-stranded antiparallel β -sheets facing each other. A disulphide linkage between two inner strands was used for the formation of this symmetric dimer. The design was based on an iterative approach by optimising the design of betabellins (a previously designed 32-residue β -sheet peptide) to improve its solubility and β -sheet character. The peptide forms a monomeric compact β -structure with high stability to thermal denaturation (**Figure 46**).

Figure 46: Model structure of Betanova. Picture adapted from [128].

4.2.2.4 Beta-4: Beta-4 is 26-amino acid four-stranded antiparallel β -sheet peptide with three ^{D} Pro regions (two ^{D} Pro-Gly, one ^{D} Pro-Ala segment) to nucleate β -turns (**Figure 47**). NMR and CD spectroscopy confirm a well-folded β -structure in methanol and 50% methanol-water solutions without aggregation effects at high peptide concentrations [129].

Figure 47: NMR derived superposed structures of Beta-4 in methanol-water solutions. Picture adapted from [129].

4.2.2.5 B5Dimer: The 70-amino acid long peptide is homo-dimer of four-stranded antiparallel β -sheet peptide (B5Cys). The peptide adopts an extended eight stranded β -sheet structure in methanol confirmed by NMR spectroscopy (**Figure 48**). However, the peptide shows a four-stranded β -sheet structure in water with stretches of unstructured segments at the N- and C- termini [130].

Figure 48: NMR structure of B5dimer in methanol. Picture adapted from Venkatraman *et al*, 2002.

4.2.2.6 TH peptide: Toxin Hand (TH) is a common β -sheet motif found in snake and scorpion venoms. A 29-amino acid long peptide was designed to emulate the TH motif after several rounds of design modifications. Even though the design shares

only 38% homology with the natural sequence, a well-folded structure resembling toxin hand was observed for the peptides (**Figure 49**) [131].

Figure 49: NMR structures the optimized TH peptides. Picture adapted from [131].

Till date, most β -sheet designs have focussed solely on structural aspects with very few reports on function. A few noteworthy functional β -sheet models designed are explained below:

4.2.2.7 Rubredoxin mimic: The sophisticated function of iron-binding in rubredoxin was mimicked by designing an all- β sheet sandwich protein computationally designed by Degradó and Group[132]. A pair of three-stranded β -sheet motifs contain the site (2-Cys each) to bind iron in a tetrahedral tetra-thiolate coordination. The design also utilized Trp-Zip linker to connect the three stranded β -sheet pairs to form an overall compact cross β -structure with enhanced stability (**Figure 50**). This peptide showcased activity for 16 redox cycles while compared to the 2-3 cycles' activity showcased by existing models.

Figure 50: Design of rubredoxin mimic by computational methods. Figure adapted from [132]

4.2.2.8 ssDNA binding: It is known that single-stranded DNA binds to the β -sheet surface of the protein (oligonucleotide-binding fold-OB fold). A β -hairpin dimer (WKWK)₂ was designed by Waters and group for this purpose [133]. The peptide binds to two ssDNA molecules with an affinity comparable to native DNA receptors. Since aromatic stacking and electrostatic interactions play a key role in ATP interaction, lysines and tryptophans were placed within the same side of the β -hairpin to facilitate ATP interaction (**Figure 51**). It was also observed that the binding of ssDNA was enhanced in the β -structured peptide compared to an unstructured peptide with a similar sequence.

Figure 51: Design of a β -hairpin that binds to ssDNA. Picture adapted from [133].

4.2.2.9 ATP binding: A 12-residue long β -hairpin with tryptophan and lysine residues are placed diagonally on one surface of the β -sheet to provide aromatic

stacking and electrostatic interactions respectively for ATP intercalation [134]. The template sequence used was a β -hairpin model with a Asn-Gly I'- β -turn designed by Gellman's research group (**Figure 52**). The peptide binds ATP in aqueous buffers with an association constant of 5800 M^{-1} and a ΔG of -5.1 kcal/mol at 1:1 stoichiometry. NMR titrations in the presence of 10 times excess ATP revealed upfield shifts for all alpha and beta protons of the peptide, with more pronounced changes observed for Lys and Trp residues indicative of its interaction with the adenine ring of ATP.

Figure 52: Design of an ATP binding β -hairpin. Picture adapted from [134].

4.2.2.10 Esterase activity: The self-aggregating tendency of peptides to form amyloid fibrillar structures was utilized by Korendovych and group to create a biocatalyst for ester hydrolysis [135]. A heptapeptide with an amyloid forming tendency was used as the starting design template (**Figure 53**). The design was modified by replacing lysine residues to create a carbonic anhydrase (CA) like active site where the Zn metal coordinates to three His residues. The optimized peptide showcased a much lower catalytic efficiency (Estimated $k_{\text{cat}}/K_{\text{m}}$: $62 \text{ M}^{-1}\text{s}^{-1}$) than CA ($\sim 2500 \text{ M}^{-1}\text{s}^{-1}$). However, the presence of several active sites on this peptide makes its activity almost comparable to CA.

Figure 53: Design of catalytic amyloids that bind zinc and hydrolyze esters. Figure adapted from [135]

4.3 Results and Discussion

4.3.1 Peptide design strategy

The four-stranded β -sheet peptides- peptide 1, peptide 4 and peptide 7 were utilized for constructing the water-soluble peptides (peptide 11, peptide 12, and peptide 13). Here, we included lysine residues (K1 and K19) at the N- and C- termini to improve the solubility of these peptides in aqueous buffer conditions (**Figure 54**). Histidine 13 of peptide 13 was replaced to Serine in peptide 14 to provide a different coordination environment for heme and investigate its effect on heme binding affinity and catalysis. Another variant of peptide 13 was prepared by replacing η -aminooctanoicacid residue with three Gly residues as a linker between strand 2 and strand 3. The Gly-Gly-Gly linker contributes the same number of atoms to the peptide backbone and therefore can be considered the homomorphous replacement of η -aminooctanoicacid residue. Furthermore, a shorter version of peptide 13 was created by deletion of Val residues (V8 and V10) in strand II and strand III respectively in peptide 16 to modify the hydrophobic heme-binding pocket.

$K^1-I-F-W-{}^D P-G-H-F-V-{}^D P-G-V-M-H-{}^D P-G-Y-I-I-K^{20}$	peptide11
$K^1-I-F-W-{}^D P-G-H-F-V-\delta\text{-aminovalericacid}-V-M-H-{}^D P-G-Y-I-I-K^{19}$	peptide12
$K^1-I-F-W-{}^D P-G-H-F-V-\eta\text{-aminooctanoicacid}-V-M-H-{}^D P-G-Y-I-I-K^{19}$	peptide13
$K^1-I-F-W-{}^D P-G-H-F-V-\eta\text{-aminooctanoicAcid}-V-M-S-{}^D P-G-Y-I-I-K^{19}$	peptide14
$K^1-I-F-W-{}^D P-G-H-F-V-G-G-G-V-M-H-{}^D P-G-Y-I-I-K^{21}$	peptide15
$K^1-F-W-{}^D P-G-H-F-\eta\text{-aminooctanoicAcid}-M-H-{}^D P-G-Y-I-I-K^{16}$	peptide16

Figure 54: Primary structure of designed peptides with residues coordinating heme (His, Ser) highlighted in red.

4.3.2 Structural Characterisation by NMR spectroscopy

β -sheet peptides in aqueous solutions tend to aggregate or form stable oligomers by forming intermolecular hydrogen bonds/stacking interactions. Therefore, design of β -sheet peptides that exist as monomers in solutions is very challenging. We carried out PFG-NMR spectroscopy experiments for peptides 11, 12 and 13 to determine their diffusion coefficients and estimate their oligomeric state (**Table 4**). We observed that all the peptides were monomeric in water solutions. Furthermore, concentration-dependent (upto 0.5 mM peptide concentration) chemical shift and sharp NMR spectra validated the non-aggregating nature of these peptides. Chemical shifts ($^1\text{H}\alpha$ and $^{13}\text{C}\alpha$) indicated the presence of β -sheet structures (four stretches of positive $\text{H}\alpha$ and complimentary negative $\text{C}\alpha$ values) for peptides 8-12 (**Figure 55**). Chemical shift and NOE analysis of peptide 11 in addition to the four β -strands also showed the presence of three β -turns ($^{\text{DP}}$ - G segment).

Table 4: Diffusion coefficients for peptides 11, 12 and 13 and estimated molecular weight using DSS as a reference.

Peptide	$D(10^{-10} \text{ m}^2/\text{s})$	Experimental MW (Da)	Molecular Weight (Da)
DSS	2.73		218.31
11	1.24	2382.30	2323.86
12	1.13	2051.70	2268.85
13	1.23	2323.80	2310.69

Figure 55: Secondary $^{13}\text{C}\alpha$ and αH chemical shift of A) peptide11 B) peptide12 and C) peptide13. Negative deviation in $^{13}\text{C}\alpha$ chemical shift can be observed for all residues except turn-residue $^{\text{DP}}$ and end-residue K20. Positive deviation in αH chemical shift can be seen for residues I2, F3, W4, H7, F8, V12, M13, H14, Y17, I18, and I19 in peptide1 indicating four stranded β -sheet conformations. Residues $^{\text{DP}}$ -G showed negative deviations for β -turn conformations. Similar secondary chemical shifts were observed for peptide2 and peptide3, except for residues I18 and I17, respectively.

Long range NOEs were observed between NH/NH, side-chain/NH and side-chain/side chain protons. These NOEs were further translated to distance constraints and utilized for structure determination. The NMR derived structure of peptide 15 revealed expected β -sheet topology with packing interactions observed between residues F8/M13, H7/H13, and V12/I19 (**Figure 56 and 57**). The N- and C-terminus

lysine residues were observed to be in random coil conformation.

Figure 56: Schematic representation of A) peptide 11 and C) peptide 12/13 showing NOE connectivity observed within β -sheets. X represents the linker residue: δ -aminovalericacid or η -aminooctanoicacid in peptide 12 and 13 respectively. Sections of two-dimensional ^1H - ^1H NOESY spectra of B) peptide 11 D) peptide 12 and E) peptide 13 showing long range NOE connectivity diagnostic of β -sheet structure.

The NMR structures of peptide-12 and peptide-13 on the other hand, have a four-stranded β -sheet with a looplike conformation for the δ -aminovalericacid / η -aminooctanoicacid residue with limited packing interactions between strand II and strand III (**Figure 57**). Packing interactions were observed between aromatic residues F3/F8 and hydrophobic residues M13/I18 on the opposite face of the β -sheet. The structure of peptide 11 has a different β -sheet structure compared to peptide 10, and it

additionally also revealed packing interactions between Gly linker residues and residues in strand II and strand III.

Figure 57: NMR-derived structures of designed peptides. Superimposed twenty low energy structures of A) peptide 11, C) peptide12, E) peptide 13 and F) peptide 15. Ribbon representation of B) peptide11 D) peptide 12 F) peptide 13 and H) peptide 15 showing side-chain packing interactions in β -sheets.

Table 5: Summary of structural statistics of the designed peptides

	11	12	13	15
Distance constraints:				
Sequential [$ i-j = 1$]	63	64	58	61
Medium range [$1 < i-j < 4$]	9	12	12	15
Long range [$ i-j \geq 4$]	23	23	25	11
Total	186	158	147	171
Dihedral-angle constraints	26	26	26	26
Constraints violations				
Minimum NOE violation (Å)	0.21	0.21	0.27	0.19
Maximum NOE violation (Å)	0.47	0.5	0.44	0.44
Minimum dihedral angle violation (°)	5.1	6.33	4.59	10.24
Maximum dihedral angle violation (°)	25.14	9.28	13.06	19.66
Deviation from mean structure (Å)				
All backbone atoms	0.05	1.47	0.4	0.44
All heavy atoms	0.5	2.1	1.03	1
Ramachandran Plot				
Most favoured region	75	73.3	73.3	57.1
Additionally allowed region	25	26.7	26.7	42.9
Generously allowed region	0	0	0	0
Disallowed region	0	0	0	0

4.3.3 Heme binding and stoichiometry analysis

Figure 58: Heme binding characterisation of designed peptides. Heme binding isotherms of A) peptide 11, E) peptide 12, I) peptide 13, M) peptide 14, Q) peptide 15 and U) peptide 16. Spectral changes of heme (10 μM) on titrating peptides (0-5 equivalents): B) peptide 11, F) peptide 12, J) peptide 13, N) peptide 14, R) peptide 15 and V) peptide 16. Job's plot indicating peptide:heme stoichiometry as 1:1: C) peptide 11, G) peptide 12, K) peptide 13, O)

peptide 14, S) peptide 15 and W) peptide 16. Reduced and oxidized spectra of heme/peptide complexes: D) peptide 11, H) peptide 12, L) peptide 13, P) peptide 14, T) peptide 15, and X) peptide 16.

The heme binding affinity of the peptides was estimated by monitoring the spectral changes of heme using UV-vis spectroscopy (**Figure 58**). Free heme shows a broad Soret band at ~395 nm, and upon addition of increasing concentrations of peptides (11,12,13,14,15,16) a red shift of the Soret band (λ_{max} - 414 nm) is observed with additional unresolved α - β bands at 500-600 nm.

Table 6: Dissociation constants of the designed peptide-heme complexes

Peptide	K_d
11	$0.8 \pm 0.4 \mu\text{M}$
12	$62.8 \pm 9.8 \text{ pM}$
13	$39 \pm 22 \text{ pM}$
14	$0.5 \pm 0.4 \mu\text{M}$
15	$1.9 \pm 0.7 \mu\text{M}$
16	$9.2 \pm 3 \text{ nM}$

The Soret band further red shifted to 428 nm with more resolved α - β bands on addition of sodium dithionite (reducing agent). These spectral changes of the peptide-heme complexes are indicative of a bis-histidine ligation where the hexacoordinated Fe(III) of heme has a low spin state. For the peptide 14-heme complex, we observed a Soret maximum at 406 nm with a weak charge-transfer band at 630 nm (**Table 7, Figure 58**). This charge-transfer band indicates a high spin state of Fe(III) in heme. All the peptides bind to heme with a 1:1 stoichiometry as indicated by Job's Plot. Heme titration curves were used to determine the dissociation constants of the peptides. As expected, the replacement of β -turn of peptide 11 yielded high affinity

binding in peptides 12 and 13 with K_d values estimated at picomolar range (**Table 6**). Further, the binding isotherms of these peptides also demonstrate a clear breakpoint in the curve at 1:1 stoichiometry. Peptide 14 on the other hand, interacted with much lower affinity (K_d -0.5 μ M) due to the presence of a different coordination for heme binding. The spectral characteristics of this peptide hint the presence a high spin Fe with His/H₂O coordination. The Gly variant peptide (Peptide 15) shows the lowest affinity to heme (K_d -1.8 μ M).

Table 7: UV-Vis characteristics of heme-proteins and peptides

Protein / peptide	Oxidized (nm)				Reduced (nm)		
	Ligation	Soret	α - β	Charge transfer band	Soret	α	β
11	His/His	414	530-560		428	532	560
12	His/His	414	530-560		428	532	560
13	His/His	414	530-560		428	532	560
14	His/Ser	406		~630	392-420	528	558
15	His/His	412	530-560		428	532	560
16	His/His	414	530-560		428	532	560
Protein 86 [33]	His/His	412	530		426	530	560
ME1 [136]	His/His	414	550		427	530	560
Cyt b5 microsome [137]	His/His	410	530		423	527	555
Cyt b5 mitochondria [137]	His/His	412	530		423	525	556
Mb (H ₂ O) [138]	His/H ₂ O	409.5	505	635			
Hb(H ₂ O) [138]	His/H ₂ O	405	500	631			

The linker residues cause major changes in the apo peptide structure which results in its reduced affinity to heme. Peptide 16 has a comparatively lower affinity than

peptide 13 ($K_d \sim 9.2$ nM). This shorter variant peptide has a smaller hydrophobic binding pocket which may affect its heme attachment.

4.3.4 Resonance Raman spectroscopy

Figure 59: Resonance Raman spectra of peptides in complex with heme. A) peptide11, B) peptide13, C) peptide14 and D) peptide14+imidazole. resonance Raman spectra of peptides 11, 13 and peptide14+imidazole signify low spin ferric complexes as indicated by bands at 1586 cm^{-1} and 1372 cm^{-1} respectively. Whereas, resonance Raman spectrum for peptide14-heme reveals the presence of high spin ferric state as indicated by bands at 1566 cm^{-1} and 1370 cm^{-1} respectively.

Table 8: Resonance Raman frequencies of heme-proteins and peptides

Peptide /Protein	Ligation	ν_4	ν_2	ν_{10}
Cyt C	Met/His	1370.56	1586.06	1637.27
11	His/His	1372.14	1584.20	1638.25
13	His/His	1372.14	1586.28	1638.25
14+ Imidazole	His/His	1372.14	1586.28	1638.25
Cyt b5 [139]	His/His	1372	1585	1639
14	His/ Ser	1370.55	1566.86	1615.93
Catalase [140]	Tyr	1373	1568	1625
Myoglobin [141]	His/ H ₂ O	1373	1565	1608

The spin and oxidation state of iron in heme when coordinated to peptides 11, 13 and 14 was further validated using resonance Raman spectroscopy (**Figure 59, Table 8**). The characteristic Raman bands of the peptide-heme complexes were compared with native heme-protein standards: cytochrome C, cytochrome b5 and myoglobin. Peptide 11 shows a Raman band $\nu_4 \sim 1372$ nm signifies a ferric heme centre while Raman bands $\nu_2 \sim 1584$ nm and $\nu_{10} \sim 1638$ nm reveal the presence of its hexacoordinated low spin state. Resonance raman spectrum for peptide 13-heme also indicated similar bands verifying the presence of low spin ferric heme iron. Peptide 14-heme complex on the other hand, displayed Raman bands $\nu_4 \sim 1370$ nm, $\nu_{10} \sim 1615.93$ nm and $\nu_2 \sim 1566.86$ nm indicating the presence of a high spin ferric heme iron. In the presence of imidazole (1 mM), we observed that the peptide 14-heme complex is converted to a low spin state. The absorption spectra of the peptide-heme complex also revealed similar trends with a red shift of the Soret band and appearance of weak

α - β bands at 500-600 nm. These results collectively suggest that a low spin hexacoordinated heme Fe with His/imidazole coordination is achieved on titration of imidazole (**Figure 61**, **Table 6**, **Table 7**).

4.3.5 Heme binding kinetics

Heme binding kinetics of peptides 11, 12, 13 and 15 were examined using stopped flow kinetics by monitoring the increase in absorbance of Soret band over time (**Figure 60**). Peptide 12, 13 show rapid rates of heme binding compared to peptides 11 and 15 (**Table 9**). The results are in complete correlation with their binding affinities and the geometry of the heme binding pocket. The flexible linker residues: η -aminooctanoicacid and δ -aminovalericacid provide an extended heme binding surface for its rapid insertion. The heme binding pockets for peptide 11 and 15 on the other hand are restricted and limited, thus resulting in slower binding rate constants.

Figure 60: Kinetics of heme binding to peptides 11, 12, 13 and 15.

Table 9: Heme binding rate constant for the peptides.

Peptide	k (M/s)
13	577.6
12	271.9
11	105.2
15	71.03

4.3.6 Peroxidase Activity

Peroxidase activity of the designed peptides was screened by monitoring the oxidation of substrate ABTS at 700nm. These peptides were compared for enzymatic activity with microperoxidase 11 (MP11), a shorted peptide derived from cytochrome C known for its peroxidase activity potential. The enzymatic parameters were derived by Michaelis-Menten plots. We observed that MP11 demonstrated a remarkably high activity when compared to the peptides (**Figure 62**). This can be attributed to its hexacoordinated heme bound state to histidine and a weaker ligand like water and a covalent attachment of its heme moiety. As expected, peptide 14, containing its heme molecule coordinated to Ser residue, displays the highest peroxidase activity and peptide 13 is found to be the least active as reflected by the enzymatic parameters (k_{cat}/K_m , K_m and V_{max}) (**Table 10**). The lower peroxidase activity of the peptide 13 may be due to its tight association with heme which prevents its binding to H_2O_2 substrate. This is also reflected in the UV-vis analysis of heme-peptide14 complex and heme-peptide13 complex with imidazole or H_2O_2 whereas heme-peptide13 participates in limited interactions with these substrates (**Figure 61**).

Table 10: Enzymatic parameters of designed peptides

Peptide	$V_{\max}$ ($\mu\text{M/s}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m ($10^3 \text{M}^{-1} \text{s}^{-1}$)
13	19.99	54.71	39.98	0.73
12	25.36	70.37	50.72	0.72
11	41.02	79.22	82.04	1.03
15	37.869	87.425	75.74	0.87
4	73.97	107.5	295.88	2.75
Heme	26.19	105.33	52.36	0.497
MP-11	15995.3	50.97	31990.6	627.635

Figure 61 : UV-vis studies of peptide 11 and 13 on titrating substrates: H_2O_2 and imidazole. Spectral changes of A) heme-peptide 13 complex (10 μM) B) heme-peptide 11 on titrating imidazole (0-1 mM). Spectral changes of heme-peptide complex (10 μM) for C) peptide 3 and D) peptide 4 on titrating hydrogen peroxide (0-100 μM).

Figure 62 : Michaelis-Menton plots for the designed peptides (A-E) and F) Microperoxidase-11. The absorbance at 700 nm for each hydrogen peroxide titration was converted to reaction rates and plotted versus the respective hydrogen peroxide concentration. Microperoxidase-11 has much higher activity than the designed heme-peptides. Out of the heme-peptides, peptide 14 is the most active and peptide 13 is the least active.

4.3.7 Apomyoglobin heme transfer experiments

Competitive heme binding experiments with a naturally occurring heme-protein (apo-myoglobin) were used to compare the heme-binding capabilities of peptides 11 and

13. The apo form of myoglobin, a native heme-protein that has the highest recorded heme binding potential (K_d estimated to around $\sim 10^{-14}$ M) was used for this study [142]. Peptide 13 showcased a reduced heme-transfer rate while compared to peptide 11 (Figure 63A, B & C). Further, the UV-vis spectrum of apo-peptide 13 and apo-myoglobin mixed in a 1:1 stoichiometry with heme indicated some spectral characteristics similar to peptide 11-heme complex. The UV-vis spectrum of apo-peptide 11, reveals a Soret band at 409 nm like myoglobin (Figure 63D & E).

Figure 63: Myoglobin/peptide heme competition experiments. A) Absorption spectra monitoring the dissociation of heme- peptide 13 complex and the formation of myoglobin-heme complex with a Soret band maximum at 409 nm. The continuous increase of absorption at 409 nm for peptide13 over the time indicated slow dissociation of heme from the complex. By contrast, absorption increase was found to be rapid and saturated for B) peptide11, due to faster dissociation of the cofactor. C) Time course of heme transfer from the peptides to apo-myoglobin monitored at 409 nm ($\lambda_{\max}$ of myoglobin-heme complex). D) Absorption spectra of heme alone (black), peptide3-heme complex (red), apo-myoglobin-heme complex (blue) and apo-myoglobin + apo-peptide with heme (green). E) Absorption spectra of heme alone (black), peptide1-heme complex (blue), apo-myoglobin-heme complex (red) and apo-myoglobin+apo-peptide1-heme (green). The absorption spectra of heme revealed efficient

binding of peptide 3 to heme in the presence of apo-myoglobin.

4.3.8 CD spectroscopy

The three-dimensional structures of heme-bound peptides were limited due to spectral line broadening and low solubility at high concentrations. Therefore, CD spectroscopy was used to probe the secondary structural characteristics of heme-bound peptides. CD studies indicate the presence of similar β -sheet structures in apo and holo forms of the peptides. An induced negative CD band ~ 420 nm was observed for all heme-bound peptides indicating that heme is experiencing a chiral environment (**Figure 64**).

Figure 64: Far-UV CD spectra of peptide alone (black) and heme-bound (red) and near-UV CD spectra (inset graph) of heme at the Soret region in peptide-heme complexes. (A) peptide11, (B) peptide12, (C) peptide13. Concentration of peptide and heme was 100 μ M in 5 mM Tris buffer, pH 9.0 at room temperature. CD studies were performed using a 0.1 mm path-length cuvette with a 2 nm bandwidth and a step size of 0.5 nm at 0.5 seconds per data

set. For the near UV CD experiments (onset graph), 11 μ M peptide +10 μ M heme was prepared in 5 mM Tris buffer, pH 9.0 using 1 cm path-length cuvette. A dichroic signal was observed for all peptide-heme complexes indicating that heme is experiencing a chiral environment.

4.3.9 Stability studies

Figure 65: Thermal denaturation curves of A) peptide 11 and B) peptide 13 (apo and heme-bound) by monitoring the changes in far-UV CD spectra.

Peptide 11 and 13 were chosen for a comparative study based on their stabilities using thermal and chemical denaturation. Thermal denaturation was probed by using CD spectroscopy and monitoring the positive band ~ 225 nm (and 230 nm for holo-peptide 10) indicating exciton coupling between aromatic residues. Apo-peptide 13 displayed a structural unfolding with an estimated $T_m \sim 30$ $^{\circ}$ C, whereas the heme-bound peptide demonstrated a significantly higher thermal stability with an estimated $T_m \sim 60$ $^{\circ}$ C. Peptide 13 showcased lower thermal stability in both apo and holo (estimated T_m for holo peptide ~ 45 $^{\circ}$ C) forms (**Figure 65**).

Heme-bound peptides 11 and 13 were also subjected to urea induced denaturation studies. Urea denaturation was probed using UV-vis spectroscopy and monitoring the Soret band (λ_{max}) at different concentrations of urea (0-9 M). The λ_{max} value of peptide 13-heme complex remained unchanged till 5 M urea, suggesting a highly stable complex. The

peptide 11-heme complex showcased a dissociation at low concentrations of urea itself (>4 M urea) (Figure 66).

Figure 66: Spectral changes of A) peptide 13 and C) peptide 11 upon titration with urea. Urea unfolding curves monitoring the change in wavelength of Soret band (λ_{max}) of heme B) peptide 13 and D) peptide 11.

4.4 Summary

Designing monomeric β -sheet mini-proteins in a water-soluble environment is a formidable task. Designing of multi-stranded functional β -sheets has not been attempted so far due to complexities in design and a tendency for self-aggregation.

Design of a monomeric high-affinity β -sheet metallo-peptide was achieved by modifying the properties of peptide 1 and 7 in the previous chapter and optimizing the binding pocket of heme by modification of a single / few residues (GGG linker, δ -Ava residue, η -aminooctanoic acid, H12S variant, $\Delta V9\Delta V11$ variant peptide). The peptides assume four-stranded β -sheet structures in solutions with no aggregation effects. Further, the incorporation of ω -amino acid linker residues (δ -Ava, η -Aoa) has resulted in a high-affinity heme-peptide complex with binding affinities in picomolar range.

The optimized peptides efficiently compete for heme with a naturally occurring protein (apo-myoglobin) scaffold. Furthermore, heme-bound peptide complexes display high stability towards thermal and chemical induced denaturation.

CHAPTER 5

5. Heme Interacts With A β In Membranes

5.1 Overview of the project

Alzheimer's disease (AD) is the most common age-related disorders; however, its pathogenesis is not clearly understood at present. The disease is characterized by the generation and accumulation of amyloid-beta plaques in the brains of patients afflicted with the disease. The 40-residue (major component) and 42-residue peptides are the major products formed from a proteolytic cleavage of a larger membrane protein APP (Amyloid precursor protein). These peptides aggregate to form neurotoxic fibrils which are hypothesized to play a vital role in Alzheimer's pathogenesis. It is important to note that these plaques are found associated with high concentrations of metal cofactors (Cu^{2+} , Zn^{2+} , heme), and A β has been recently shown to bind to these metals in sub-micromolar affinity. Such an association results in the formation of Reactive Oxygen Species (ROS) further resulting in oxidative stress and damaging neurons in the brains of AD patients.

Recently, it has been proven that the A β 40 peptide binds to heme with high affinity (estimated $K_d \sim 140$ nM) in solution. Interestingly, the A β -heme complex can efficiently inhibit A β aggregation, but it also contributes to oxidative damage to cells by forming a peroxidase. A proper understanding of this complex in a membrane-like environment is necessary as the C-terminus stretch of A β 40 (29-40 residues) is believed to be a part of the transmembrane segment of APP. Furthermore, cholesterol and lipids were also found to be present near the amyloid plaques in AD.

For the A β -heme studies in membrane, DPC micelles were used to facilitate a membrane mimicking environment. Furthermore, a shorter N-terminus A β (1-16) peptide was utilized for comparative studies with the full-length A β 40 peptide. The shorter non-aggregating peptide contains all the heme coordinating residues: three histidines (H6, H13 and H14) and Tyrosine (Y10). Heme is hypothesized to bind to two regions within the peptide in aqueous solutions: a high affinity region with a bis-His coordination for the 1-16 portion and a low affinity hydrophobic stacking interaction with the aromatic residues in 17-40 region. However, some other reports have suggested entirely different coordination modes (single-His, Tyr10). Therefore, we performed heme binding experiments with peptides dissolved in aqueous solutions. Overall, these experiments could contribute to a better understanding of the role of A β -heme complex in AD.

5.2 Introduction

5.2.1 A brief overview on AD

Dementia is an age-related neurodegenerative syndrome that affects nearly 50 million people worldwide (according to the WHO Alzheimer's Report). Alzheimer's disease (AD) is the most common form of dementia accounting for almost 60-70% of the total cases. The primary clinical symptoms of AD are a progressive decline of cognitive functions such as memory-loss, comprehension skills, an ability to perform day to day roles and behavioural changes such as mood swings [143]. Aging is an important risk factor of AD [144], one in eight people over 65 years' age acquire the disease, and nearly 45% of the total population above 85 years' age have AD. Its prevalence among elderly people makes AD an important health issue with the rise of aged population over the years. AD is classified into two types: the first type is majorly influenced by genetic factors and is known as early onset familial Alzheimer's disease (FAD). This type accounts for almost 25% of the total AD cases, and it can be attributed to the mutations in genes encoding AD proteins: amyloid precursor protein (APP) and presenilins 1 and 2. Mutations in these genes result in an increased generation of an APP-derived peptide called A β 42, a key component of senile plaques found in AD. The second and most common type is sporadic Alzheimer's disease (sAD) or late-onset AD, and it is influenced by genetic as well as non-genetic contributions. Mutations at the gene encoding Apolipoprotein E (APOE) is believed to be responsible for sAD.

The brain of patients afflicted with AD are characterized by the presence of neurofibrillary tangles (NFTs), senile plaques (SP), inflammation and neuron loss. While NFTs are derived from a hyper-phosphorylated tau protein, the SPs majorly consist of proteinaceous deposits of amyloid- β peptides. The mechanism of AD is still an

ongoing debate and the cause and initiating event of the disease is not well understood. However, a lot of emphasis in AD research has been given to amyloid cascade hypothesis [145]. Amyloid cascade hypothesis was proposed in 1991 by Hardy and Allsop (**Figure 67**), which suggests that the initiating event in AD pathology is the deposition and aggregation of A β , specifically A β 42 peptide. This would be followed by other pathological events, including NFT formation, cell-damage, and dementia [146].

Figure 67: The sequence of pathogenic events leading to AD proposed by the amyloid cascade hypothesis. Figure adapted from [147].

5.2.2 Amyloid precursor protein

The amyloid precursor protein (APP) is a 110-135 kDa highly conserved membrane protein. The protein can be processed alternatively by either α/γ or β/γ secretases to result in a non-amyloidogenic or amyloidogenic pathway respectively [148].

Figure 68: A β processing and cleavage products. The non-amyloidogenic APP processing pathway (right/blue) involves cleavages by α - and γ -secretases resulting in the generation of a long-secreted form of APP (sAPP α) and C-terminal fragments (CTF 83, p3 and AICD50). The amyloidogenic APP processing pathway (left/red) involves cleavages by β - and γ -secretases resulting in the generation of a long-secreted form of APP (sAPP β), C-terminal fragments (CTF 99 and CTF 89) and A β s. A β fragments oligomerize and fibrillize leading to AD pathology (left and upper panel). Figure adapted from [149].

In the non-amyloidogenic pathway, APP is cleaved by α -secretase at the middle of the A β domain, to generate a large N-terminus fragment and a shorter C-terminus

fragment (C-83 residues) (**Figure 68**). The membrane-bound C83 fragment is further processed by γ -secretase to generate a 3kDa peptide fragment (which subsequently undergoes rapid degeneration) and the APP intracellular domain (AICD). In the amyloidogenic pathway, APP is cleaved by β -secretase to generate a different product: an N-terminus fragment and a longer C-terminus fragment (C-99 residues). The membrane-bound C-terminus is subsequently processed by γ -secretase to release A β peptides and AICD. A β peptides range from 37-43 amino acids length. A β 40 is the major fragment produced in the absence of any gene mutations in APP and γ -secretase (a multi-protein complex which consists of presenilin 1 and 2). The biological function of APP is not well understood despite a few studies having found its significance in the neurite outgrowth and synaptic function. However, it has been recently proven that an APP knockout mice model did not show a significant change in any of these functions, but instead showed decreased locomotion activity [150].

5.2.3 A β peptide

Amyloid β peptide (A β), is a 37-43 amino acid peptide, formed as a by-product due selective processing of amyloid precursor protein (APP) by β - and γ -secretases. A β monomers have a high tendency to aggregate or self-associate to form soluble oligomers (dimer to octamer) that eventually lead to fibril formation (protofibril, fibrillar stage) (**Figure 69**).

Amyloid plaques primarily constitute the fibrillar aggregates of A β peptides (specifically A β 42 peptide). The A β 42 peptide is considered to be the more lethal peptide form, due to its rapid aggregation tendency [151, 152], and elevated presence in diseased patients [153, 154]. However, recent studies have argued that the intermediate oligomeric forms of A β peptides (dimer, trimer till hexamer stage) are

considered to be the prime reason of A β 's toxicity, resulting in neuronal death. These soluble oligomeric species aid in the initiation and progression of AD by : 1) formation of membrane pore/channel [155-157] leading to an altered ion homeostasis [158, 159] in the membrane, 2) Binding to membrane receptors and as a result affecting its primary function [160]; 3) Forming a metal-bound species and resulting in oxidative stress [161, 162], 4) inflammatory response activation by interacting with the membrane [163], and 5) alteration of DNA structure [164].

Amyloid aggregation has always been associated as a causative effect of the disease, however, a recent study has demonstrated the protective role of A β in innate immunity by functioning as a potent antimicrobial peptide (AMP) [165].

Figure 69: Nucleation-dependent polymerization model of amyloid aggregation. Figure adapted from [166].

5.2.4 Structural evolution during aggregation

X-ray crystallography, solution-state and recently solid-state NMR have been utilized to derive structural information of A β peptides in water and membrane-like environment. Monomeric A β is intrinsically disordered in aqueous solutions and it transitions between various intermediate oligomeric states (α -helical, β -sheet conformation) to form a stable cross β -fibrillary structure. An important structural feature of A β peptides is its polymorphic nature which has resulted in innumerable PDB depositions of the same peptide at various salt and pH conditions.

Table 11: PDB Structures of A β peptides using different experimental techniques

Year	PDB ID	Author	Region	Experimental Technique
1994	1AMB	Talafoos et al.[167]	A β (1-28)	Solution NMR
1996	1QCM	Kohn et al.[168]	A β (25-35)	Solution NMR
1998	1BA4	Coles et al.[169]	A β (1-40) in SDS	Solution NMR
1998	1BA6	Watson et al.[170]	A β (1-40) M35ox	Solution NMR
2000	1HZ3	Zhang et al.[171]	A β (10-35)	Solution NMR
2000	1BJB	Poulsen et al.[172]	A β (1-28) in SDS	Solution NMR
2002	1IYT	Crescenzi et al.[173]	A β (1-42)	Solution NMR
2004	1QWP	D'Ursi et al.[174]	A β (25-35)	Solution NMR
2004	1QXC	D'Ursi et al.[174]	A β (25-35) in HFIP	Solution NMR
2004	1QYT	D'Ursi et al.[174]	A β (25-35) in SDS	Solution NMR
2004	1NMJ	Huang et al.[175]	Rat A β (1-28)-Zn	Solution NMR
2005	2BEG	Luhers et al.[176]	A β (1-42) fibril	Solution NMR
2006	1ZE7	Zirah et al.[177]	A β (1-16)	Solution NMR
2006	1ZE9	Zirah et al.[177]	A β (1-16)-Zn	Solution NMR
2006	2BP4	Zirah et al.[177]	A β (1-16)-80%TFE	Solution NMR
2006	1Z0Q	Tomaselli et al.[178]	A β (1-42)	Solution NMR

2007	2ONV	Sawaya et al.[179]	A β (37-42)	X-ray Diffraction
2007	2ONA	Sawaya et al.[179]	A β (35-40)	X-ray Diffraction
2011	3PZZ	Colletier et al.[180]	A β (29-34)	X-ray Diffraction
2011	3Q2X	Colletier et al.[180]	A β (27-32)	X-ray Diffraction
2011	2Y29	Colletier et al.[180]	A β (16-21)	X-ray Diffraction
2011	2Y3J	Colletier et al.[180]	A β (30-35)	X-ray Diffraction
2011	2Y3K	Colletier et al.[180]	A β (35-42)	X-ray Diffraction
2011	2LFM	Vivekanandan et al.[181]	A β (1-40)	Solution NMR
2012	2LNQ	Qiang et al.[182]	A β (25-35) D23N	Solid-State NMR
2012	2LI9	Istrate et al.[183]	Rat A β - Zn complex	Solution NMR
2013	2M4J	Lu et al.[184]	A β (1-40)	Solid-State NMR
2013	2M5N	Fitzpatrick et al.[185]	A β (1-42) protofilament	Solid-State NMR
2014	4Q8D	Pham et al.[186]	A β (15-23)	X-ray Diffraction
2014	2MJ1	Fonar et al.[187]	A β (17-34)	Solution NMR
2015	2MXU	Xiao et al.[188]	A β (1-42)	Solid-State NMR
2015	5AEF	Schmidt et al.[189]	A β (1-42) fibril	Solution NMR
2015	2MVX	Schutz et al.[190]	A β (1-42) fibril	Solid-State NMR
2015	2MPZ	Sgourakis et al.[191]	A β (1-42) fibril D23N	Solid-State NMR
2016	5KK3	Colvin et al.[192]	A β (1-42)	Solid-State NMR
2016	5HOX	Kreutzer et al.[193]	A β (17-36)	X-ray Diffraction
2016	2NAO	Walti et al.[194]	A β (1-42) fibril	Solution NMR
2016	5HOW	Kreutzer et al.[193]	A β (17-36)	X-ray Diffraction

Most PDB structures have captured the initial unstructured and end-point fibrillar conformations of A β peptides (**Table 11**). The α -helix to β -sheet conformational conversion is a key event that needs to be characterised to understand the formation of neurotoxic oligomers. However, obtaining structural information of these transient species is difficult as they are unstable in solutions.

Interestingly, it was observed that A β peptides adopt a predominant helical

conformation in membrane-like environment. NMR derived structure of A β 40 peptide in SDS detergent micelles (**Figure 70**) demarcated the presence of two helical regions (Q15-V24 and K28-V40), a short hinge like segment between the two helices (G25-N27) and an extended N-terminus portion (D1-G9) [169, 195]. NMR structures of A β peptide in apolar environments (HFIP and TFE) that mimic lipid membranes also show a predominant α -helix conformation [173, 174, 177].

Figure 70: NMR derived structure of A β 40 in SDS micelles. Figure adapted from [169].

A β peptides are known to interact with various membrane receptors (lipoproteins, transthyretins), form ion channels and affect metal ion homeostasis in the membrane. The peptides also influence membrane fluidity by interacting with membrane components such as cholesterol, gangliosides and phospholipids and accelerate its own aggregation. Recent studies have shown that statin, a cholesterol lowering drug can lower the risk of AD. However, the mechanism of the drug's inhibitory action is not well understood [196, 197]. Therefore, these events highlight the role of cell membranes in amyloidosis.

Furthermore, A β peptides are a part of the transmembrane region of APP and are generally released in the extracellular space after proteolytic cleavage. However, some of these cleaved peptides due to their hydrophobic characteristic attach to the membrane surface after cleavage and form fibrils. An understanding of the peptide's neurotoxic mechanism requires a detailed knowledge of the possible structural conformations it can adopt in a eukaryotic membrane-like environment.

5.2.5 Metal binding and its role in AD pathogenesis

The AD plaques were observed to contain an accumulation of redox metals like Cu (around 0.4 mM concentration), Fe (around 1 mM concentration) and Zn (around 0.2 mM) [198, 199]. A β peptides are known to coordinate with these metals in high affinity and affect their homeostasis in the brain [200]. The interaction of redox metals with amyloids has been hypothesized to result in the production of Reactive Oxygen Species (ROS) [201-205]. The formation of hydrogen peroxide and oxidative free radicals cause lipid peroxidation and disrupts protein/DNA structure. It has been found that sub-micromolar concentrations of Cu (II) are sufficient enough to generate a high amount of ROS [162, 206-210]. It has been observed that amyloid beta plaques consist of an oxidized M35 residue; this residue may be participating in the redox reduction reaction of Cu(II) (in the presence of oxygen and reducing agents like ascorbic acid) to generate ROS [211-215].

The N-terminus hydrophilic region of A β (1-16 residues) contains the metal binding site: three histidines (H6, H13 and H14) and a tyrosine (Y10) while C-terminus hydrophobic region is responsible for its aggregation and fibril-forming properties. It is important to note that most of the metal binding characterisation of A β peptides have been carried out with the non-amyloidogenic N-terminus (1-16) segment to

prevent aggregation effects for binding analysis (ITC, NMR experiments which generally require high concentrations of peptide).

Zinc and Copper ions have been found to promote the aggregation of A β peptides. Zinc was shown to facilitate amyloid formation in transgenic mice (rodent species generally do not exhibit an AD pathology). Zinc requires four-six ligands to satisfy its coordination. NMR studies of diamagnetic Zn (II) ion indicate the axial coordination of residues: H6, H13, H14 and E11. N-terminus residue D1 and water are the probable additional sites for formation of six-coordinate geometry [216-218]. Some studies have shown that the histidine residents participating in zinc binding are from different peptide units; facilitating its subsequent aggregation [219, 220]. Zinc is estimated to have a binding affinity of around 1-60 μ M range. A higher binding affinity was observed at long incubation times indicating that zinc preferentially binds to the oligomeric species of A β [221, 222].

Copper (II) is known to bind A β with high affinity (Estimated K_D – 0.01-1 nM range) [223-227]. The histidine residues (H6, H13 and H14) are known to coordinate Cu(II) [220, 224, 228]. It adopts different coordination modes at low and high pH respectively [208, 224, 229, 230]. At low pH ($\sim$ pH 7.0) a three nitrogen/ one oxygen coordination is observed where the oxygen is contributed by a carboxylate sidechain or carbonyl group, one nitrogen group from the N-terminus and the other nitrogens are contributed by two histidines [208, 227, 230, 231]. At high pH condition ($\sim$ pH 9.0), D1 amide nitrogen, A2 carbonyl oxygen and amide nitrogen, and one histidine nitrogen are the coordinating groups [230]. It is observed that Zn and Cu ions compete for the same binding site at pH 7.0 condition [232].

Heme, an iron containing metabolite was recently shown to bind A β peptides with

high affinity. Interestingly, heme can prevent aggregation of A β by forming a heme-A β complex [233]. However, the formation of this complex triggers a chain of cytopathological events in AD namely: 1) regulatory heme deficiency [234-238] 2) decreased activity in heme containing complex IV protein- a key protein complex in ETC responsible for ATP production and simultaneous reduction of mitochondrial oxygen to water preventing PROS formation [234, 239]. 3) Reduction of monomeric APP protein levels to almost 50% [235] 4) increased levels of heme forming protein, ferrochelatase [234] 5) increased levels of heme degrading protein, heme oxygenase [237, 238] 6) increased level of heme degradation products such as bilirubin [240] 7) formation of a peroxidase, resulting in ROS and oxidation of neurotransmitters: serotonin and dopamine [241, 242].

The putative binding site for heme is located at the 1-16 region of the peptide, where heme can coordinate in various possibilities: His/water coordination, His/His coordination or a catalase like Tyr coordination. UV-vis spectroscopy data of peptide-heme complex showed a red shift in the Soret band of heme and low intensity α - β peaks at 530 and 560 nm suggested the presence of low-spin complex with His/His coordination. However, another report suggested a high-spin complex with His13/water coordination by comparing spectroscopic data of selective His mutated peptides. The binding information has been solely derived from spectroscopic techniques (UV-vis and EPR studies). NMR binding titrations could provide a better indication of the heme coordinating residues.

Human A β 40 peptide possesses two heme binding sites: 1) a high affinity site at the hydrophilic (1-16) region (K_d estimated to be 140nM) 2) a low affinity site at the hydrophobic (17-40) region (K_d estimated to be around 210 nM). Rodent Abeta

peptide has a similar sequence when compared to human A β except for residues Arg5, Tyr10 and His13 and lacks AD pathology. Interestingly, the peptide was found to bind heme at lower affinity (K_d estimated to be 1 μ M) and show a weak peroxidase activity. The latter can be attributed to a lack of Arg5 residue to facilitate in heterolysis of the O-O bond.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Synthetic A β 16 and A β 40 was purchased from GL-Biochem (Shanghai,China). DPC was purchased from Avanti polar lipids. DPC-d38, D₂O and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were purchased from Cambridge Isotope Laboratories Inc. (Massachusetts, USA).1,1,1,3,3,3-Hexafluoro-2-propanol was purchased from Merck. Other chemicals such as Sodium dithionite, Hemin, ABTS, thioflavin T were purchased from Sigma-Aldrich.

5.3.1 Purification and expression of ¹⁵N and ¹⁵N ¹³C A β 40.

His ₆ -tag	Furin tag	D	P	AB40

Figure 71: Schematic representation of A β 40 gene

A synthetic, codon-optimized gene of A β 40 peptide (Sequence: D-A-E-F-R-H-D-S-G-Y-E-V-H-H-Q-K-L-V-F-F-A-E-D-V-G-S-N-K-G-A-I-I-G-L-M-V-G-G-V-V) was sub-cloned into the pET-14b vector (Novagen) containing furin pro-domain (along with 6x-His), as N-terminus sequence [110]. An Asp-Pro cleavage site was introduced between furin prodomain and A β peptide to separate the peptide from its fusion tag. The recombinant plasmid (containing ampicillin resistance) was transformed into Rosetta BL21(DE3) cells (containing chloramphenicol resistance). A single isolated colony was picked and cultured in LB (Luria bertani broth) overnight (around 14-16 hours) containing ampicillin (100 μ g/ml) and chloramphenicol (32 μ g/ml) as a starter culture. The starter culture was then transferred to a 4 litre TB broth (Terrific Broth) containing the antibiotics in 1:100 volume ratio. The large-scale culture was then placed in a 37°C shaker incubator at 180 rpm till OD was 0.6-0.8. For producing labelled peptide, the cells after reaching 0.6-0.8 OD were collected by centrifugation

at 5000 rpm for 20-30 mins at 30°C, and re-suspended in 1 litre isotope enriched minimal media (M9) containing ^{15}N ammonium chloride with/ without ^{13}C glucose. The cells were induced with 1 mM IPTG and kept at 37°C overnight. Cells were harvested by centrifugation at 5000 rpm for 30 mins, 4°C. Expression was verified using 15% SDS-PAGE. As the A β -furin fusion protein is localized to inclusion bodies, protein purification is carried out in denaturing conditions (8 M urea). The cells were re-suspended in 8 M urea, 50 mM phosphate buffer, 300 mM NaCl buffer pH 8.0 and incubated for 30 mins while shaking at room temperature. The cells were then lysed by sonication and followed by centrifugation at 18000 rpm for 30 mins at 4°C to eliminate cell debris. The supernatant containing the fusion protein was then loaded on the Nickel-NTA column (QIAGEN) and eluted using 8 M urea buffer containing 500mM imidazole. Eluents containing pure protein was verified by SDS/PAGE and kept for dialysis against water for around 1 day (to remove urea and salts). This resulted in the formation of a protein precipitate solution which was further lyophilized to obtain protein powder. The lyophilized protein was treated with 50% formic acid, purged with nitrogen gas, and kept in the dark at around 45-50°C for 24 hours to cleave the Asp-Pro bond. Formic acid was removed using rotary evaporation and the film obtained was re-dissolved in 8 M urea, 50 mM phosphate buffer, 300 mM NaCl buffer pH 8.0. In-order to separate the peptide from furin tag (containing 6x-His), Nickel-NTA purification was carried out where the flow-through and wash fractions contain A β peptide and the imidazole eluents contain furin and the uncleaved fusion protein. The fractions containing monomeric A β peptide were confirmed using a 16% Tris-Tricine SDS PAGE. These were pooled and dialysed against water (to remove urea and salt) for 1 day. The precipitate obtained was treated with 100 mM NaOH followed by dilution in 10% acetonitrile, 0.1% TFA. This was

further purified using reverse phase HPLC using an acetonitrile gradient, and the major peaks were confirmed by MALDI-TOF and NMR (1-D NMR for unlabeled peptide / ^{15}N - ^1H HSQC for labelled peptide).

5.3.2 Preparation of A β peptide

A β peptide powder was dissolved in HFIP and lyophilized. The film obtained after HFIP treatment was further dissolved in 100 mM NaOH and then diluted in MQ water on ice. The working concentration was prepared by dilutions with 2 mM DPC, 50 mM sodium phosphate buffer pH 7.5.

5.3.3 Thioflavin T fluorescence

A β stock concentration of $\sim 500\mu\text{M}$ was prepared in 2mM DPC, 50mM phosphate buffer pH 7.5 after prior treatment with HFIP and NaOH. A freshly prepared stock of 3mM thioflavinT (TfT) was prepared in water. This was further diluted to $200\mu\text{M}$ in 2mM DPC, 50mM phosphate buffer pH 7.2 and syringe filtered. The final concentration of A β used is $50\mu\text{M}$ (with / without heme prepared in the same buffer) and TfT used was 10mM.

The aggregation of A β was monitored by assaying the fluorescence of TfT, which increases upon binding to A β aggregates [excitation filter of 435 nm (bandwidth, 10 nm) and emission filter of 485 nm (bandwidth, 10 nm)] at 37°C.

5.3.4 UV-vis studies (K_d determination, sodium dithionite reduction and stoichiometric analysis)

The binding affinity of heme to A β 40 was characterized using a multi-well plate reader (Tecan Infinite M200 PRO) by titrating increasing concentrations of peptide (upto five equivalents) to a fixed concentration of heme prepared in 50mM sodium

phosphate buffer, 2 mM DPC, pH 7.2. The Soret band at 412 nm was monitored for complex formation for each aliquot after a three-hour long incubation. Wavelength scans from 350-600 nm was further recorded to monitor the shift in the absorption maxima of heme on binding to the peptides. Binding isotherms were obtained by plotting the absorbance at 412 nm versus peptide concentration. The binding data was analysed using Hill's equation for cooperative binding.

Peptide-heme stoichiometry was determined using method of continuous variation or Job's plot. 50 μ M peptide and heme stocks were prepared in 2 mM DPC, 50 mM sodium phosphate buffer, pH 7.2. Different ratios of the two stock solutions were mixed while keeping the total volume constant. The absorbance values at 412 nm and 356 nm were recorded for the various mole fractions of heme using a multi-well plate reader (Tecan Infinite M200 PRO). This difference in absorbance was then plotted against the mole fraction of heme.

The ferrous heme spectra were obtained by addition of sodium dithionite (from a freshly prepared 5mM stock solution) to the peptide-heme complex (20 μ M peptide, heme concentration) in 2 mM DPC 50 mM sodium phosphate buffer pH 7.2 (de-aerated for 30 minutes by purging nitrogen).

5.3.5 Stopped-flow kinetics

The oxidation of ABTS as a measure of peroxidase activity was recorded at 700 nm versus time at room temperature. 8 μ M A β peptide solution containing 1 μ M heme and 5 mM ABTS were mixed with 20 mM hydrogen peroxide in 5 mM Tris, pH 9.0. The absorbance values at 700 nm was converted to concentration ($\epsilon_{700\text{nm}} = 1.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) and the reaction rate was calculated by linear regression analysis. In-order to obtain the kinetic parameters for the peptide-heme complexes (V_{max} , K_m , k_{cat}/k_m),

reaction rates were obtained by varying hydrogen peroxide concentrations. . These reaction rates were plotted versus H_2O_2 concentration and fit to the Michaelis-Menton equation using Origin 9.0 software.

$$V = \frac{V_{max}[H_2O_2]}{K_m + [H_2O_2]}$$

V is the reaction rate ($\mu\text{M/s}$), V_{max} is the maximal velocity for the enzymatic reaction ($\mu\text{M/s}$), K_m is the Michaelis-Menton constant and $[H_2O_2]$ is the concentration of H_2O_2 used. Turnover numbers (k_{cat}) were calculated by dividing the maximal velocity by the concentration of heme-peptide complex (ie; $0.5\mu\text{M}$).

5.3.6 NMR Spectroscopy

NMR samples for A β 40 / A β 16 were prepared by dissolving 0.2-0.3 mM peptide (after NaOH pre-treatment) in 20 mM sodium phosphate buffer / 10 mM DPC, 20 mM sodium phosphate buffer pH 6.5. DSS was used as an internal reference. NMR spectra were acquired in a Bruker Avance II 600 / 700 MHz spectrometer equipped with a cryoprobe at room temperature (298 K). For NMR titrations of A β 40 with heme, 2D- TOCSY and ^{15}N - ^1H HSQC experiments were acquired by addition of heme from a stock solution of 1mM. NMR data were processed using TopSpin 3.0 (Bruker), and the chemical shifts were directly / indirectly referenced to the frequency of DSS (^1H). Spectral analysis was done using SPARKY 3.113.

5.4 Results and Discussion

5.4.1 Peptide purification

Figure 72: Expression and Purification of A β 40 peptide. A) Purification profile of A β 40-furin B) Re-purification of A β after formic acid cleaving C) HPLC purification of the wash fraction shows a sharp band at 50% acetonitrile gradient / 25 minutes elution D) MALDI spectrum of A β 40 showing the expected molecular weight (4.3kDa).

To carry out heteronuclear NMR experiments, the A β 40 was expressed using furin as a fusion tag. The N-terminus of the furin also contains a His-tag for ease in purification. The fusion protein was successfully overexpressed in inclusion bodies using TB (Terrific Broth) and M9 media and purified using Ni-NTA affinity chromatography under denaturation conditions (8 M urea). The fusion protein was cleaved at the D-P site using 50% formic acid for 24 hours. The furin and uncleaved protein present (both contain His-tag) along with A β peptide was removed by using Ni-NTA affinity purification. The A β peptide collected in the flow-through is

dialyzed and further purified using reverse phase HPLC. The major peak obtained during purification was confirmed by MALDI-ToF and NMR spectroscopy (**Figure 72**).

5.4.2 Heme binding studies

Figure 73: Spectral characteristics of A β peptides in phosphate buffer. UV-vis absorption spectra of heme alone (red), A β 16-heme complex (blue), A β 16-heme reduced (purple), A β 40-heme complex (orange) and A β 40-heme reduced (cyan) in 50mM sodium phosphate buffer pH 7.2.

UV-vis spectroscopy was used to probe Soret band of heme in the presence of excess of peptides A β 16 and A β 40 (4 peptide: 1 heme stoichiometry is used). Heme alone in phosphate buffer shows a broad absorbance at $\sim$ 395 nm (**Figure 73**). We observed a very broad and less shift of the Soret band $\sim$ 400 nm indicating weak affinity binding of the A β 16 peptide. Furthermore, reduction of heme-A β 16 complex with sodium dithionite also did not indicate spectral characteristics of any known coordinations of heme. Interestingly, the A β 40-heme complex in phosphate buffer displayed a red shift of the Soret band of heme $\sim$ 412 nm with low intensity α - β bands at around 500-600 nm. The bis-His coordination of heme is confirmed by observing α , β bands at 530 and 560 nm after reduction with sodium dithionite. This also confirms the presence of

a low spin hexacoordinated low spin Fe (III) heme in A β 40. It is interesting to note that all the three histidines (H6, H13 and H14) available for binding to A β are located near the N-terminus (1-16) portion of peptide.

Figure 74: Spectral characteristics of A β peptides in DPC micelles. UV-vis absorption spectra of heme alone (black), A β 16-Heme complex (blue), A β 16-heme reduced (purple), A β 40-Heme complex (red) and A β 40-Heme reduced (dark cyan) in 2 mM DPC, 50 mM phosphate buffer at pH 7.2.

We also screened the peptides in a membrane mimicking environment using DPC micelles (**Figure 74**). We observed that heme alone has a Soret band at ~380 nm and when it binds to A β 16, we observe a shift in the Soret band to 402 nm indicating a low affinity heme binding complex. The reduction of A β 16-heme complex in DPC is indicative of a low spin Fe (III) with a bis-histidine coordination with α , β bands at 530 and 560 nm. Heme in the presence of A β 40 peptide shows a clear red shift of the Soret band to 412 nm with α - β bands at 500-600 nm. The α - β bands in the reduced A β 40-heme complex clearly reflect the presence of a bis-histidine coordination. All these results collectively suggest that heme binds to A β 40 peptide via bis-histidine

coordination in membrane and aqueous solutions and it needs the presence of its C-terminus portion (17-40) to ligate heme efficiently.

Figure 75: Heme binding characterisation of Aβ peptides in DPC micelles. A) Spectral changes of heme (10 μM) on addition of increasing concentration of Aβ40 peptide (0-50 μM). A red shift and a Soret maximum at 412 nm with low intensity peaks at 500-600 nm is observed. B) Spectral changes of heme (10 μM) on addition of increasing concentration of Aβ16 (upto 5 equivalents) does not get altered much indicating weaker affinity. C) Heme binding isotherm of Aβ40 peptide monitored at Soret maximum-412 nm. D) Job's plot of Aβ40 peptide indicating peptide:heme stoichiometry 2:1

Atamna *et al.* [242] have reported a bis-histidine coordination of heme-Aβ40 complex with a binding affinity of around 140 nM and two binding sites within the peptide. The heme binding affinity of the Aβ40 in DPC micelles was estimated by titrating increasing concentrations of peptide to fixed concentration of heme (2 μM). It was observed that Aβ40 binds to heme in a cooperative manner with dissociation constant

estimated as 15 μ M (**Figure 75**). The stoichiometry determined by Job's plot indicated a 2 peptide: 1 heme ratio. These results suggest a completely different mode of heme binding in membrane where a pre-dimerization of the A β 40 peptide is required for heme coordination.

5.4.3 Interaction analysis of A β with heme using NMR spectroscopy

The NMR spectra of A β 16 and A β 40 in the amide region were indicative of sharp and less dispersed peaks indicative of random coil conformations. The interactions of heme and A β 16/A β 40 peptides were monitored in phosphate buffer at 298 K. The peptides were titrated with various concentrations of heme in ratios of 1:0.25, 1:0.5, 1:1 and 1:2. The presence of a Fe centre in heme would affect chemical shifts and causes paramagnetic line broadening of NMR amide signals upon binding.

Figure 76: NMR titrations of heme with A β 16 peptide in phosphate buffer, pH 6.5. Maroon peaks represent the TOCSY peaks of the untitrated spectrum while orange peaks represent the titrated spectrum.

As observed by the 2D-TOCSY spectra of the A β 16 peptide, there is not much chemical shift changes observed in the amide signals and only a reduction in signal intensity confirming weak interactions as observed in the UV-vis data (**Figure 76**). The resonances of A β 16 and A β 40 peptide were assigned by utilizing 2D-TOCSY and NOESY experiments. At 1 peptide: 2 heme ratio, residues A2, Y10 and H6 showed the maximum peak shift changes. This result suggests that the residues Y10 or H6 residues might be one of the coordinating residues for heme in A β 16 peptide (**Figure 76**).

Figure 77: NMR titrations of heme with A β 40 peptide in phosphate buffer, pH 6.5. Blue peaks represent the TOCSY peaks of the untitrated spectrum, maroon peaks represent the titration at 1 peptide: 0.5 heme stoichiometry, orange peaks represent the titration at 1 peptide:1 heme stoichiometry, cyan peaks represent the titration at 1 peptide:2 heme stoichiometry.

Conversely, the full length A β 40 peptide showed major chemical shift perturbations and peak disappearance (due to peak broadening) confirming a stronger affinity to heme. For the titration experiments, cobalt porphyrin was used to prevent line

broadening due to a paramagnetic Fe in heme. The residues in the 1-16 region of A β 40 (A2, S8, Y10, H6, H13, H14, Q15, L17) showed the maximum perturbations (**Figure 77**). The data clearly establishes the bis-histidine coordination of A β 40 which may be satisfied by histidine residues (H6, H13 or H14). We also carried out ^{15}N - ^1H HSQC titrations of A β 40 with heme in phosphate buffer. Each signal in an HSQC spectrum represents a single amino acid, and a total of about 26 peaks of the 40 residues were observed. This may be due to signal overlap or conformational exchange with water at high pH (6.5). Even at 1 peptide: 1 heme ratio, we can observe major peak disappearance of residues present in the N-terminus. However, at 1:2 ratio, only the V40 residue is retained while all the other peaks have disappeared due to line broadening in the presence of heme Fe (III) (**Figure 78**). The significant reduction in intensity of peaks is indicative that this interaction occurs at intermediate exchange rate.

Figure 78: Interaction analyses of ^{15}N A β 40 with heme in phosphate buffer. A) HSQC titrations of heme with ^{15}N A β 40 peptide in phosphate buffer, pH 6.5. Maroon peaks represent the peaks of the untitrated spectrum, orange peaks represent the titration at 1 peptide: 0.5 heme stoichiometry, yellow peaks represent the titration at 1 peptide: 1 heme stoichiometry and green peaks represent 1 peptide:2 heme stoichiometry. B) Relative change in intensity of the heme titration with ^{15}N A β 40 at the ratio 1:0.5.

Figure 79: Interaction analyses of ^{15}N A β 40 with heme in DPC micelles. A) HSQC titrations of heme with ^{15}N A β 40 peptide in 10mM DPC phosphate buffer, pH 6.5. Maroon peaks represent the peaks of the untitrated spectrum, orange peaks represent the titration at 1 peptide: 0.5 heme stoichiometry, yellow peaks represent the titration at 1 peptide: 1 heme stoichiometry and blue peaks represent 1 peptide: 2 heme stoichiometry. B) Relative change in intensity of the heme titration with ^{15}N A β 40 at the ratio 1:0.5.

The ^{15}N - ^1H HSQC spectrum of A β 40 peptide at 10mM DPC showcased only 20 peaks out of the expected 40 peaks. The assignment of the ^{15}N - ^1H HSQC spectrum in DPC

micelles was done by utilizing a previously assigned spectrum in DPC micelles [243]. The lesser peaks observed in HSQC may be due to the presence of a dimer or higher oligomeric state. We observed significant peak perturbations and disappearance on addition of heme (**Figure 79**). It is indicative of the peptide binding to heme in the N-terminus (1-16) region suggests that the histidines (H6, H13 and H14) are playing a key role in this interaction.

In-order to proceed with a structural characterisation of A β 40 peptide in membrane, DPC and LMPG micelles were used to provide a membrane mimicking media. The quality and number of cross peaks are representative of the oligomeric state of the peptide/protein. Both DPC and LMPG micelles produced HSQC spectra with broadened peaks or more signals than expected, respectively (**Figure 80**). Peak broadening is typically a consequence of oligomerization due to a slower tumbling rate of the protein-DPC complex. Furthermore, three dimensional heteronuclear experiments (HNCACB, CBCACONH, 3D-TOCSY, 3D-NOESY) employed for structural calculations require high concentrations and stability of peptides in their membrane mimicking environments for longer durations.

Figure 80: ^{15}N - ^1H HSQC spectrum of A β -40 in LMPG micelles (200mM). The peaks

observed are more than the expected 40 for each residue.

5.4.4 Circular Dichroism

Figure 81: CD spectroscopy of apo and heme bound A β peptides in DPC micelles. A) A β 16 and B) A β 40.

Secondary structure of A β peptides in DPC micelles are investigated using far-UV CD Spectroscopy (**Figure 81**). The apo and holo conformations of A β 40 peptide demonstrated a positive maximum at 190nm and two negative minima at 208 nm and 220 nm respectively indicating that A β 40 and A β 40-Heme adopt a helical conformation in the presence of DPC micelles. Conversely, the apo and holo forms of A β 16 peptide displayed a negative minimum at 200 nm indicating the presence of a random coil conformation. The difference in structures of A β 40 and A β 16 peptides in DPC could explain the low affinity binding observed in the latter.

5.4.5 Thioflavin T Fluorescence

Figure 82: Aggregation kinetics of Aβ40 monitored by Thioflavin fluorescence. A) Fluorescence intensity change of Thioflavin T when it binds to Aβ40 fibrils in phosphate buffer (red) and DPC micelles (blue). B) Monitoring aggregation of Aβ40 by thioflavin T fluorescence in the absence (black) and presence of heme (red).

Thioflavin T is a benzothiazole dye that recognizes and binds to the β -sheet structure of amyloid fibrils. Upon binding to the fibrils, it emits an enhanced fluorescence and it shows low basal fluorescence in the presence of unstructured/ α -helical state of Aβ40 in aqueous and DPC solutions. Spontaneous aggregation of 50 μ M Aβ40 peptide prepared in DPC micelles and aqueous buffer conditions was monitored by measuring the fluorescence emission at 485 nm wavelength (and 435 excitation wavelength) for three days. The Aβ40 aggregation curve is characterized by a nucleation phase ~20 hours followed by an elongation phase initiated after 30 hours and it reaches a plateau after formation of fibrillar structures are completed (**Figure 82A**). We observed the Aβ40 in DPC micelles undergo a faster and increased fibrillar formation than in aqueous solutions. On studying the aggregation trends with increasing concentrations of heme, we observed that heme caused a delayed nucleation and fibril formation of Aβ (**Figure 82B**). At 2 heme: 1 peptide ratio, the aggregation is completely abrogated by heme and there is no increase in fluorescence for the entire duration of the

experiment. Heme inhibits A β aggregation effectively with an IC₅₀ value of 1.5 μ M (Figure 83).

Figure 83: Heme inhibits A β 40 aggregation monitored by thioflavin fluorescence. A) Aggregation assay of A β 40 peptide monitored at various concentrations of heme. B) The elongation rate of A β aggregation monitored at various heme concentrations gives an IC₅₀ fit value of 1.5 μ M.

5.4.6 Peroxidase activity

Figure 84: Peroxidase activity of A β 40 peptide. A) Time course of ABTS oxidation at 700 nm for A β 40-Heme complex at 20 mM H₂O₂ concentration at phosphate buffer and DPC micelles. B) Steady-state kinetics of ABTS oxidation as a function of hydrogen peroxide concentration of A β -40/heme complex in phosphate buffer and DPC micelles. The data was fitted to a Michaelis-Menten equation to derive catalytic parameters.

A β 40-Heme complex in aqueous solutions acts as a peroxidase and as a result causes the oxidation of neurotransmitters such as serotonin and dopamine. Here, we

investigated the peroxide dependent oxidation of the organic substrate-ABTS for the peptides in DPC using stopped flow kinetics. **Figure 84** shows the oxidation of ABTS substrate as a function of time. A β 40 peptide in DPC micelles shows a low peroxidase activity when compared to A β 40 in aqueous solutions. Furthermore, the low k_{cat}/K_m , and high K_m values clearly reflect its inability to function as a peroxidase and contribute to oxidative stress (**Table 12**).

Table 12: Enzymatic parameters of A β peptide in solution and membrane.

Peptide	V_{max} ($\mu\text{M/s}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m ($10^3 \text{M}^{-1} \text{s}^{-1}$)
A β 40-DPC	56.72	42.87	113.44	2.64
A β 40 phosphate	140.7	3.4	281.44	140.72

5.5 Summary

Alzheimer's disease (AD) is one of the most prevalent age-related disorders and its pathogenesis is not clearly understood at present. The generation and deposition of amyloid-beta peptide is thought to play a prominent role in AD. Furthermore, this peptide binds to heme and results in depletion of regulatory heme and oxidative stress to cells. In-order to dissect heme binding and identify the key residues involved, we utilized NMR spectroscopy to study heme binding to A β 16 and A β 40 peptides. Even though the three histidine residues that could potentially bind heme are all present in the shorter fragment amyloid-beta 16 peptide, the full-length peptide shows a greater affinity to heme.

Further, to understand the role of this interaction in the cell membrane, we performed heme binding studies of A β 16 and A β 40 in a membrane mimicking environment (DPC micelles). We observed that A β 40 binds to heme in micromolar affinity via bis-histidine coordination. The peptide-heme complex functions as a weaker peroxidase in micelles than aqueous media and does not contribute to oxidative stress. These findings could help us understand the implications of A β /heme complex in AD.

6. FUTURE DIRECTIONS

Some of the directions of this project can be:

1. Studying multiple heme binding sites by extending the β -sheet structure.

β -barrels in nature consist of atleast 6-8 antiparallel β -strands and all β -sheet heme proteins bind to a single heme molecule.

2. Using the design to incorporate a covalently linked heme with pentacoordinated heme (single-His binding site).

The covalent linkage could help in NMR structure determination of peptide-heme complexes. Further, both these properties would help in creating better biocatalysts for peroxidase activity, oxygen binding etc.

3. Structure determination of A β -40 in a membrane-like environment.

Use of bicelles or nanodiscs as membrane mimics can help in understanding the peptide's structure in membrane.

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