CHAPTER III

Intrinsically Disordered Caveolin-1 Binding
Motif of α-HL: Implication in Assembly and Membrane Penetration.
Bacterial pore forming toxins (PFTs) serve as primary virulence factor during bacterial pathogenesis. They initiate the pathological means by pore formation on target cell membranes such as plasmamembrane [1,8]. Pore formation by PFT goes through a characteristic transition of water soluble protein into a transmembrane channel, in which membrane binding segment initiates the conformational changes for the insertion of transmembrane domain, thus permeabilizing the target cells [12,127]. Besides transmembrane domain insertion, evidences support a secondary domain (especially, the membrane recognition and oligomerisation domain that richs in basic and aromatic amino acids) of β-PFTs also incorporates into the membrane to stabilize oligomeric pore [25,48,127-129]. Hence, it is important to know how these stretches of amino acids of β-barrel PFTs undergo changes for efficient penetration of target cell membranes for their assembly.

Staphylococcal α-hemolysin (α-HL), a potent β-pore forming toxin (β-PFT), can assemble on erythrocytes, human monocytes, lymphocytes, platelets, and endothelial cells but with varied degree of susceptibility [21,36]. The assembly of α-HL (nucleated or non-nucleated) on target cells is proposed to include atleast three distinct stages viz. (i) the binding of water soluble monomers to target membrane, (ii) oligomerization of the membrane bound monomer into a heptameric pre-pore, and (iii) the final conversion of the pre-pore into a functional pore. Thus the activity of α-HL can be defined as the conversion of water-soluble monomer to an amphipathic transmembrane channel and pore on target cell, which results in permeabilization of cell membrane[12]. The susceptibility of target cells can vary over many orders of magnitude suggesting the existence of a ‘receptor’ that might facilitate its assembly. We have earlier provided some biochemical and modeling based evidences in which we have shown that the α-HL can assemble on mammalian cells with the help of functional form of Caveolin-1, the major structural protein of caveolae (flask shaped invaginations) present on mammalian cell membrane and its function is dependent on cholesterol [121,130,131]. In addition, the α-HL cannot assemble on cell membranes devoid of Caveolin-1 such as HT29 colon cancer cells or human granulocytes, which are few examples. For this assembly, the details mentioned in the previous chapter on the role of Caveolin-1 binding motif of α-HL appears to be crucial [121,125,130,131]. In summary the Caveolin-1 binding motif initiates the functional oligomerization after binding to Caveolin-1 of mammalian cells.
The interaction between $\alpha$-HL and Caveolin-1 was suspected to occur involving the scaffolding domain (amino acids 81–101) of Caveolin-1 and the Caveolin-1 binding motif of $\alpha$-HL (amino acids 179–187) through likely ionic and $\pi$–$\pi$ stack interactions (Figure 3.1) [131]. This mode of binding might initiate some conformational changes for an efficient insertion of its transmembrane $\beta$-barrel. But the question remained unsolved as to how $\alpha$-HL can establish contact with the Caveolin-1, which is present in the cytoplasmic face of membrane?

![Figure 3.1 Proposed model of interaction between $\beta$-barrel of $\alpha$-HL and Caveolin-1 scaffolding domain: Simple modeling of the interaction of the predicted $\alpha$-helix region of Caveolin-1 with $\beta$-barrel and Caveolin-1 binding motif of alpha hemolysin ($\alpha$-HL). Only the relevant portions of the interacting $\alpha$-HL (residues 109–187) and the predicted structure of Caveolin scaffolding domain (82–101) from each heptamer assembly are used for modeling using QUANTA (Molecular Simulations, USA). Possible stacking interaction of the aromatic residues F89, F92, and F99 of Caveolin with Y118, F120, and W179, respectively, of $\alpha$-HL are shown. The tryptophan W85 at the N-terminal side of Caveolin (bottom) is juxtaposed with hydrophobic amino acids iso-leucines and valine belonging to different chains of $\alpha$-HL. $\alpha$-HL's residues are numbered in white while the Caveolin-1 residues are in red [131].]

For facile penetration and interaction with Caveolin-1, the Caveolin-1 binding motif should show its ability to insert into lipid bilayer. There are ample evidences to suggest the molecular basis of insertional capability of the Caveolin-1 binding motif (or the rim domain, the membrane recognition and oligomerization domain, which contains Caveolin-1 binding motif)
of α-HL. The basic and aromatic amino acids cluster, the characteristic feature of rim domain of α-HL, might be responsible for targeting to eukaryotic membranes for penetration (Chapter 1). Several other assumptions have been put forward that membrane recognition and oligomerization domain/loop of other members of β-PFTs can penetrate into the membrane in order to establish contact with their respective receptor or for β-barrel stabilization. One study suggests the membrane recognition domain of cholesterol dependent cytolysins (CDCs) penetrates into the membrane to interact with its cholesterol receptor (presents in upper lipid layer of lipid raft) the for subsequent oligomerization step, which is pre-requisite for channel formation [128]. Moreover, this membrane recognition domain has cholesterol recognition consensus motif (CRAC motif) [70]. CRAC motif, the general consequence motif for cholesterol binding is \( L/V-X (1-5)-Y-X (1-5)-R/K \) and should be within aromatic amino acids rich region. Caveolin-1, the proposed receptor of α-HL, scaffolding domain (at cytoplasmic surface) interacts with cholesterol as it has cholesterol reorganization amino acid consensus motif [73]. The interaction promotes lipid raft formation and membrane penetration of Caveolin-1 scaffolding domain. Although α-HL does not bind to cholesterol \( \textit{in vitro} \) but its assembly process is regulated by cholesterol in model membranes. Hypothetically, the rim domain of α-HL also possesses CRAC like motif \(({^{175}V-N-Q-N-W-G-P-Y-D-R}^{185})\) [70,113]. Thus, Caveolin-1 binding motif of α-HL may insert inside the membrane to interact with Caveolin-1. Recently proposed “A rivet model for channel formation by aerolysin like pore forming toxins” suggests that in addition to the normal β-barrel domain, a lipid binding loop (like rim domain of α-HL), which present at the mouth of β-barrel, inserts into the membrane though β-hair pin and provides stability to oligomers or β-barrel. Interestingly, this hydrophobic loop (with \( W^{247}PLVG \)) of aerolysin also drives membrane insertion of the developing channel [31]. Although above theoretical evidences support that the aromatic amino acids rich Caveolin-1 binding motif of α-HL has membrane inesertional capability but experimental proofs are still needed.

Hence, it was important to understand in detail, the relevance of \( \pi \)-stack interaction that provided by aromatic amino acids of Caveolin-1 binding motif in assembly and membrane penetration. Therefore, we studied membrane penetration needed for Caveolin-1 binding and functional oligomerization of α-HL on RBCs membrane by cysteine scanning mutagenesis of aromatic amino acids of Caveolin-1 binding motif (aromatic amino acids are the signature residue of membrane penetrable Caveolin-1 binding motif), and chemical modification with
membrane impermeable reagent and environmental sensitive fluorophore. Such information will help us to generalize whether or not the Caveolin-1 binding motif sequences of other toxins/proteins can penetrate eukaryotic cell membranes for defining the pathological consequences.
3.2. MATERIALS

3.2.1 Chemicals
Standard chemical reagents were of analytical purity grade and were mainly obtained from the companies: MERCK, Sigma-Aldrich (USA)

3.2.2 Enzymes and inhibitors
Enzymes for cloning purposes: restriction enzymes, T4-DNA ligase, Pfu and Taq polymerases were obtained from New England Biolabs. Respective buffer were supplied by manufacture

3.2.3 Kits
DNA cleanup and plasmid purification were carried out with kits from Qiagen (Germany): QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit and QIAprep Spin Miniprep Kit. Protein Purification by Ni-NTA agarose was purchased from Qiagen.

3.2.4 Bacterial strains and plasmids
*E.coli* JM109: Promega, USA; *E.coli* BL21 (DE3): Novagen, USA; *E.coli* DH5α: Promega USA., pT7Nc: Constructed in lab from pET23d+ (Novagen USA), and pGEMEX1 (Promega, USA); pET28a+ (Novagen USA)

3.2.5 Medium and antibiotics
Ampicillin: Sigma, USA(100µg/ml);Kanamycin: Sigma USA (30µg/ml)

3.2.6 Primers: All primers were obtained from Geno Mechanix, USA.

T7 promoter primer: 5’ TAATACGACTCACTATAGGG-3’

K SX20: 5’-ATTTAAGCTTTATTTGTCATTCTCTCTCTCTCTTCC-3’

W179C sense: 5’-ATGGTCAATCAAAATTGTGGACCATATGAT-3’

W179C antisense: 5’-ATCATATGGTCCACAATTTTGATTGACCAT-3’

Y182C Sense: 5’-CAAAATTGGGGACCATGCGATAGAGATTCT-3’

Y182C Antisense: 5’-AGAATCTCTCTCTATCGCATGGTCCCCAATTTTG-3’

W187C Sense: 5’-GATAGAGATTCTTGTAACCCGGTATGCGGCAATCAACTT-3’

W187C Antisense: 5’-TCTTGGAAACCCTTGATGCAGGCAATCAACTT-3’
3.3. METHODS

3.3.1 Site directed mutagenesis of aromatic amino acids of Caveolin-1 binding motif (W-G-P-Y-D-R-D-S-W) of α-HL

Active α-HL gene was cloned previously as pT7Nc/α-HL in our laboratory and that used as the template for sub-cloning in pET28a+ as pET28a+/α-HL. T7 (forward primer) and KSX20 (reverse primer) were used for the amplification of full length α-HL (\(^1\)ADSD….EMTN\(^{293}\)) to get fragment size of 0.930kbp.

For amplification, PCR mixture (50µl) contained 50pmol of each primer, 0.1µg of pT7Nc/α-HL, 0.1mM concentration of each deoxynucleoside triphosphate, 2U of Vent DNA polymerase, and the corresponding reaction buffer (New England Biolab). The hot start amplification involved an initial denaturation cycle (95º C for 5 minutes), then 20 cycles of denaturation (95º C for 1minute), annealing (58º C for 2 minutes) and extension (72º C for 3 minutes) was carried

Figure 3.2 Cloning strategies of pET28a+/α-HL (Described in text)
out by final extension (72º C for 10 minutes). The amplified DNA was purified from gel by using Qiagen DNA purification kit.

The PCR product (0.933kbp) was digested with restriction enzymes and sub-cloned into expression vector pET28a+ at NcoI and HindIII sites. 1µg of the pET28a+ plasmid was digested with 10U of NcoI and 20U of HindIII for 6hours. After complete digestion, sample was incubated at 70ºC for 10minutes for inactivation of restriction enzymes. The amplified and gel purified PCR product was also digested overnight with same restriction enzymes used for the vector (NcoI and HindIII). Both digested vector and PCR product ran in 1% agarose gel and purified from gel by using Qiagen gel extraction purification kit. Ligation was performed using T4 DNA ligase and a 5:1 insert to vector ratio at 16°C overnight. Chemical transformation was done using JM109. 1µl of ligated plasmid DNA was mixed with 100 µl JM109 E. coli competent cells, kept on ice for 30 minutes, heat shocked at 42ºC for 60 seconds and returned to ice for 5 minutes. 1ml LB was added to transformation and cells were allowed to grow without antibiotic selection for 1 hour at 37°C. The entire volume was pelleted down at 2000rpm and plated in LB kanamycin plate. Colonies were resuspended in 50µl of deionized water. Colony PCR was performed using 10 µl of colony re-suspension and condition previously used for the amplification of PCR product 15µl of each PCR reaction was run on 1% agarose gel to check for bands of the correct size for confirmation of clones. For positive clones, 10µl colony re-suspension was transformed into JM109. Single colony from transformed plate then inoculated in 10 ml LB/Kanamycin and grown overnight at 37°C. After overnight growth, glycerol stocks were prepared. The pellet was collected after centrifugation and the plasmid DNA was purified using the Qiagen Miniprep Spin Column. Plasmid DNA was eluted in 50µl of elution buffer and stored at -20ºC. To confirm the correct orientation of insert ligation, 2µg of purified DNA was used as double digestion with vector specific BglII and insert specific HindIII. Correct reading frame of all positive clones were examined by DNA sequencing.

The following mutants: W179C, Y182C and W182C (here after used as Single Cysteine Caveolin-1 Binding Motif Mutants (SCCBMM)) of α-HL were constructed. Site directed mutagenesis of the aromatic amino acids of Caveolin-1 binding motif of α-HL (W179-G-P-Y-D-R-D-S-W187) was performed using the Quick-Change kit (Stratagene). pET28a+/α-HL (here after referred as α-HL) clone was used as template for mutagenesis. For mutagenesis, the
reactions were carried out in 50 µL of supplied reaction mixture containing 400nM of each oligonucleotide primer, and 50ng of plasmid DNA. Reactions were performed on ependroff thermal cycler with 1 cycle of 95°C for 1min and 17 cycles of the following profile: 95°C for 1min; 55°C for 1min; 72°C for 3min. The reaction mixture (3µL) was used to transform *Escherichia coli* XL-1 Blue competent cells. Plasmid DNAs were prepared from cultures of the transformants using QIA prep Spin Miniprep kit (Qiagen). The sequence with mutation was confirmed by automated DNA sequencing.

**3.3.2 Expression and purification of α-HL and SCCBMMs**

After confirmation of correct ORF of clones and mutation, α-HL and SCCBMMs recombinant plasmids were transformed in *E. coli* BL2 (DE3) expression host. Single colony from each freshly transformed plates were inoculated in 10ml LB medium with kanamycin (30µg/ml) and the culture was grown at 37°C till OD<sub>600nm</sub> reached 0.25-0.35. The cells were collected by centrifugation and washed three times with LB kanamycin to remove β-lactamase. 100µl of washed pallet re-inoculated in 10ml of LB-kanamycin and grown at 30 ºC until the OD<sub>600nm</sub> reached 0.4 and then induced with 1mM IPTG. After 4hours of growth cells were harvested and suspended in 1X lamelie sample buffer. The samples were electrophoresed to examine the expression level of α-HL and SCCBMMs.

For purification, one liter culture for α-HL and SCCBMMs were expressed and induced as described above. The cell pellet was resuspended in 200ml of lysis buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub> 300 mM NaCl, 10mM imidazole , pH 8). The cells were then lysed by sonication and the insoluble cellular debris was removed by centrifugation (12,000rpm for 15minutes). The supernatant constituted the soluble fraction of expressed proteins were passed into 2ml bed volume of Ni-NTA agarose column. Unbound proteins were washed with wash buffer (100mM NaH<sub>2</sub>PO<sub>4</sub> 300mM NaCl, 10mM imidazole , pH 8). Proteins were finally eluted with 1ml aliquots of the elution buffer (10mM NaH<sub>2</sub>PO<sub>4</sub> buffer pH 8, 250mM imidazole and 10% glycerol). Purity of fractions was examined by 12% SDS-PAGE and commassie blue staining.

**3.3.3 Processing of Rabbit Blood**

Heparin inhibited and freshly drawn blood (2ml) of New Zealand rabbit was washed three times with five volume of sodium phosphate buffer saline (NaPBS, 20mM NaH<sub>2</sub>PO<sub>4</sub>, 150mM
NaCl pH 7.4). After each wash, RBCs were pelleted down by centrifuging of wash buffer dissolved RBCs at 1600rpm for 10min. After final wash, 10% RBCs was made by making its final volume by 10ml in storage buffer (KPBSA, 20mM KH₂PO₄, 150mM NaCl pH 7.4) and stored at 4ºC. As per experimental need 10% RBCs were diluted with KPBSA.

3.3.4 Kinetics of hemolysis of rRBCs by α-HL and SCCBMMs
The time courses of lysis by different SCCBMMs were compared with α-HL in reducing and non-reducing condition. Purified SCCBMMs (5μg/ml) were incubated (in the presence or absence of 5mM DTT) in 200μl of 1% rRBC in KPBSA. At regular intervals of time, light scattering was recorded at 595 nm as reported earlier [120].

3.3.5 Limited Proteinase K digestion
Purified mutants (30μg) were incubated with 500ng Proteinase K and at different time points an equivalent of 4 μg of digested mutant removed and processed for SDS–PAGE after inactivation of the protease (by 1XLaemmli Sample Buffer containing 1mM PMFS) [120].

3.3.6 Measurement of Intrinsic and ANS fluorescence
Fluorescence emission spectra in the absence and presence of 1-anilino-8-naphthalene sulfonic acid (ANS) of the SCCBMMS and α-HL were acquired with Perkin–Elmer LS50B spectrofluorimeter at 25ºC by using 30μg/ml of protein in MOPS buffer (10mM MOPS, pH 7.4). The excitation wavelength was 280 nm for intrinsic fluorescence (without ANS) and for ANS studies it was 380 nm. The slit width was 5 nm for all measurements [121].

3.3.7 Binding, oligomerisation and oligomer stability of SCCBMM on rRBCs
For binding and oligomerisation assay, SCCBMMs (5μg/ml) and α-HL (1μg/20μl) were incubated in 0.5% of rRBCs for 1hour. Protein (SCCBMMs) bound membranes were collected by centrifugation at 1600rpm. The rRBCs pellets were washed three times with KPBSA and dissolved in 20μl of 1XLSB containing 1% SDS and heated at 50ºC or 90ºC for 10min. Samples were resolved by 7.5% SDS–PAGE and transferred to nitrocellulose paper, which was then probed with HRP-conjugated anti-His-tag antibody (1:2000 dilution). The degree of binding and oligomerization was measured by densitometry. Oligomer stability assay was essentially carried out described as above. But instead of 5 μg/ml, 10μg/ml of SCCBMMs used for
incubation with rRBCs, then rRBCs pellets were lysed with hypotonic buffer (5mM sodium phosphate buffer pH 8) and samples were heated at 50 or 65°C for 10 min [120,121].

3.3.8 Digestion of membrane-bound SCCBMMs with Proteinase K
Limited proteolysis was performed for α-HL and SCCBMMs bound to rRBCs. α-HL or SCCBMMs (5μg/ml) were incubated for 15 min or 1 hour in 1 ml of 1% rRBCs respectively and rRBCs pellets were isolated by centrifugation. Protein bound rRBCs membranes were prepared by lysing the rRBCs with 5mM of phosphates buffer pH 8. The membrane pellet was resuspended in 20μl of KPBS and incubated with Proteinase K (1μg). Reaction was stopped by the addition of 1X laemmli’s sample buffer containing 1 mM PMSF, heated at 95 °C, 5 min and subjected to 15% SDS–PAGE. The digestion pattern by Proteinase K was examined by immuno blot analysis using HRP conjugated anti-His tag antibody (Dilution 1:2000).

3.3.9 In Vitro Binding of SCCBMMs to Caveolin-1
Enzyme-linked immunosorbent assay (ELISA) was performed to assess the in vitro binding of Caveolin-1 to SCCBMMs. The 96-well plates (Nunc Maxisorp) were coated with α-HL or SCCBMMs (4 pmoles/well) in 100mM sodium bicarbonate, pH 8.5 for 16-20 h at 4°C. The wells were then blocked with TBST buffer (10mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.05% Tween 20) containing 10mg/ml bovine serum albumin for 2 hours at room temperature. After blocking, the wells were incubated with Caveolin-1 (16pmoles/well) for 2 hours and washed thoroughly with TBST. The plates were then incubated with anti-Caveolin-1 (N20) for 1 hour, then with HRP-conjugated secondary antibody for 1 hour and washed with TBST. The color reaction was initiated by the addition of ABTS (2, 2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) Roche, Germany). The plates were quantified on a microplate reader at 405 nm. Controls employed were (i) blank well blocked with TBST containing 0.1% BSA. (ii) α-HL or SCCBMMs coated wells without the Caveolin-1 in a similarly blocked well. (iii) Caveolin-1 in the absence of α-HL or SCCBMMs..

3.3.10 In vivo Binding of SCCBMMs to Caveolin-1 by immunoprecipitation
PBS starved cells (10^7 cells) were treated with 200mM of toxins (α-HL and SCCBMMs) for 1 hour and then lysed in an immunoprecipitation assay (IPA) buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.5% deoxycholate, 0.05% SDS, 1 mM Na3VO4, 1 mM phenylmethylsulfonylfluoride, 1μg/ml leupeptin and 1μg/ml aprotinin). The cell lysates
were centrifuged at 10,000rpm for 10min at 4°C and the supernatants were pre-cleared by incubating with protein-A agarose beads for 1hour at 4°C, followed by centrifugation at 10,000rpm for 1 min at 4°C. The supernatants were subjected to immunoprecipitation using 2µg of anti-His antibody. The samples were incubated overnight, followed by incubation with protein A-agarose beads for 2hours at 4°C. After washing the beads three times with ice-cold IPA buffer containing detergent and three times with IPA buffer without detergent, the bound proteins were eluted by the addition of 1X SDS-PAGE sample buffer followed by electrophoresis and immunoblot analysis with Caveolin-1 and phospho-Caveolin-1 antibody.

3.3.11 Assembly study with SCCBMMs on A431cell
This assay was performed as described above for binding and oligomerization on RBCs membrane. α-HL and SCCBMMs (1µg) were incubated with A431cells (1X10⁴) for 1hours at 37°C. At the end of incubation, cells were washed PBS (150mM NaCl, 20 mM KH₂PO₄, pH 7.4) to remove unbound toxins and the cell pellet was dissolved in 15µl of 1X laemmlí sample buffer. Samples were heated at either 50°C or 90°C before and subjected to 8% SDS-PAGE. The resolved proteins transfer to nitrocellulose paper and immunoblot with anti-His antibody (1:1000 dilutions).

3.3.12 Hetero-oligomerization Assay
Purified SCCBMMs were incubated with wild type α-HL as per the indicated molar ratios in Figure. To the protein mixers, rRBCs (1% final) was added and subsequently all the samples were incubated for 1hour. Erythrocyte suspensions were centrifuged briefly and the supernatant was removed. Release of hemoglobin was detected spectro-photometerically by measuring absorbance at 545nm against a blank (KPBSA) [121].

3.3.13 Cysteine modification by IASD and behavior assessment of SCCBMMs on rRBCs membrane
Each SCCBMM was labeled with 4-acetamido-40-(iodoacetyl) amino) stilbene-2,2’-disulfonate (IASD) (1:5 molar ratio) at room temperature for 1hour in presence of 10 mM DTT and reaction was stopped with 200mM DTT [48]. The IASD labeled and purified protein labeling efficiency was assessed by 16% SDS-PAGE along with non-labeled protein as per reference [48]. Quantitative analysis of binding, oligomerisation and kinetics of lysis of IASD modified
proteins was examined on RBC membrane using 5μg/ml labeled and unlabeled protein as described earlier [132]. Percentage of binding loss of IASD labeled SCCBMMs was measured by densitometry.

3.3.14 Cysteine modification by Badan and membrane penetration assessment of SCCBMMs on rRBCs membrane

Modifications of SCCBMMs were carried out essentially like IASD modification described above. Inactivation of unreacted badan was achieved by addition of 200mM DTT and removed by passing through desalting column. Efficiency of labeling was calculated by nano-drop and lysis kinetics and oligomerization were measured as described above (for unlabeled SCCBMMs) using an aliquot of 5μg of labeled protein. For membrane penetration measurement after badan modification, 5μg of labeled SCCBMMs were incubated for 6 hours in 1 ml 0.25% RBCs in dim light, washed three times with KPBSA and excited at 408 nm and emission spectra was recorded between 400 and 600 nm. For controls spectra were recorded immediately after addition to rRBCs.
3.4 RESULTS

3.4.1 Cloning of Single Cysteine Caveolin-1 binding motif mutants of \(\alpha\)-HL

For the convenience of purification and site specific labeling (with select fluorophores) of SCCBMMs described in this chapter, we sub-cloned wild type \(\alpha\)-HL and SCCBMM mutants with a carboxy terminus 6-histidine tag. The extension of carboxy terminus (due to the his residues) of \(\alpha\)-HL does not affect the behavior of \(\alpha\)-HL polypeptide [120]. The \(\alpha\)-HL was cloned in pET28a\(^+\) that has an option to add six his sequence to C-terminal of polypeptide between NcoI and HindIII (Figure 3.2 and 3.3A).

![Figure 3.3 Cloning of pET28a\(^+\)/ \(\alpha\)-HL and Single Cysteine Caveolin Binding Motif Mutants (A-I) Preparation of vector and insert for pET28a\(^+\)/ \(\alpha\)-HL cloning: Ethidium bromide stained 1% agarose gel, showing the restriction analysis of the 0.930Kbp PCR fragment and linearized vector. Lane 1: Un-cut pET28a+ vector, lane 2: NcoI and HindIII cut vector, lane 3: NcoI and Hind III cut insert, lane M: DNA ladder (MBI Fermenta). (A-II) Identification of recombinant Plasmid pET28a\(^+\)/ \(\alpha\)-HL by colony PCR: Three colonies picked from ligation mixture transformation plate and selected clones were amplified with T7 and insert specific KSX20 primer. Expected size of ~0.930Kbp insert was checked by 1% agarose gel. Lane 1: clone 1, lane 2: clone 2, lane 3: clone 3. 20\(\mu\)l sampled loaded in each lane. All clones were positive (A-III) Recombinant Plasmid pET28a\(^+\)/ \(\alpha\)-HL confirmation by double digestion: Plasmid prepared by alkaline lysis methods for all those clones were confirmed positive and digested with BglII and HindIII to check the insert ligation. Expected size of ~0.930Kbp insert fall out was checked by 1% agarose gel. Lane 1: inset used for ligation, lane 2: Clone 1, lane 3: clone 2, lane 4: clone 3, lane 5: only cut vector lane 6: DNA ladder.](image-url)
After confirmation pET28a+/α-HL clone, we used this construct as template for site directed mutagenesis. Site-directed mutagenesis of aromatics amino acids (179 W, 182 Y and 187W) of Caveolin-1 binding motif (179W-G-P-Y-D-R-D-S-W187) of α-HL into cysteine was carried out by the oligonucleotide-based method as described in the manual provided with the kit. (Quick Change Mutagenesis Kit) (Figure 3.3(B)). Constructs with respective mutation were verified by automated DNA sequencing.

3.4.2. Expression and purification of α-HL and SCCBMMs

Production and purification of recombinant proteins is an important step in the biochemical analysis and functional characterizations of proteins. For high yield expression, the pET series of vectors, is under the control of T7 RNA promoter and contains C-terminal his region that can be used for easy purification. Thus, only E. coli strains engineered to express the T7 RNA polymerase upon IPTG induction can be used for expression. BL21 (DE3), which is protease deficient and IPTG inducible host used for all constructs (α-HL and SCCBMMs) expression. All the proteins were expressed at 28°C after induction (Figure 3.4A) and purified from the E. coli cell lysate with the help of Ni-NTA agarose resin as described in the experimental section. Eluted proteins were analyzed on SDS-PAGE and which showed strong protein bands of molecular mass around 33 kDa (Figure 3.4(B)). Greater than 95% of purity was obtained per construct.

Figure 3.4 (A): Expression status: After cloning and mutation confirmation, all constructs (α-HL and SCCBMMs) were used for expression as described in methods section. The expression statuses of proteins were as follows: Lane 1: vector control
(pET28a+), lane 2: α-HL, lane 3: W179C, lane 4: Y182C, lane 5: W187C, lane 6: low molecular weight marker (BioRad). (B) **Purified recombinant α-HL and SCCBMMs:** The recombinant constructs (α-HL and SCCBMMs) were expressed in E. coli BL21 (DE3) and the respective soluble fractions were prepared and processed as described in methods section and purified using Ni-NTA agarose. Lane 1: α-HL, lane 2: W179C, lane 3: Y182C, lane 4: W187C, lane 5: low molecular weight marker (BioRad).

### 3.4.3. SCCBMMs do not lyse target cells

The hemolytic activities of α-HL and SCCBMMs produced in E. coli were assayed as reported earlier [132]. Interestingly, none of the SCCBMMs were able to lyse rRBCs in 24 hours even at a concentration of 5μg/ml, as α-HL requires only 20ng/ml in a few minutes (Figure 3.5(A)).

![Figure 3.5](image)

**Figure 3.5  Quantitative hemolysis assay of α-HL and SCCBMMs in absence and presence of DTT:** Final concentration of each protein 5 μg/ml was diluted in 200μl 1% rRBCs and lysis was measured at 595 nm in absence(A) and presence of 5mM DTT(B). The data represents the average of three independent measurements. Symbols correspond to α-HL (○), W179C (■), Y182C (●), and W187C (▄).

In view of the possibility of a disulfide bond formation between two molecules of α-HL after binding to rRBCs membrane that might result in loss of hemolytic activity, all SCCBMMs were reduced with 5mM DTT at room temperature and their hemolytic activities were assessed. The data in Figure 3.5(B) shows that the DTT treatment did not result in eliciting the hemolytic activity of SCCBMMs. In summary, point mutations in Caveolin-1 binding motif of α-HL abolished the hemolytic activity not due to intermolecular disulfide bond formation but the aromatic residues probably play a decisive role in membrane penetration during the assembly of α-HL on target cells.
3.4.5 Limited proteolysis by Proteinase K of SCCBMMs

Proteinase K cleavage of α-HL results in a two half like domains (primary cleavage occurs between 131 and 136 amino acids) and very useful for understanding the overall folding of α-HL. Based on this logic, the relative overall folding of SCCBMM were evaluated by limited proteolysis. From figure 3.6, it is clear that all SCCBMMs have nearly similar folding like α-HL as they have yielded the characteristic two half like pattern. However, subtle differences exist between the SCCBMMs. For example, W179C was more susceptible than the other two mutants. These observations were consistent with our earlier report in which we have created α-HL mutants devoid of Caveolin-1 binding motif which were highly susceptible to Proteinase K [121].

Figure 3.6 Susceptibility of SCCBMMs to ProteinaseK: Each SCCBMM (30 μg) in solution was treated with 500 ng of Proteinase K as described in Materials and methods section. The samples were resolved by 15% SDS–PAGE and stained with coomassie brilliant blue. The lanes labeled with U and M represents the undigested protein and low range proteins markers, respectively.

3.4.5 In vitro stability of SCCBMMs

To access relative folding of SCCBMMs, the intrinsic fluorescence emission (IFE) was examined as we converted two tryptophans (179 and 187) and one tyrosine (182) into cysteine in case of SCCBMMs. The data in figure 3.7A is a representative profile of intrinsic fluorescence emission spectra of α-HL and three SCCBMMs. Overall, only negligible red shift in the emission maximum (λmax) was observed for all SCCBMMs. Specifically W179C and
Y182C showed 1nm shift (at 337 nm instead of 336 nm observed for α-HL) and 2.5 nm shift was recorded for W187C 338.5 nm) in comparison to α-HL at 336 nm [120,121]. From these data it is clear that no large scale conformational or structural destabilizations were observed due to the point mutations at the Caveolin-1 binding motif. We had earlier reported that the α-HL upon complete denaturation exhibited an emission maximum at 356 nm. In comparison, α-HL(1–289) (devoid of 4 carboxy terminal amino acids) had exhibited an emission maxima at 345 nm and was completely digested by Proteinase K (no two half like pattern, Ref.[120]). Hence, it is reasonable to assume that the SCCBMMs described here are fairly well folded and very much similar to α-HL.

Figure 3.7 (A) Intrin sic fluorescence of αHL and SCCBMMs: SCCBMMs and α-HL (30μg/ml) in MOPS buffer (10mM, pH 7.4) were equilibrated at 25°C in 1 cm path length quartz cuvettes. Intrinsic fluorescence measurements were performed at an excitation wavelength of 280 nm and the emission spectra were recorded from 300 to 500 nm. All measurements were averaged over three scans at a scanning speed of 500 nm/min .The various spectra represent the fluorescence intensities at 356 nm of buffer (1), Y182C (2), W179C (3), W187C (4) and α-HL (5)

(B) Fluorescence spectrophotometry of ANS binding to SCCBMMs and α-HL: The final concentration of 5µM ANS was homogeneously mixed with protein solutions (30μg/ml) MOPS buffer as reported earlier. ANS fluorescence measurements were at an excitation wavelength of 380nm and the emission spectra were recorded from 400 to 600 nm. The fluorescence intensity of ANS were in presence of buffer (1), W179C (2), Y182C (3), W187C (4) and α-HL (5).

The surface hydrophobicities of the α-HL and SCCBMMs were compared (Figure 3.7 B) by their ability to bind ANS as it binds only to hydrophobic pockets, if any, of proteins. The ANS fluorescence emission was unchanged when compared with α-HL suggesting that SCCBMMs at neutral pH do not bind ANS. Thus, point mutations in this region do not affect the overall surface hydrophobicity where as deletion does. This clearly indicates that the Caveolin-1
binding region of $\alpha$-HL acts as water insulator to hydrophobic residues that bind to ANS after removal of Caveolin-1 binding motif containing loop [121]. Hence, modifications of Caveolin-1 binding motif, whether point mutation or loop deletions do not affect the global folding of $\alpha$-HL and mutants are fold like native $\alpha$-HL.

3.4.6 Mutations do not abolish SCCBMM binding to target cells

In view of the loss of hemolysis of SCCBMMs, we examined whether or not lack of hemolysis was due to impairment in efficient binding and/or oligomerisation and/or membrane penetration. Interestingly, all the SCCBMMs bound to target membranes and oligomerized but with certain degree of variability (Figure 3.8B). Among the SCCBMMs, W179C showed highest binding but lowest oligomerization where as W187C showed lowest binding but efficient oligomerization (Figure 3.8 A and C). Oligomers formed by the mutants are stable at 50°C and 1% SDS like its wild type counterpart which are stable upto 65°C in 1%SDS [48]. Noticeably, the oligomers of SCCBMMs showed reduced stability (Figure 3.8D) with the exception of the oligomer formed by W187C which was behaved like $\alpha$-HL. In view of absence of lysis and oligomer formation, the oligomers bands can be classified as pre-pore oligomers. In principle, the pre-pore can be subdivided into three categories viz. (i) A pre-pore which is closer to the membrane bound monomer, (ii) a pre-pore closer to the functional pore, and (iii) an intermediate between (i) and (ii) [59]. For example, the H35C mutant appears to belong to category (ii) while the H35N mutant can be classified into category (i). In our observation, the pre-pores formed by the SCCBMMs belong to category (iii) based on the data shown in figure 3.8. Hence, it is clear that the SCCBMMs could not fulfill the final step for pore formation and the mutated aromatic residues play an important role at later stage of pre-pore formation also in addition to the membrane binding.

3.4.7 Proteolytic stability of SCCBMMs on RBCs membrane: $\alpha$-HL, which assembled partially or arrested at pre-pore stage is susceptible to cleavage at its N-terminus by Proteinase K [59]. Based on resistance to cleavage at the amino terminus, it is possible to identify whether or not $\alpha$-HL mutant proceeded beyond pre-pore stage. The proteolytic susceptibilities of SCCBMM were compared with $\alpha$-HL and H35N in presence of RBC membrane (Figure 3.9A) [59]. The time dependent proteolytic resistance was found to be in the order $\alpha$-HL $\geq$ W187C $>$ Y182C $>$ W179C $\geq$ H35N All SCCBMMs (expect W179C) assembled beyond the susceptible pre-pore stage as they are quite resistance to ProteinaseK. W179C which showed highest
susceptibility to Proteinase K, probably assembled like H35N mutant. It is probable that the mutants form a pre-pore like intermediate, which differ in terms of degree of inter-protomer interactions. This data clearly indicates that the Caveolin-1 binding motif has a significant role in membrane penetration for functional pore formation.

Figure 3.8 (A) Binding and oligomer formation on RBCs membrane by SCCBMMs: Binding and oligomerization to RBCs was determined by incubation of 5μg/ml of each SCCBMMs and 1μg/20μl of α-HL for 1hours with rabbit erythrocytes (1%RBCs). Toxin treated RBCs were by pelleted down at membranes at 1700rpm and dissolved in 1% SDS. To SDS dissolved samples 5X LSB were added and heated either at 50°C or at 90°C. The samples were subjected to electrophoresis (7.5% polyacrylamide gels) monomer and oligomers position were analyzed by immunobloting with HRP conjugated anti his antibody (dilution 1:2000). (B) Various lanes represent: Lane1: α-HL (50°C), lane2: W179C(50°C), lane 3: W179C(90°C), lane 4: Y182C(50°C), lane 5: Y182C(90°C), lane 6: W187C9(50°C), lane 7: W187C9(90°C). O7 corresponds the heptameric oligomer formed mutants and M1 represent the monomers of corresponding mutants. Relative binding (A) and oligomerization (C) were measured by densitometry. (D): Heat stability of SCCBMMs: Binding and oligomerization to RBCs was performed by incubation of 10μg/ml of each SCCBMM with rabbit erythrocytes (1%RBCs) for 1hour at room temperature. Toxin treated samples were pelleted down by centrifuging at 1700rpm for 5minutes. RBCs membranes were prepared by lysing pallet two times with sodium phosphate buffer (5mM, pH 8). Samples were either heated at 50°C and 65°C with 1X LSB. Rest procedure as per above. The lane represents for various proteins and temperature are as follows: Lane1: W187C(50°C), lane2: W187C(65°C), lane 3: Y182C(50°C), lane 4: Y182C(65°C), lane 5: W179C(50°C) lane 7: W179CC(65°C).
Figure 3.9(A) ProteinaseK digestion of membrane bound α-HL and SCCBMMs: The toxin-treated membranes were digested with Proteinase K (0.05 mg/ml) as described in materials and methods section. Samples were electrophoresed on 15% SDS–PAGE and transferred to nitrocellulose paper followed immunoblot with anti-his tag antibody. U represents the control (without Proteinase K treatment), and the numbers represent the length of Proteinase K treatment in minutes. (B) Hetero-oligomerization assay: Hetero-oligomerization assay in the presence of wild type α-HL as described in materials and methods section. Increasing molar concentration of SCCBMM was incubated with α-HL as indicated in 1ml of 1% rRBCs. The percentage lysis caused by mixture proteins was measured as reported earlier[121]. The data represents the average of four independent measurements.

Figure 3.10 Assembly of SCCBMMs on A431 cells: SCCBMMs (1µg) were added to the A431 cells (1 x 10⁴) in 50µl of PBS and incubated for 1hours. Then cells were washed with PBS to remove unbound protein. Samples were process described in methods. M and O7 respectively, represent the monomer and oligomer of α-HL and SCCBMMs. The data represents one of the three independent experiments. Individual protein treated and tempature used for dissociation are depicted in figure.
3.4.8 SCCBMMs target on A431 cells like RBCs membrane

Above observations on rRBCs illustrate the importance of the aromatic amino acids residues of Caveolin-1 binding motif of α-HL for its assembly and activity on the target cells. For further proof, assemblies of SCCBMMs were examined on A431 cells (Figure 3.10). SCCBMMs bind and oligomerize on A431 cells like rRBCs membrane, when compared to α-HL [130]. This observation indicates that the SCCBMMs form oligomers and have undergone conformational changes like observed on RBCs membrane.

![Figure 3.11 Interaction of SCCBMMs and α-HL with Caveolin-(1-101): The assay for the interaction of SCCBMMs and α-HL with caveolin-1 was carried out as described in material and methods section by ELISA (A) and immunoprecipitation (B). (A) For ELISA assay, SCCMMMS and α-HL were coated at a concentration of 4pmoles per well. Coated wells were probed with 16pmole of Caveolin-1. Binding of the Caveolin-1 was observed with the help of horseradish peroxidase conjugated anti-Caveolin-1 antibody at 1:10,000 dilutions. The colour development was initiated with the addition of 2, 2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) Roche, Germany. Black bar represents wells coated with either SCCBMMs or α-HL. Grey bar represents colour contributed by Caveolin-1. Master grey bar represents the interaction between Caveolin-1 and coated protein depicted in figure. The data represent the average of three independent measurements. (B) Immunoprecipitation was carried out as described in the methods section using A431 cell line and anti-his tag antibody after treatment with α-HL or SCCBMMs. The immunoprecipitates were processed and SDS-PAGE was carried put on a 12% gel. Proteins were transferred onto nitrocellulose membrane, blocked and probed with Anti-Caveolin-1 antibody (Directed against C terminal of the protein) and reprobed with Anti-His tag antibody.]

3.4.9 Single point mutation reduced binding affinity of α-HL both in vitro and in vivo

We have established that Caveolin-1 interacts with α-HL in vitro and in vivo. The role of aromatic amino acids of α-HL in Caveolin-1 binding was examined. The effect of mutation on Caveolin-1 affinity of SCCBMMs was compared with α-HL in vitro by conventional ELISA assay and in vivo by immunoprecipitation (A431cell). The data in figure 3.11A shows
SCCBMMs have ~50% reduction in binding affinity of Caveolin-1 in a significant manner. Further proof of the reduction interaction of SCCBMMs and Caveolin-1 was obtained by pull down assay, shown in figure 3.11B. The data in figure 3.14B was obtained by treating A431 cells with His tag α-HL and SCCBMMs and the cell lysate was immunoprecipitated with anti-His antibody and immunoblot with anti Caveolin-1 antibody. This clearly indicates that the SCCBMMs interact with Caveolin-1 but not that strongly like α-HL and Caveolin-1 interacts with each other on A431 cells. This result was contrary to EGFR and Caveolin-1 binding where single amino mutation could not prevent the interaction Caveolin 1 binding motif of EGFR and Caveolin-1 [116]. All these results, prove that the aromatic amino acid mutation of α-HL result in affinity loss, which probably as a result of loss of second step interaction (Chapter II, Figure 2.7) between scaffolding domain of Caveolin-1 and Caveolin-1 binding motif of α-HL that was assumed to occur through π-π stack interaction.

3.4.10 SCCBMMs do not form hetero oligomers: We have previously shown that Caveolin-1 binding motif directly involved in oligomerization of α-HL and its deletion totally abolished the co-operative oligomerization property [51,121]. All SCCBMMs oligomerized reasonably well, however, they surprisingly, could not form hetero-oligomers with α-HL (Figure 3.9B). The lysis caused by α-HL was not affected by an increase in SCCBMM concentration. It is also clear that the co-operative oligomerization defective in case of SCCBMMs. Interestingly, the oligomers formed by the SCCBMMs, to a certain degree, are fairly resistant to heat and SDS which is a characteristic property of α-HL.

3.4.11 IASD modification of SCCBMMs and membrane penetration: Utility of IASD has been well established for the study of topology of membrane binding proteins. IASD labeled SCCBMMs showed no lytic activity (Figure 3.12B) and reduced membrane binding (Figure 3.12C) and no oligomerization in contrast to IASD labeled cysteine mutants of the amino acids D\(^{183}\)-R-D\(^{185}\) of Caveolin-1 binding motif. This suggests that aromatic amino acids of Caveolin-1 binding motif are involved in the binding and membrane penetration for β-barrel formation [48].
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Figure 3.12 (A) Cysteine Modification by IASD and Gel-shift Electrophoresis: Each SCCBMM was incubated in 0.05M sodium phosphate, pH 7.5, containing 10 mM DTT for 5min at room temperature in the molar ratio of 5:1 as described in materials and methods section. Modification was analyzed by gel-shift assay (16%) as per the reference [48]. Each lane represents 2µg of labeled or unlabeled protein. (B) Kinetics of lysis with IASD labeled SCCBMMs: Activity of IASD labeled proteins was examined as described in Figure 3.5B in presence of 5mM DTT. Symbols correspond to α-HL (▼), W179C-IASD (▲), Y182C-IASD (●), and W187C-IASD (◇). (C) RBC membrane binding and oligomerization with IASD labeled proteins: SCCBMMs were labeled with IASD as described above and the membrane binding and oligomerization assay were carried out as done for figure 3.8B by using 5 µg/ml of IASD labeled or unlabeled protein as control. The binding represents as W179C (lane 1), Y182C (lane 2), W187C (lane 3), W179C-IASD (lane 4), Y182C-IASD (lane 5), and W187C-IASD (lane 6). Lane C represents α-HL as a marker. Figure 12B and C were obtained by immuno-bloting with anti-His antibody.

3.4.11 IASD modification of SCCBMMs and membrane penetration: Utility of IASD has been well established for the study of topology of membrane binding proteins. IASD labeled SCCBMMs showed no lytic activity (Figure 3.12B) and reduced membrane binding (Figure 3.12C) and no oligomerization in contrast to IASD labeled cysteine mutants of the amino acids D183-R-D185 of Caveolin-1 binding motif. This suggests that aromatic amino acids of Caveolin-1
binding motif are involved in the binding and membrane penetration for β-barrel formation [48].

Figure 3.13 (A, B) Badan modification of SCCBMMs: All SCCBMMs were modified with badan as mentioned in Materials and methods section and electrophoresed on 12% SDS–PAGE. The panel (A) represents the coomassie stained version of the gel and the panel (B) the badan emission detected with UV light. Lane 1: W179C, lane 2: W179C-Bd, lane 3: Y182C, lane 4: Y182C-Bd, lane 5: W187C, lane 6: W187C-Bd and lane 7: Protein marker. (C) Hemolytic activity of badan labeled SCCBMMs was carried out as described for Figure 3.5. The symbols respectively represent (▲), (▼), (□), and (○) for W179C, Y182C, W187C, and α-HL. (D) Relative emission spectra of badan labeled SCCBMMs on rRBCs were measured as described for material and method section. Various spectra represents all labeled SCCBMMs at 0 min (C), W179C (1), Y182C (2), and W187C (3) after 6 h time points. (E) Binding and oligomerization of badan labeled SCCBMMs: Binding and oligomer patterns for respective proteins are depicted in figure. M and O7 represent monomer and oligomer respectively.

3.4.12 Membrane penetration of SCCBMMs: We have examined the membrane penetrability of the SCCBMMs by badan modification, which is an environmentally sensitive fluorophore. We have been able to achieve high labeling efficiency of all
SCCBMMs (W179C (99.34%), W187C (98.45%), and Y182C (96.11%)) which indicate the high degree of accessibility of the aromatic amino acids to solvent (Figure 3.13A and B). Badan labeling did not change the silent nature of SCCBMM towards lytic activity. We anticipated that badan can retrieve back the activity by fulfilling the aromatic surface requirement that was lost due to mutation (Figure 3.5). To determine relative penetration, badan emission spectra (emission spectra shift and increase intensity corresponds to membrane penetration) were recorded for all SCCBMMs after membrane binding. Interestingly, there was a marked increase in fluorescence emission intensity of badan as seen in Figure 3.13D. All SCCBMMs have yielded blue shifts upon oligomerization but major shift was obtained for W187C. It is straightforward to interpret the apolar environment experienced by badan labeled mutants of the hydrophobic core of the lipid bilayer. All labeled SCCBMMs were also oligomerized like it unlabeled counterpart (Figure 3.13E). All the data together indicate that the water soluble, Caveolin-1 binding motif of α-HL penetrates well inside the membrane to interact with Caveolin-1 or Caveolin-1 like molecule(s), plays an important role in oligomerization and conformational changes necessary to form transmembrane pore.
3.5 DISCUSSION

Assembly of staphylococcal $\alpha$-HL varies from cell type to cell type and this variation compels us to believe the existence of a receptor that might facilitate its assembly. The target cell susceptibility can vary as a function of the presence of Caveolin-1. However, Caveolin-1, per se, is absent on red blood cells of most species but enough evidence exits for its presence on mammalian cells [73]. The Caveolin-1 binding motif of $\alpha$-HL appears to play a crucial role in its assembly. Most intriguing question that remained unanswered is how the $\alpha$-HL establishes its contact with Caveolin-1, which is present in the cytoplasmic side of cell membrane?

It has been proposed that Caveolin-1 binding motif (the basic and aromatic amino acids cluster) has ability to penetrate into membrane. Therefore, we mutated aromatic amino acids of Caveolin-1 binding motif into cysteine to study membrane penetration and functional oligomerization on RBCs membrane by $\alpha$-HL. Comparative study with previously reported (chapter II) Caveolin-1 binding motif deficient mutants ($\alpha$-HL-SD, $\alpha$-HL-LD) and a carboxy terminal deletion mutant, support the view that SCCBMMs are reasonably folded and Caveolin-1 binding motif is structurally flexible. All SCCBMMs appear to have normal binding to rabbit RBC membrane but have lost their co-operative oligomerization property, i.e., all SCCBMMs arrested as membrane bound pre-pore stage, although with varying degree of oligomer stability (Figure 3.8 and 3.9). These pre-pore oligomers have established a certain degree of interprotomer contacts but not sufficient enough to form $\beta$-barrel assembly needed for cell lysis (3.5). This observation in conjunction with the absence of hetero-oligomer formation clearly indicates that the SCCBMMs have not undergone necessary conformational changes that associate with the $\beta$-barrel penetration (Figure 3.9). In order to test the membrane penetration really needed for $\beta$-barrel formation we modified the SCCBMMs with badan, an environmentally sensitive fluorophore (Figure 3.13). Interestingly enough, we have observed differences in the intensity and blue shift of the badan emission signifying the change in the environment of the aromatic residues of the Caveolin-1 binding motif of $\alpha$-HL as membrane penetration is expected to result in an increase in emission intensity associated with blue shift (Figure 3.13D). Cysteine scanning mutagenesis of charged residues of $\alpha$-HL followed by site specific chemical modification of the cysteines by IASD have revealed that the charged residues of Caveolin-1 binding motif (W-G-P-Y-D-R-D-S-W) have interesting properties [48]. Firstly, single cysteine mutants have normal
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hemolytic activity but upon IASD modification have lost the same. This indicates that the penetration of D-R-D segment into the membrane is important. In contrast, the mutations at the aromatic residues of the Caveolin-1 binding motif (W-G-P-Y-D-R-D-S-W) led to loss of hemolytic activity (Figure 3.12). This clearly indicates that the aromatic residues play an important role in establishing ‘apt cellular contacts’ for binding and initiation of conformational changes. We anticipated that the badan modification (of the cysteine mutants) might restore the activity as the badan can substitute for the aromatic amino acids. However, the badan modification has not restored the hemolytic activity of \( \alpha \)-HL, indicating the importance of proper penetration and conformational changes initiated by this amino acid segment. It has been argued that the W179 of Caveolin-1 binding motif can interact with preclustered head groups of sphingolipids in natural erythrocytes and artificial membranes, hence, might contribute for the clustering of \( \alpha \)-HL at cell surface before penetration of its \( \beta \)-barrel [49,50]. This Caveolin-1 binding motif, as shown by us earlier, is structurally flexible, hence, can elicit differential mode of target binding, i.e., it can interact with Caveolin-1 of mammalian cells as well as phospholipids head groups like phosphotidyl choline (Chapter II). Depending upon the target membrane viz. red blood cells, artificial membranes, and nucleated cells, this segment may exhibit the desired flexibility for membrane binding and penetration. In summary we examined, nature of the Caveolin-1 binding motif and its relevance in membrane binding and \( \beta \)-barrel formation. We have been able to demonstrate that the intrinsically flexible Caveolin-1-binding motif co-operatively regulates assembly of\( \alpha \)-HL.