Addition of a small hydrophobic segment from the head region to an amphipathic leucine zipper like motif of *E. coli* toxin hemolysin E enhances the peptide-induced permeability of zwitterionic lipid vesicles

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Abstract

To find out the sequence requirement of the H-205 peptide, containing an amphipathic leucine zipper motif corresponding to the amino acid (a.a.) region 205–234 of hemolysin E (HlyE) to induce efficient permeation in zwitterionic lipid vesicles, the peptide was extended at the N-terminal after the addition of seven amino acids from the predicted transmembrane region in the head domain of the protein-toxin. The new peptide, H-198 (a.a. 198–234) and a scrambled mutant peptide of the same size were synthesized, fluorescently labeled and characterized functionally and structurally. The results showed that H-198 induced significantly higher permeation in the zwitterionic PC/Chol lipid vesicles than its shorter version, H-205. H-198 formed large aggregates in the PC/Chol vesicles unlike H-205 and also adopted more helical structure in the membrane mimetic environments compared to that of H-205. Fluorescence energy transfer experiments by flow cytometry indicated that only H-198 but not its mutant or H-205 oligomerized in the zwitterionic lipid vesicles, while in the negatively charged lipid vesicles both H-198 and H-205 formed oligomeric assembly. The results suggest a probable role of the hydrophobic residues of the head domain of HlyE in inducing permeability in the zwitterionic lipid vesicles by the peptide derived from the a.a. 198–234 of the toxin.

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1. Introduction

Cytolytic proteins and peptides play an important role in performing offensive and defensive actions in a number of organisms [1]. Pathogenic bacteria often express virulence factors that are of fundamental importance for the interactions between the microorganism and the host [2]. Hemolysin E, also known as silent hemolysin A (SheA) or cytolysin A (ClyA) by different groups, is a 34-kDa novel pore-forming protein toxin, which is present in several pathogenic strains of *Escherichia coli* (including O157), *Salmonella typhi* and *Shigella flexneri* and represents the first member of a new class of cytotoxin [3]. This protein toxin is also considered as a virulence factor in the pathogenic strains of these bacteria. In *E. coli* K-12, expression of hlyE gene is activated by the action of the global transcription factors FNR and CRP (cAMP receptor protein), in response to oxygen starvation and glucose starvation, respectively [4,5]. Thus, the expression of this gene occurs in response to environmental conditions related to infection. Purified HlyE or *E. coli* expressing HlyE exhibit cytotoxic and apoptogenic activities towards human and murine macrophages, human peripheral monocytes and HeLa cells [5,6].

HlyE has neither N-terminal signal sequence nor undergoes posttranslational processing like other hemolysins. Recently Wai et al. have shown that outer membrane vesicles (OMVs) released from the bacteria contain HlyE protein [7].

Osmotic protection assays and lipid bilayer experiments show that HlyE forms moderately cation-selective water permeable pores of diameter 25–30 Å [8,9]. On the basis of hydrophobicity calculations HlyE has one predicted transmembrane segment ranging from residue 177 to 203; in addition, there is a second
shorter hydrophobic sequence from residues 89 to 101 [9]. The crystal structure of hemolysin E shows the rod shape of the molecule consisting of a bundle of four long helices [10].

However, neither the mechanism of membrane-interaction nor the exact membrane-interacting segment(s) of this toxin is known. Recently an amphipathic leucine zipper like motif located in the α-F region has been characterized [11] which interacts with phospholipid vesicles. The results showed that the peptide (H-205) corresponding to the amino acid region (amino acid 205–234 region) although bound to both zwitterionic (PC/Chol) and negatively charged (PC/Pg/Chol) lipid vesicles destabilized the negatively charged lipid vesicle very efficiently and the zwitterionic vesicles comparatively much weakly. Since the outer membrane of eukaryotic cells is mostly composed of zwitterionic lipids, in order to look into the potential of this motif to interact with eukaryotic membrane our objective was to find out the sequence requirement of the H-205 segment to induce efficient permeation in the zwitterionic lipid vesicles.

It was anticipated that probably due to lack of sufficient hydrophobic amino acids (it contains less than 40% hydrophobic amino acid residues), H-205 did not perturb the zwitterionic lipid vesicle as efficiently as the negatively charged one. After looking into the sequence of hemolysin E, it was observed that a stretch of conserved and hydrophobic residues is present at the looking into the sequence of hemolysin E, it was observed that a stretch of conserved and hydrophobic residues is present at the

2.1. Materials

Rink amide MBHA resin (loading capacity: 0.4–0.8 mmol/g) and all the N-α Fmoc and necessary side-chain protected amino acids were purchased from Novabiochem, Switzerland. Coupling reagents for peptide synthesis like 1-hydroxybenzotriazole (HOBT), diisopropylcarbodiimide (DIC), 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N,N-diisopropylethylamine (DIEPA) were purchased from Sigma, USA. Dichloromethane, N,N dimethyl formamide (DMF) and piperidine were of standard grades and procured from reputed local companies. Acetonitrile (HPLC grade) was procured from Merck, India while trifluoroacetic acid (TFA), trifluoroethanol (TFA) and sodium dodecyl sulfate (SDS) were purchased from Sigma. Egg phosphatidylethanolamine (PC) and egg phosphatidylglycerol (PG) were obtained from Northern Lipids Inc., Canada while cholesterol (Chol) was purchased from Sigma. Calcein was purchased from Sigma and used without further purification. NBD-fluoride (4-fluoro-7-nitrobenz-2-oxa-1, 3-dizole) and tetramethylrhodamine succinimidyl ester were procured from Molecular probes (Eugene, OR). The other reagents used in the present study were of analytical grade and procured from local companies; buffers were prepared in mill Q water.

2.2. Peptide synthesis, fluorescent labeling and purification

All the peptides were synthesized manually in solid phase. Stepwise syntheses were carried out on rink amide MBHA resin (0.15 mmol) utilizing the standard Fmoc chemistry, employing DIC/HOBt or TBTU/HOBt/DIEPA coupling procedure [12–14]. Deprotection of α-amino group and the coupling of amino acids were checked by Kaiser test [15] for primary amines and chloranil test for secondary amine [16]. After the synthesis was over, each peptide was cleaved from the resin with simultaneous de-protection of the side chains by treatment with a mixture of TFA/pheno/hoodsaliol/o/1,2-ethanediol/water (82.5:5:5:2.5:5 v/v) for 6–7 h. Labeling at the N-terminus of a peptide by fluorescent probes was achieved by a standard procedure as reported earlier [11,17–19]. In brief, 15–20 mg of resin bound peptide was treated with 25% piperidine (in DMF) to remove the Fmoc group from the N-terminal amino group. The resin was washed and dried. Then Fmoc de-protected resin-bound peptides were incubated either with two–three equivalents of tetramethylrhodamine succinimidyl ester in dimethylformamide in the presence of 5% diisopropylethylamine or with NBD-fluoride for 48–72 h, which ultimately results the formation of N-α-Rhophospho- or N-α-NBD-peptides. After sufficient labeling, the resin was washed with DMF and DCM in order to remove the unreacted fluorescent probes. The peptides were cleaved from the resin as above and precipitated with dry ether. All the peptides were purified by RP-HPLC on an analytical Vydac C4 column using a linear gradient of 0–80% acetonitrile in 45 min with a flow rate of 0.6 ml/min. Both acetonitrile and water contained 0.05% TFA. The purified peptides were ~95% homogeneous as shown by HPLC. Each peptide was subjected to ES-MS analysis for detection of molecular mass.

2.2.1. Preparation of small unilamellar vesicles (SUVs)

SUVs were prepared by a standard procedure [11,17–19] as follows. Dry lipids [either PC/cholesterol (8:1 w/w) or PC/Pg/Chol (4:4:4 w/w)] of required amounts were dissolved in CHCl3/MeOH (2:1 v/v) in small glass vials. Solvents were evaporated under a stream of nitrogen, which resulted in the formation of a thin film on the wall of glass vial. The glass vials containing lipid films were further kept under vacuum for ~20 min until it became transparent. The lipid concentration was determined by phosphorus estimation [20].

2.2.2. Circular dichroism (CD) experiments

CD spectra of the peptides were recorded in phosphate buffered saline (PBS, pH 7.4), 40% TFE and 1% SDS by utilizing a Jasco J-710 spectropolarimeter. The spectropolarimeter was calibrated routinely with 10-camphor sulphonlic acid. The samples were scanned at room temperature (~30 °C) with the help of a capped quartz cuvette of 0.2 cm path length in the wavelength range of 250–195 nm. An average of 4–6 scans were taken for each sample with a scan speed of 20 nm/min and data interval of 0.5 nm for peptide concentration of ~20 μM. The fractional helicities were calculated by the following formulae [21,22].

\[ \Phi_0 = (\theta_0^0)/\theta_{222}^0 - (\theta_0^0)/\theta_{138}^0 \]

where \( \theta_{138}^0 \) was the experimentally observed mean residue ellipticity at 222 nm. The values for \( \theta_{138}^0 \) and \( \theta_{222}^0 \) that correspond to 100 and 0% helix contents were considered to have mean residue ellipticity values of ~32,000 and ~2,000 respectively at 222 nm [22].

2.2.3. Membrane-binding experiments

The affinity of the peptides for phospholipid vesicles was determined by binding experiments as reported earlier [11,18,23–25]. Briefly, small
unilamellar vesicles were added gradually to 0.2–0.3 μM of NBD-labeled peptide at room temperature. Fluorescence intensities of NBD-labeled peptides alone and after each addition of lipid vesicles were recorded on a Perkin Elmer spectrofluorimeter LS-50B, with the excitation wavelength set at 467 nm and emission at 520 nm. The excitation and emission slits were fixed at 8 nm and 6 nm respectively. The contributions of lipid to any of the recorded signal were measured by titrating the unlabelled peptide (concentration same as NBD-labeled peptide) with the same amount of lipid vesicles and were subtracted from the original signal. The binding isotherms were analyzed by the following equation.

\[ X_b = K_f C_t \]

where \( X_b \) is defined as the molar ratio of bound peptide per 60% of the total lipid, assuming that the peptides were initially partitioned only over the outer leaflet of the SUV as suggested by Beschiasvilli and Seelig [24]. \( K_f \) represents the partition coefficient and \( C_t \) indicates the concentration of the free peptide at equilibrium. \( X_b \) was calculated by extrapolating the fluorescence signal, \( F_x \) (fluorescence signal when all the peptide is bound to lipid) from a double-reciprocal plot of \( F \) (peptide fluorescence in the presence of lipid) versus \( C_t \) (concentration of lipid). Fraction of peptide bound (\( f_b \)) was determined by the following equation.

\[ f_b = (F - F_0)/(F - F_0) \]

where \( F \) is the fluorescence of the peptide when it is bound to lipid and \( F_0 \) is the fluorescence of the peptide in its unbound state. If \( f_b \) is known, \( C_t \) can easily be calculated for each concentration of the lipid. \( K_f \) can be determined from the slope of the plot of \( X_b \) and \( C_t \).

2.2.4. Calcein release from the calcein-entrapped lipid vesicles

In order to determine the pore-forming activity of H-198 and its mutant, peptide-induced release of calcein from calcein-entrapped lipid vesicles was measured. Calcein-entrapped lipid vesicles were prepared with a self-quenching concentration (60 mM) of the probe in 10 mM HEPES buffer at pH 7.4 as reported earlier [26–28]. Briefly, thin film of lipid (either PC/PG/Chol or PC/Chol) was re-suspended in calcein solution, vortexed for 1 min and then sonicated in a bath-type sonicator. The non-encapsulated calcein was removed by the liposome suspension by gel filtration using a sephadex G-50 column. Usually lipid vesicles are diluted to approximately 10 fold after passing through a G-50 column. The eluted calcein-entrapped vesicles were diluted further in the same buffer to a final lipid concentration of ~3.0 μM for the experiment. Peptide-induced release of calcein from the lipid vesicles was monitored by the increase in fluorescence due to the dilution of the dye molecules from its self-quenched state. Fluorescence was monitored at room temperature with excitation and emission wavelengths fixed at 490 and 520 nm respectively with excitation slit of 8 nm and emission slit of 6 nm. Calcein release as measured by the fluorescence recovery is defined by the equation:

\[ F_t = [(I_t - I_0)/(I_0 - I_t)] \times 100\% \]

where \( I_t \) is the observed fluorescence after the addition of a peptide at time \( t \) (after 5 min of the addition of the peptide), \( I_0 \) is the initial fluorescence before addition of the peptide and \( I_t \) is the total fluorescence, which was determined after the addition of triton X-100 (0.1% final concentration) to the dye-entrapped vesicle suspension.

2.2.5. Flow cytometric studies to detect the binding and assembly of the peptides in lipid vesicles

Fluorescence resonance energy transfer (FRET) experiments with a donor-labeled peptide and an acceptor-labeled peptide in the presence of lipid vesicles were also performed as reported earlier [29] by using a Becton Dickinson FACSCalibur flow cytometer and CellQuest Pro software. The excitation and emission wavelengths were set at 488 and 530 nm respectively. Equimolar amounts (2.7 μM) of NBD-labeled peptide (donor) and Rho-labeled peptide (acceptor) were added to the lipid vesicles. Changes in NBD fluorescence due to FRET were determined by comparing NBD-fluorescence of the donor in the presence of Rho-labeled peptide to that when Rho-peptide was replaced with the respective unlabeled peptide. 10^6 Events were counted for each experiment.

2.3. Results

In order to find out the amino acid sequence requirement of the amphipathic leucine zipper motif (H-205 peptide, corresponding to the amino acid region 205–234) to induce efficient permeation in the zwitterionic membrane and also to understand any possible role of this segment in the membrane-interaction of hemolysin E, one wild type and one mutant peptide derived from the amino acid region of 198–234 were designed and synthesized. Fig. 1A shows the schematic localization of the extended version of this amphipathic leucine zipper like motif of hemolysin E under investigation. The panel A of Fig. 1 also schematically shows both the putative transmembrane segments with two identified and partially characterized heptad repeats [11] as marked. The sequence alignment of the corresponding amino acid region of the proteins of hemolysin E toxin family indicates that a considerable number of amino acids belonging to all four proteins have identical sequences (Fig. 1B). Fig. 1C depicts the amino acid sequences of the unlabeled and fluorescent-labeled wild type peptide H-198 and the mutant peptide, which has the same size and composition as the wild type peptide but positions of lysine at 206 with leucine at 215 and isoleucine at 208.

Fig. 1. Schematic representation, sequence alignment and amino acid sequences of H-198 and its analog. (A) Schematic representation of hemolysin E and the localization of H-198 segment in it. H-198, shown as extended version of amphipathic leucine zipper like motif (amino acid 198–234) and other important segments like both the putative transmembrane domain TM1 (a.a. 88–101) and TM2 (a.a. 177–203) and two heptad repeats at a.a. 130–157 and 205–234 have been shown as marked. (B) Sequence alignment of H-198 (a.a. 198–234) derived from hemolysin E of E. coli with homologous regions of hemolysin sequences of avian E. coli (EcAv), Salmonella typhi (Styphi) and Shigella flexneri (Shflex). Identical amino acids have been marked as bold letters. (C) Designations and sequences of the peptides used in this study. The mutated amino acid is underlined.
with serine at 217 were interchanged with each other. The shorter version (Mu1-H-205) of the mutant Mu-H-198 was reported earlier (11). In order to understand the importance of amino acid sequence in the assembly and functional activity of the peptide H-198, Mu1-H-205 was also extended by adding the seven hydrophobic amino acids at the N-terminal like the wild type peptide.

### 2.3.1. H-198 induced stronger permeability in the zwitterionic phospholipid vesicles than its shorter version H-205

In order to check the ability of H-198 and its analogue to destabilize the lipid bilayer, release of calcein from calcein-entrapped lipid vesicles in the presence of these peptides was measured. As shown in the panel A of Figs. 2 and 3, the extended leucine zipper like peptide (H-198) triggered the release of liposome-encapsulated calcein in a dose-dependent manner from both the zwitterionic PC/Chol and negatively charged PC/PG/Chol lipid vesicles respectively. However, the mutant was less efficient than the wild type H-198 in releasing calcein from either of the PC/Chol (Fig. 2B) or PC/PG/Chol (Fig. 3B) lipid vesicles indicating the requirement of specific sequence in destabilizing the lipid vesicles as was also observed in case of the smaller version of the wild type peptide H-205 [11]. The profiles (Fig. 2C) of H-205-induced weak permeability of PC/Chol lipid vesicles [11] clearly indicated that H-205 was much less active than its extended version, H-198 (Fig. 2A) in destabilizing the zwitterionic lipid vesicles. The plots of fluorescence recovery, which is a measure of peptide-induced permeability of lipid vesicles, clearly indicated that H-198 was significantly more active than the mutant in inducing permeability in zwitterionic PC/Chol (Fig. 2D) and negatively charged PC/PG/Chol (Fig. 3C) lipid vesicles. It is to be mentioned that the shorter version, H-205, induced a calcein fluorescence recovery of only 20% at a peptide/lipid molar ratio of 0.7 and fluorescence recovery of only 8% at a peptide/lipid ratio of 0.2 [11]. However, the present data indicated that the extended version, H-198 induced a fluorescence recovery ~100% at peptide/lipid ratio of ~0.20 in PC/Chol lipid vesicles. On the other hand, H-205 [11] and H-198 showed comparable permeability in the negatively charged PC/PG/Chol lipid vesicles. For example, both H-205 and H-198 induced 100% fluorescence recovery in PC/PG/Chol vesicles at a peptide/lipid molar ratio range of 0.14–0.15. Thus the results suggested that the addition of seven hydrophobic amino acids at the N-terminal of H-205 did not show much effect on its permeability towards the negatively charged lipid vesicles.

Also, it was observed that NBD- or Rho-labeled H-198 induced very similar calcein release from the calcein-entrapped lipid vesicles as that of the unlabeled H-198, which indicated that labeling of H-198 did not disturb its pore-forming activity significantly (data not shown). Thus the results clearly suggest that H-198 induced appreciably stronger permeation in the zwitterionic PC/Chol vesicles than its shorter version H-205. Or in other words the addition of seven hydrophobic amino acids at the N-terminal of H-205 enhanced the ability of the peptide to destabilize the zwitterionic membrane.

### 2.3.2. Both H-198 and its mutant peptides bound to phospholipid membrane

In order to understand the basis of enhanced permeability of zwitterionic lipid vesicles in the presence of H-198 compared to that of the shorter version H-205, the interaction of the peptides to lipid vesicles was studied in detail. The binding of H-198 and its mutant to phospholipid vesicles was examined by employing their NBD-labeled analogs. The sensitivity of the NBD probe to the dielectric constant of the medium has been exploited extensively to detect the binding of the NBD-labeled peptide to membrane [11,23,30–32]. Fluorescence emission spectra of NBD-labeled H-198 were recorded in aqueous buffer and in the presence of phospholipid vesicles. The fluorescence spectra of the wild type and mutant peptides exhibited broad emission maxima at around 540 nm in phosphate buffer indicating the location of the NBD-probe, attached to the N-terminal of the peptides in the hydrophilic environment [32]. However, in the

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**Fig. 2.** Calcein release induced by H-198, H-205 and the Mu-H-198 from the zwitterionic PC/Chol lipid vesicles entrapped with calcein. Panels A, B, and C show the profiles of calcein release induced by increasing concentrations of H-198, Mu-H-198 and H-205 respectively as measured by the increase in fluorescence with respect to time. In panel A, a, b, c, d and e are 0.15, 0.30, 0.375, 0.450 and 0.60 μM of H-198; in panel B, a, b and c are 0.15, 0.30 and 0.6 μM of Mu-H-198 and in panel C, a, b and c, are the profiles of H-205 induced calcein release from calcein entrapped PC/Chol lipid vesicles (3.0 μM) with peptide concentration 0.69, 1.38, and 2.07 μM respectively. Panel D depicts the relative pore-forming activity of the peptides in zwitterionic lipid vesicles as shown by the plots of percentage of fluorescence recovery with respect to peptide to lipid molar ratio. The concentration of PC/Chol lipid vesicles was 2.5 μM. Symbols: closed squares, H-198, closed circles, Mu-H-198.
emission maxima in the wavelength ranges of 529–532 nm in the negatively charged phospholipid vesicles (PC/PG/Chol) also indicating the presence of zwitterionic PC/Chol and PC/PG/Chol lipid vesicles respectively [11]. Thus a comparison of emission maxima of H-198 and H-205 in both kinds of lipid vesicles indicated that the addition of seven hydrophobic amino acids at the N-terminal of H-205 probably assisted in its insertion in the zwitterionic lipid vesicles although without showing much effect on its localization in the negatively charged lipid vesicles. These results clearly demonstrated that the synthetic wild type and the mutant peptide derived from the amino acid region 198–234 of HlyE bound to both zwitterionic and negatively charged lipid vesicles but their localization was influenced by their amino acid sequence and also lipid composition of the membrane.

2.3.3. H-198 exhibited slightly higher affinity than the mutant toward phospholipid vesicles

The affinity of H-198 and its mutant to the phospholipid vesicles was determined by the binding experiments utilizing their NBD-labeled analogues and SUVs with different lipid composition. Fluorescence signals of the NBD-labeled peptides as a result of their binding to the phospholipid membrane were plotted with respect to lipid/peptide molar ratio. Fig. 5A depicts the binding curve for H-198 in PC/Chol vesicles, while Fig. 5B and C describe such plots for H-198 and Mu-H-198 in PC/PG/Chol lipid vesicles. All the plots show a gradual increase in fluorescence with increase in lipid concentration indicating a progressive binding of the NBD-labeled peptides to the lipid vesicles. Binding isotherms were generated by plotting $X_b$ with respect to $C_r$ as has been described in Materials and methods. Partition coefficients of the NBD-labeled peptides to the phospholipid vesicles were estimated from the slope of the binding isotherms after extrapolating to zero. NBD-labeled H-198 exhibited an appreciable affinity for PC/Chol lipid vesicles as indicated (panel D) by its partition coefficient of $2.0 \pm 0.1 \times 10^4$ M$^{-1}$, which is similar to that of the surface-active peptides, derived from anti-microbial peptides, bacterial toxins and viral fusion proteins [18,25,34]. The estimated partition coefficient for H-198 in PC/PG/Chol lipid vesicles was $2.3 \pm 0.1 \times 10^4$ M$^{-1}$, and the mutant peptide exhibited a partition coefficient of $1.66 \pm 0.1 \times 10^4$ M$^{-1}$ in the same kind of lipid vesicles. It is to be mentioned that H-205 exhibited a partition coefficient of $1.5 \pm 0.1 \times 10^4$ M$^{-1}$ in PC/Chol vesicles [11], which suggested that the extended version, H-198 possesses a slightly higher affinity for zwitterionic lipid vesicles than its shorter version H-205. The nature of the binding curve also provides an idea about the assembly of a peptide in membrane. The linear nature of binding isotherm of NBD-labeled mutant peptide in PC/PG/Chol vesicles (Fig. 5F) suggests that the binding of the peptide to lipid vesicles was a simple adhesion process and NBD-Mu-H-198 peptide molecules did not form large aggregates in the negatively charged lipid vesicles. However, the binding isotherm of NBD-labeled H-198 in PC/Chol (Fig. 5D) and PC/PG/Chol lipid vesicles (Fig. 5E) bent downward appreciably and deviated from linearity. As suggested earlier, this kind of curve

![Fig. 3. Calcein release induced by H-198 and the mutant Mu-H-198 from the negatively charged PC/PG/Chol lipid vesicles entrapped with calcein. Panels A and B show the profiles of calcein release induced by increasing concentrations of H-198 and Mu-H-198. In panel A, a, b, c, d and e are 0.03, 0.06, 0.08, 0.16 and 0.4 μM of H-198. In panel B, a, b, c, d and e are 0.16, 0.32, 0.64 and 0.8 μM of Mu-H-198. Panel C represents the plots of percentage of fluorescence recovery induced by the peptides with respect to peptide to lipid molar ratio in PC/PG/Chol (2.5 μM) lipid vesicles. Symbols: closed squares, H-198 and closed circles, Mu-H-198.

![Fig. 4. Binding of NBD-labeled H-198 and its mutant Mu-H-198 to negatively charged PC/PG/Chol and zwitterionic PC/Chol lipid vesicles. Squares and triangle represent NBD-labeled H-198 and NBD-labeled Mu-H-198 respectively. Closed symbols, 0.2 μM of NBD-labeled peptides in PBS; open symbols, NBD-labeled peptides in the presence of 412 μM of PC/PG/Chol and plus centered symbols, NBD-labeled peptides in the presence of 412 μM of PC/Chol lipid vesicles.
indicates the co-operativity in binding of the peptides to membrane [11,25,34].
Furthermore, these binding isotherms revealed that NBD-labeled H-198 formed
large aggregates in both zwitterionic and negatively charged lipid vesicles. It is
to be pointed out that the nature of binding of H-198 to zwitterionic lipid vesicles
was contrasting to H-205 in the sense that while the former peptide formed
larger aggregates there in, the latter only formed smaller aggregates [11].

2.3.4. H-198 adopted significant helical structures in the membrane mimetic
environments
Circular dichroism studies were carried out in order to determine the
secondary structures of these peptides. Secondary structures of H-198 and its
mutant were determined in aqueous buffer (PBS), and in membrane mimetic
environments like SDS micelles and 40% TFE in water with the help of mean
residual ellipticity values at 222 nm. Both the peptides exhibited mostly random
coil structure in PBS and therefore not presented. However, the extent of their
helical structures increased significantly in 40% TFE and 1% SDS (Fig. 6). The
mean residual ellipticity values at 222 nm of the CD spectra of wild type H-198
in 40% TFE and 1% SDS were −31,554 and −25,868 which correspond to
98.5% and 79.5% helical structures respectively. For Mu-H-198 mean residue
ellipticity values at 222 nm in 40% TFE and 1% SDS were −24,866 and
−21,986 which correspond to helix contents of 76.2% and 66.6% respectively.
As reported earlier the helix contents of H-205 in 40% TFE and 1% SDS were
77.8 and 68.7% respectively [11]. Thus the results indicated that the addition
of seven amino acids at the N-terminal of H-205 resulted an appreciable increase
in its helix contents. Altogether, these results indicated that the wild type H-198
adopted significant helical structure in both 40% TFE and 1% SDS and
mutations resulted in a slight decrease in helical structure.

2.3.5. H-198 exhibited higher affinity than its analogs to phospholipid vesicles
and assembled therein as evidenced by flow cytometric studies
In order to evaluate any possible role of this extended leucine zipper like
motif in the assembly of hemolysin E in membrane, fluorescence energy transfer
experiments were performed with the help of a flow cytometer as described in
Materials and methods. Zwitterionic PC/Chol lipid vesicles treated with NBD-
H-198 peptide showed higher fluorescence (the X-axis values) compared to that
treated with NBD-H-205. Furthermore, the lipid vesicles treated with Mu-H-198
exhibited even slightly lower fluorescence. Probably, the results indicate that H-
198 possesses higher affinity than H-205 toward the zwitterionic lipid vesicles.

Fig. 5. Determination of affinity of the NBD-labeled (0.2 μM) wild type and mutant peptides to phospholipid vesicles as determined by titration with PC/Chol and PC/
lipid vesicles; Panel D, binding isotherm of NBD-H-198 in PC/Chol vesicles; panels e and F are binding isotherms of NBD-H-198 and Mu-H-198 in PC/PG/Chol lipid
vesicles.
and the affinity of the mutant is the least among the three peptides. The data also support the binding studies of the NBD-labeled peptides to phospholipid vesicles. However, when the corresponding Rho-labeled peptide was added to membrane-bound NBD-H-198 significant decrease in NBD-fluorescence was observed (Fig. 7B) indicating an energy transfer event, which could be due to oligomeric assembly of the peptide in zwitterionic lipid vesicles as was reported for perfringolysin O in the human red blood cells [29]. The extent of energy transfer was less for the peptide H-205 and almost no energy transfer was observed for Mu-H-198. The data probably indicated either negligible ability (H-205) or inability (Mu-H-198) of the peptide molecules to form oligomeric assembly in the zwitterionic lipid vesicles. However, the picture was a bit different in the negatively charged lipid vesicles. Not much difference was observed between the fluorescence levels of the PC/PG/Chol lipid vesicles treated with NBD-labeled H-198 or H-205. Also, a significant energy transfer was observed when Rho-H-205 or Rho-H-198 was added to PC/PG/Chol lipid vesicles, bound to the corresponding NBD-labeled peptides indicating that both the peptides formed oligomers in the negatively charged lipid vesicles. However, no appreciable energy transfer was observed between NBD-Mu-H-198 and Rho-Mu-H-198 in PC/PG/Chol vesicles as was observed in the PC/Chol lipid vesicles. Altogether, the flow cytometric studies indicated that in the negatively charged PC/PG/Chol vesicles both H-205 and H-198 and in the zwitterionic PC/Chol vesicles only H-198 formed oligomeric assembly.

3. Discussion

The results depicted here clearly indicated that the synthetic H-198 segment of E. coli toxin hemolysin E induced zwitterionic PC/Chol lipid vesicle permeation much more efficiently than its shorter version, H-205 [11], a peptide corresponding to an amphipathic leucine zipper motif located in the a.a. region 205–234 and the mutant peptide, which has the same composition as H-198 but with four amino acids interchanged their positions. Another alanine-substituted mutant (Mu2-H-198) of H-205 (heptadic leucine at position 215 replaced by alanine), which was reported earlier [11], was also extended to the same size as the H-198. However, since this alanine-substituted mutant exhibited very similar and significantly reduced membrane permeability (Supplementary Fig. 1) like the scrambled mutant (Mu-H-198), detailed structural studies of this mutant were not performed. The results described here further suggested that the addition of the hydrophobic amino acid residues from the predicted transmembrane domain in the head region of HlyE to the H-205 enhances the peptide-induced permeability of zwitterionic lipid vesicles and also assists in self-assembly of the peptide in the same kind of phospholipid vesicles.

The binding and assembly of a peptide in a particular kind of lipid vesicles are the key issues related to its efficiency to induce permeation therein. Binding experiments employing the NBD-labeled peptides indicated that H-198 possesses slightly higher affinity than H-205 towards the zwitterionic lipid vesicles (Fig. 5). Flow cytometric studies, which showed that the zwitterionic

![Graph](image_url)

**Fig. 6.** Determination of secondary structures of wild type H-198 and Mu-H-198 in SDS micelles and 40% TFE/water by recording their CD spectra in the corresponding environment. Symbols: solid line, H-198 in 40% TFE; dash, H-198 in 1% SDS; dot, Mu-H-198 in 40% TFE, dash dot, Mu-H-198 in 1% SDS. The concentration of H-198 and its mutant was 21.0 μM.

![Graph](image_url)

**Fig. 7.** Detection of oligomeric assembly of H-205, H-198 and Mu-H-198 onto both PC/Chol and PC/PG/Chol lipid vesicles (825 μM) by employing energy transfer experiments with the help of a flow cytometer. Equimolar amounts (2.7 μM) of donor (NBD) labeled H-205, H-198 or Mu-H-198 (D) and either the corresponding unlabeled peptide (U) or acceptor (Rho) labeled peptide (A) incubated with PC/Chol (panels A, B and C) or PC/PG/Chol (panels D, E and F) lipid vesicles at room temp. for ~5 min and then analyzed by flow cytometry. Grey lines represent (D+U), while black lines represent (D+A) with lipid vesicles. For each experiment 10,000 events were counted.
lipid vesicles treated with NBD-H-198 exhibited maximum fluorescence followed by the lipid vesicles treated with NBD-H-205 and NBD-Mu-H-198 also support the binding experiments (Fig. 7). The higher fluorescence of zwitterionic vesicles in the presence of NBD-H-198 could be due to the binding of more fluorescent peptide molecules to the lipid vesicles and/or as a result of interaction of the peptide molecules with more hydrophobic part of the phospholipid membrane. That H-198 could interact with more hydrophobic environment of the zwitterionic lipid vesicles than H-205 is consistent with the lower emission maximum (~527 nm, Fig. 4)) of NBD-H-198 in the same kind of lipid vesicles than that of its shorter version (529–530 nm) [11].

Energy transfer experiments by flow cytometric approach suggested that the extended version, H-198 formed oligomeric assembly in both zwitterionic and negatively charged lipid vesicles whereas H-205 formed oligomers only in the negatively charged lipid vesicles (Fig. 7). Similar oligomeric assembly of cholesterol dependent toxins in human red blood cells was detected by flow cytometric studies as was reported earlier [29]. The result was also consistent with the shape of the binding isotherm (Fig. 5), which showed clear deviation from linearity for H-198 in both zwitterionic and negatively charged lipid vesicles. It has already been reported that H-205 formed large aggregates only in the negatively charged lipid vesicles but not in zwitterionic vesicles [11]. Thus the result indicated the role of seven hydrophobic amino acids from the head region that were added to the N-terminal of H-205 in altering its assembly in zwitterionic lipid vesicles. Circular dichroism studies (Fig. 6) indicated that H-198 peptide adopted almost a complete helical structure in membrane mimetic environment suggesting that this segment may contribute in the secondary structure of the whole protein.

Altogether the results suggested that the ability of H-198 to induce higher permeability in the zwitterionic lipid vesicles could be due to the stronger binding and oligomeric assembly of the peptide in the lipid vesicles compared to that of H-205 and H-198 mutant. Moreover, H-198 adopted higher helical structure in membrane mimetic environment than H-205. Thus the results also indicated the role of seven amino acids (a.a. 198–204) from the predicted transmembrane segment of the head region of hemolysin E in increasing the membranedestabilization potency of H-205 region in zwitterionic lipid vesicles. Interestingly, the similar oligomeric assembly of H-198 and H-205 in negatively charged lipid vesicles is consistent with their similar permeability in the same kind of membrane. Both H-205 [11] and its extended version induced comparable fluorescence recovery at similar peptide/lipid molar ratio in the negatively charged lipid vesicles. The results probably indicated that the amino acid region 205–234 corresponding to the peptide H-205 already possess the amino acid sequence to induce permeation in the negatively charged lipid vesicles and assembly there in and therefore, the addition of seven amino acids from the predicted transmembrane domain to the N-terminal of H-205 peptide did not show a significant effect on the activity of the peptides in the same kind of vesicles.

The actual membrane-interacting segment of hemolysin E is still not known. However, the site directed mutagenesis [8] and model building studies [10,35] suggested that the predicted transmembrane segment in the head domain, which also contains the β-tongue region, could play an important role in the membrane-interaction and toxic activity of the protein toxin. Since the eukaryotic membrane is composed of zwitterionic lipids the studies described here indicated a possible contribution of the segment consisting of a leucine zipper like motif and a small part of the transmembrane segment of the head region in the interaction and assembly of the protein to the target cell membrane. Importantly, the date also suggests that a peptide probably needs at least a certain number of hydrophobic amino acids in order to efficiently destabilize the zwitterionic lipid vesicles. In conclusion the results depicted here suggest that the addition of a small hydrophobic stretch from the predicted transmembrane segment of head region to the H-205 assists in the self-assembly of the peptide in zwitterionic lipid vesicles and enhances the peptide-induced permeability of the same kind of lipid vesicles. Probably, H-198 segment with a.a. 198–234 of HlyE may contribute in the binding and assembly of the protein toxin in target cell membrane.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.03.019.

References
